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MicroRNA-30c-2-3p negatively regulates NF-κB signaling and cell cycle progression through downregulation of TRADD and CCNE1 in breast cancer

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ABSTRACT

Nuclear Factor kappa B (NF-κB) signaling is frequently deregulated in a variety of cancers and is constitutively active in estrogen receptor negative (ER-) breast cancer subtypes. These molecular subtypes of breast cancer are associated with poor overall survival. We focused on mechanisms of NF-κB regulation by microRNAs (miRNAs), which regulate eukaryotic gene expression at the post-transcriptional level. In a previous genome-wide miRNA screen, we had identified miR-30c-2-3p as one of the strongest negative regulators of NF-κB signaling. Here we have uncovered the underlying molecular mechanisms and its consequences in breast cancer. *In vitro* results show that miR-30c-2-3p directly targets both TNFRSF1A-associated via death domain (TRADD), an adaptor protein of the TNFR/NF-κB signaling pathway, and the cell cycle protein Cyclin E1 (CCNE1). Ectopic expression of

Abbreviations: CSF2, colony stimulating factor 2; CCNE1, cyclin E1; CCND1, cyclin D1; CDK, cyclin-dependent kinase; CTRL, control; CXCL, chemokine (C-X-C motif) ligand; ER, estrogen receptor; I.B, intensity of band; IL, interleukin; LPS, lipopolysaccharide; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; miRNA, microRNA; mRNA, messenger RNA; MMPs, matrix metalloproteinases; MUT, mutant; MYC, v-myc avian myelocytomatosis viral oncogene homolog; NF-κB, nuclear factor kappa B; RNAi, RNA interference; TCGA, The Cancer Genome Atlas; TGF, transforming growth factor; TLR, toll like receptor; TRADD, TNFRSF1A-associated via death domain; TNF-α, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor; s.d., standard deviation; siRNA, small interfering RNA; UTR, untranslated region; WT, wild type.

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miR-30c-2-3p downregulated essential cytokines IL8, IL6, CXCL1, and reduced cell proliferation as well as invasion in MDA-MB-231 breast cancer cells. RNA interference (RNAi) induced silencing of TRADD phenocopied the effects on invasion and cytokine expression caused by miR-30c-2-3p, while inhibition of CCNE1 phenocopied the effects on cell proliferation. We further confirmed the tumor suppressive role of this miRNA using a dataset of 781 breast tumors, where higher expression was associated with better survival in breast cancer patients. In summary we have elucidated the mechanism by which miR-30c-2-3p negatively regulates NF-κB signaling and cell cycle progression in breast cancer.

1. Introduction

Breast cancer is a heterogeneous disease which can be divided into molecular subtypes that are associated with distinct gene expression patterns and clinical outcomes. The main molecular subtypes based on gene expression are luminal A, luminal B, human epidermal growth factor receptor 2 (HER2) enriched, basal-like tumors and normal breast like (Parker et al., 2009; Perou et al., 2000; Sorlie et al., 2001). Despite major improvements in diagnosis and treatment, breast cancer is still the second most leading cause of cancer deaths among women worldwide (Siegel et al., 2012). Estrogen receptor negative (ER-) breast cancer is characterized by the lack of estrogen receptor expression, encompasses the HER2 enriched as well as basal subtypes, and is associated with poor prognosis (Engstrom et al., 2013; Parker et al., 2009; Sorlie, 2004). NF-κB signaling is frequently active in ER-breast tumors (Biswas et al., 2004; Singh et al., 2007) and is associated with increased cell proliferation, inhibition of apoptosis and metastasis. These tumorigenic effects of NF-κB signaling as well as the constitutive activation of this pathway, particularly in the aggressive ER-breast cancer subtypes, make NF-κB signaling an attractive target for therapeutic intervention.

NF-κB is a conserved family of transcription factors with five members that are structurally related: RelA (p65), RelB, c-Rel, NFKB1 (p105 and p50), and NFKB2 (p100 and p52) (Hayden and Ghosh, 2004). In the unstimulated state, NF-κB proteins are retained in the cytoplasm through interactions with inhibitory proteins IκB (inhibitor of NF-κB). In response to stimuli such as tumor necrosis factor-α (TNF-α) or lipopolysaccharide (LPS), the IKK (IκB kinase) complex becomes activated (Cao and Karin, 2003), leading to phosphorylation and subsequent degradation of IκB. This results in nuclear translocation of activated NF-κB dimers (Karin and Ben-Neriah, 2000) and subsequent transcription of target genes (Chakraborty et al., 2013).

TNF-α is a pro-inflammatory cytokine that plays an important role in the regulation of inflammation, proliferation, immune regulation, and cell death (Jaattela et al., 1991; Walczak, 2011). It can bind specifically to TNF receptors (TNFR1 and TNFR2) that have a death domain (DD) and are expressed by most cell types (Remouchamps et al., 2011). The anti-apoptotic effect of TNF-α is caused by engagement of the adaptor protein TNFRSF1A-associated via death domain (TRADD) (Wilson et al., 2009). TRADD can then interact with TNF receptor–associated factor 2 (TRAF2) which ultimately leads to activation of NF-κB signaling (Walczak, 2011; Wilson et al., 2009).

MicroRNAs (miRNAs) are non protein-coding RNAs that modulate gene expression at the post-transcriptional level (Filipowicz et al., 2005). Mature miRNAs are between 21 and 24 nucleotides long and bind mostly within the 3′UTRs of their target mRNAs (Bartel, 2004; Cullen, 2004). This interaction is mediated by the RNA induced silencing complex (RISC) and leads to degradation or translational repression of targeted mRNAs (Valencia-Sanchez et al., 2006). More than half of the human protein coding genes are predicted to be regulated by miRNAs (Friedman et al., 2009). Their role in carcinogenesis and specifically in breast cancer is being increasingly uncovered (Keklikoglou et al., 2012; Korner et al., 2013; Uhlmann et al., 2012; Ward et al., 2014). We previously carried out a genome-wide screen for miRNAs regulating NF-κB signaling and identified the miR-520/373 family to coordinately regulate NF-κB signaling and TGF-beta signaling in breast cancer (Keklikoglou et al., 2012).

In the current study, we have found miR-30c-2-3p to be a negative regulator of NF-κB signaling. We identified *TRADD* as a direct target of miR-30c-2-3p responsible for effects on NF-κB signaling, and cyclin E1 (*CCNE1*) as a second target mediating effects on cell cycle regulation. Finally, we found miR-30c-2-3p expression to be associated with better disease specific survival as well as with a range of clinicopathological features in breast cancer patients.

2. Materials and methods

2.1. Cell culture and reagents

Breast cancer cell lines MDA-MB-231 (HTB-26), MDA-MB-468 (HTB-132), MCF-10A (CRL-10317), MCF-7 (HTB-22) and T-47D (HTB-133) were obtained from ATCC (Manassas, VA, USA). HEK293FT (R700-07) cells were obtained from Invitrogen (Carlsbad, CA, USA). Cell lines were regularly validated for lack of mycoplasma contamination and verified at the cell line authentication service at the DKFZ Core Facility. All cell culture media and reagents were obtained from Invitrogen (Carlsbad, CA, USA) and used according to the ATCC (American Type cell culture) recommendations. Human recombinant TNF-α was purchased from Sigma–Aldrich (St.

Louis, MO, USA) and used at a final concentration of 20 ng/ml. Lipopolysaccharide from *Escherichia coli* 0127:B8 (Sigma–Aldrich, St. Louis, MO, USA) was used at a final concentration of 10 ng/ml.

2.2. Transfections

All transfections were performed in antibiotic free media using Lipofectamine 2000 transfection reagent from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instructions. Short interfering (si) RNAs for *TRADD*, *RELA* and *CCNE1* (for sequences, Supplementary Table 1) were purchased from Ambion (Invitrogen, CA, USA). Si negative control (siNTC) was used as a non-targeting control from the same company. miRNA-30c-2-3p mimic and negative controls were obtained from Dharmacon (Lafayette, CO, USA). SiRNAs and miRNAs were used at a final concentration of 30 ns each. MicroRNA inhibitors from Exiqon (Vedbaek, Denmark) were transfected at a concentration of 100 nM.

2.3. NF-κB reporter luciferase and 3′ UTR targeting luciferase assay

Luciferase reporter assays were performed as previously described (Keklikoglou et al., 2012). Briefly, for the NF-κB reporter assay, HEK293FT cells were seeded in 96-well plates and transfected with miRNA mimic or siRNAs, together with the NF-κB reporter 3xKBL plasmid (provided by Dr. Mosialos, Aristotle University of Thessaloniki, Greece) and pMIR-REPORT β-gal vector (Ambion, Austin, TX, USA). Cells were stimulated with TNF-α (20 ng/ml) or LPS (10 ng/ml) 48 h post-transfection, and luciferase activity was measured after 5 h using a luminometer (Tecan, Linz, Austria). β-galactosidase activity was used for normalization of luciferase values, and measured with beta-glo® Luminescent Assay Kit (Promega, Madison, WI, USA). Luciferase values were expressed as fold change compared to control transfected cells. To prove direct targeting by miRNAs, psiCHECK2 vectors (Promega, Madison, WI, USA), containing the respective wild type or mutated 3′UTRs, were co-transfected with miRNA mimics in MDA-MB-231 cells. *Renilla* and firefly luciferase activities were measured 48 h post-transfection. *Renilla* luciferase values were normalized to firefly luciferase values and expressed as fold change compared to control transfected cells. Primers used for the cloning of 3′UTRs and mutagenesis are given in Supplementary Table 2.

2.4. Gene expression profiling

MDA-MB-231 cells were transfected with miR-30c-2-3p or mimic control and RNA was isolated 48 h post-transfection. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quality control of total RNA (Agilent bioanalyzer, Santa Clara, CA, USA) and gene expression profiling (Illumina whole genome Bead Chip® Sentrix arrays HumanHT-12 v4, San Diego, CA, USA) were performed at the DKFZ microarray core facility. Expression profiling data was normalized and analyzed for changes in expression levels using the vsn bioconductor package in R (http://www.bioconductor.org/packages/2.12/bioc/html/vsn.html). The gene expression dataset has been submitted to the ArrayExpress database with the Accession number E-MTAB-3083.

2.5. Real-time PCR

Real time PCR for miRNAs and protein coding genes was performed as previously described (Keklikoglou et al., 2012). RevertAid™H Minus First Strand cDNA synthesis kit from Fermentas (Karlsruhe, Germany) was used for the synthesis of cDNA according to the manufacturer's instructions. Primer sequences used for mRNAs of interests and corresponding probes from the Universal Probe Library (Roche, Penzberg, Germany) are given in Supplementary Table 3. *GAPDH* and *HPRT* were used as normalizing controls for mRNA expression analysis. 48 h post-transfection, cells were stimulated with TNF-α (20 ng/ml) for 5 h and total RNA isolated to evaluate changes on NF-κB target genes at mRNA levels. miR-30c-2-3p specific primer set, SYBR master mix and Universal RT kit were from Exiqon (Vedbaek, Denmark). SNO38b and SNO48 were used for normalization in miRNA expression analysis. Gene expression results were analyzed using 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001) and shown as fold changes compared to control transfected cells.

2.6. Western blotting

Cell lysate collection and western blotting were performed as described previously (Keklikoglou et al., 2012). Antibodies against TRADD (sc-7868) and CCNE1 (sc-247) were purchased from Santa Cruz Biotechnologies, (California, CA, USA). Antibodies for phospho-p105 (Ser933) (18E6) and total p105/p50 (3035) were from Cell Signaling Technology (Danvers, MA, USA). Antibody for phospho-IKKα (Ser176)/IKKβ (Ser177) (C84E11) was from Cell Signaling Technology (Danvers, MA, USA). The antibodies for total protein, IKKβ (2C8) and IKKα (3G12) were also from the same company. The anti-beta actin antibody was from Sigma Aldrich (Saint-Louis, MO, USA). Anti GAPDH antibody (14C10) was from Cell Signaling Technology (Danvers, MA, USA). Actin and GAPDH were used as loading controls. The dilution used for primary antibodies was 1:1000 (CCNE1 was used at a dilution of 1:200) and for secondary antibodies was 1:10,000. The secondary antibodies used for detection were InfraRed Dye 680 or InfraRed Dye 800 conjugated antibodies from LI-COR (Lincoln, NE, USA) and bands were visualized using an Odyssey scanner from LI-COR (Lincoln, NE, USA). Cells were stimulated with TNF-α (20 ng/ml) for 10 min after 48 h of transfection with siRNAs or miRNAs to evaluate effects on phosphorylation of p105 and IKKα/IKKβ.

2.7. Cell viability and apoptosis assays

Cell viability was assessed 72 h after transfection with miRNAs or siRNAs using either WST reagent from Roche (Penzberg, Germany) or CellTiter-Blue from Promega (Madison, WI, USA). After 3 h of incubation with respective reagent, absorbance was measured at 460 nm for WST and fluorescence for CellTiter-Blue at 560Ex/590Em. Apoptosis was measured using a Caspase-Glo 3/7 assay kit from Promega

(Madison, WI, USA). All experiments were performed according to the manufacturer's recommendations. Cell cycle assays with 7-AAD and BrdU staining were carried out according to the manufacturer's protocol (BD Pharmingen San Diego, CA, USA). Briefly, 72 h after transfection with miRNA or siRNAs, the cells were incubated with BrdU at a final concentration of 1 μM for 1 h at 37 °C. Cells were later harvested and treated with DNase I for 1 h with incubation at 37 °C. Cells were washed and resuspended in anti-BrdU, FITC-conjugated antibody purchased from BD Pharmingen (San Diego, CA, USA). Half an hour later, cells were washed and resuspended in 7-AAD solution. Stained cells were then measured by flow cytometry using a FACS Calibur (BD Biosciences, Heidelberg, Germany) and Cell Quest Pro software (BD Biosciences, Heidelberg, Germany) was used for data analyses. No stimulus was applied for cell viability, apoptosis and cell cycle progression experiments.

2.8. Invasion assay

Invasion assays were performed using a Real Time Cell Analyzer (RTCA, xCELLigence, Roche, Germany). CIM plates were used to record the impedance of invading cells. MDA-MB-231 cells were transfected in 6-well plates. 24 h after transfection, cells were starved overnight in 0.5% FBS medium. Next day, cells were trypsinized, washed once with starvation medium, counted and adjusted to a cell number of 75,000 cells/100 μl in starvation medium. The upper chamber was coated with growth factor reduced matrigel matrix from Corning (Tewksbury, MA, USA) at a dilution of 1:30 in starvation media. Matrigel was allowed to solidify in the upper chamber of CIM plate for 4 h at 37 °C. Afterwards, 100 μl of cell suspension was seeded on top of the matrigel. The lower compartment was filled with 175 μl full growth medium with TNF-α at a concentration of 10 ng/ml. Cell index values were recorded with a time-interval of 15 min for a total of 15 h.

2.9. Patient data analysis

Normalized and matched mRNA and miRNA expression data from the METABRIC study (Curtis et al., 2012; Dvinge et al., 2013) was used to analyze differential expression of miRNA and target genes in ER subgroups. We analyzed 781 patients for whom the ER status was known and for whom both mRNA and corresponding miRNA expression data was available. Welch's *t*-test was performed to detect significant differential expression changes between the two ER subgroups. Normalized miRNA expression data from The Cancer Genome Atlas (TCGA) was used for analysis of miR-30c-2-3p expression in an independent breast cancer patient dataset (Cancer Genome Atlas, 2012). To evaluate association between mRNA and miRNA, a linear regression analysis was performed on scale normalized miRNA and mRNA expression data according to Spearman rank correlation. MiRNA expression levels were categorized based on distribution quartiles into low (<Q1), medium (Q1–Q3) and high (>Q3) expression group. Fisher's exact test was used to test association between miRNA groups and categorical clinicopathological factors. Kruskal–Wallis test was used to test association between miRNA groups and quantitative clinicopathological factors. For outcome analysis, disease-specific survival (DSS) was analyzed from METABRIC study (Dvinge et al., 2013), with disease-unrelated deaths being censored. Univariate and multivariate Cox regression model was used to assess the potential prognostic impact of miRNA expression levels and clinicopathological factors for DSS. Survival analysis was done using the 'survival' R package, from CRAN (http://CRAN.R-project.org/package=survival). All statistical analyses were carried out using R version 3.0.1 (http://www.R-project.org) or GraphPad Prism 5.0 software. All *t* tests were two-sided tests. P values below 0.05 were considered statistically significant.

3. Results

3.1. miR-30c-2-3p negatively regulates NF-κB signaling and is downregulated in ER negative breast cancer patients

We previously carried out a genome wide miRNA screen to identify novel miRNA regulators of NF-κB signaling (Keklikoglou et al., 2012). Among the 810 miRNAs tested (Supplementary Table 3), miR-30c-2-3p emerged as the fourth strongest negative regulator of the signaling pathway with a z-score of −2.031 (Figure 1A). To validate the screening results, we repeated the NF-κB luciferase reporter assay in HEK293FT and indeed, miR-30c-2-3p overexpression significantly abrogated NF-κB activity, which was similar to RNAi induced knockdown of *RELA* (Figure 1B). Since NF-κB signaling was previously shown to be constitutively active in ER-breast cancer tumors (Nakshatri et al., 1997; Singh et al., 2007) we overexpressed the miRNA in MDA-MB-231 cells (Supplementary Figure 1), an established model for ER-breast cancer (Park et al., 2007). We observed decreased phosphorylation of p105 upon TNF-α stimulation, however the total protein levels (p105 and p50) remained unchanged when miR-30c-2-3p was overexpressed confirming the effect on NF-κB signaling in MDA-MB-231 cells (Figure 1C). This effect on phosphorylation of p105 was not seen in case of MCF-7, an ER+ breast cancer cell line (Supplementary Figure 2). Because of the known association of NF-κB signaling and ER-breast cancer (Singh et al., 2007), we next asked whether endogenous expression levels of miR-30c-2-3p were associated with the ER status of breast cancer patients. To this end we analyzed data from the METABRIC study (Molecular Taxonomy of Breast Cancer International Consortium), a publically available cohort of 781 breast tumors (Dvinge et al., 2013) for differential expression of miRNAs in ER- compared to ER+ tumors. This analysis identified miR-30c-2-3p as a significantly downregulated miRNA in ER- versus ER+ breast cancer patients (Figure 1D and E). We observed the same relation of miR-30c-2-3p expression and ER status (Supplementary Figure 3) in The Cancer Genome Atlas (TCGA) which has miRNA expression data from breast tumors (Cancer Genome Atlas, 2012).

3.2. Ectopic expression of miR-30c-2-3p decreases cell proliferation, invasion and reduces expression of genes relevant in inflammation

To further investigate the role of miR-30c-2-3p in NF-κB signaling, we assessed the expression of proinflammatory

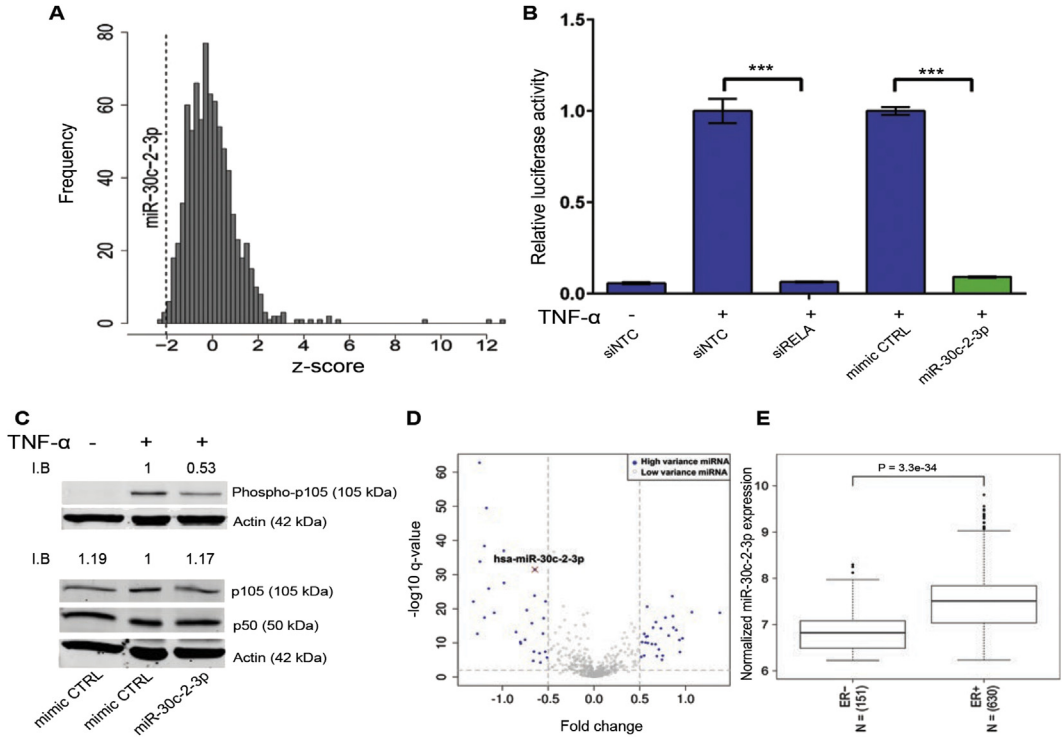

Figure 1 – miR-30c-2-3p regulates NF-κB signaling in breast cancer. (A) z-score distribution of 810 miRNAs screened for effects on signaling based on 3xKBL luciferase assay where miR-30c-2-3p was the fourth strongest negative regulator with a z-score of −2.031 (Keklikoglou et al., 2012). (B) Validation of the screen results in HEK293FT cells to measure NF-κB activity upon stimulation of the signaling by TNF-α (20 ng/ml) for 5 h β-galactosidase was used for normalization. siRELA was used as a positive control (*P ≤ 0.001 compared to respective stimulated control, *t* test). Data are shown as mean ± s.d of two biological and three technical replicates. (C) Phospho-p105 protein levels are downregulated upon treatment of MDA-MB-231 cells with TNF-α (20 ng/ml) for 10 min in cells transfected with miR-30c-2-3p mimic compared to mimic CTRL. Total protein levels (p105 and p50) remain unchanged. Cells were harvested 48 h post-transfection. Actin was used as a loading control. Intensity of bands is expressed as a ratio with respect to actin from the same blot. (D) Volcano plot showing miR-30c-2-3p as a significantly deregulated miRNA based on the fold change and q-value (P value corrected for multiple testing, Benjamini-Hochberg) out of the 853 miRNAs analyzed in the METABRIC study (Dvinge et al., 2013). Blue dots in the figure represent high variance miRNAs between ER- and ER+ breast cancer patients and gray dots represent low variance miRNAs. (E) miR-30c-2-3p is lower expressed in ER- compared to ER+ breast cancer patients from METABRIC study (Dvinge et al., 2013) (P = 3.3e-34).**

cytokines *IL8*, *IL6*, and *CXCL1* upon overexpression of the miRNA. These cytokines were significantly downregulated in miR-30c-2-3p transfected compared to mimic control transfected MDA-MB-231 cells upon TNF-α induction (Figure 2A). Additionally, the expression of *MYC*, *CCND1* and *CSF2*, which are NF-κB transcriptional targets involved in cell proliferation (Hynes and Stoelzle, 2009; Li et al., 2013; Oida et al., 2014), was significantly decreased in miR-30c-2-3p transfected cells after stimulation with TNF-α (Figure 2B). Furthermore, miR-30c-2-3p overexpression significantly decreased cell viability in MDA-MB-231 cells (Figure 2C). This result was independently verified in two other ER-breast cell lines (MDA-468 and MCF-10A) as well as in two ER+ breast cancer cell lines (MCF-7 and T-47D) (Supplementary Figure 4A). Moreover, miR-30c-2-3p significantly induced apoptosis as determined by measuring caspase 3/7 activity compared to the mimic control, 72 h post-transfection in MDA-MB-231, MDA-468, MCF-10A. However, no such effect of the miRNA was observed in the ER+ T-47D cell line while in MCF-7 cells, a second ER+ cell line tested, apoptosis was induced when the miRNA was overexpressed (Supplementary Figure 4B). Finally, we observed that overexpression of miR-30c-2-3p significantly blocked cell cycle progression in MDA-MB-231 cells with only 2.65% (+/− 0.3%) of cells in S-phase compared to 17.10% (+/− 2.6%) in the control (Figure 2D). This effect was reversed in presence of antagomirs used to block expression of endogenous miR-30c-2-3p in MCF-7 cells (Supplementary Figure 5). Next we investigated if miR-30c-2-3p was also capable of abrogating cell invasion, since NF-κB signaling is known to regulate this phenotype (Takada et al., 2006). Indeed, we observed a decreased invasive capacity of MDA-MB-231 cells upon miR-30c-2-3p overexpression (Figure 2E). Since NF-κB signaling had previously been shown to facilitate cell invasion/migration through regulation of matrix metalloproteinases (MMPs), like MMP-9 (Bera et al., 2013), we speculated that decreased invasion might be accompanied by decreased expression of *MMP-9* levels upon miR-30c-2-3p overexpression. Indeed we observed a downregulation of *MMP-9* expression at the mRNA level in the presence of miR-30c-2-3p upon TNF-α stimulation (Figure 2F).

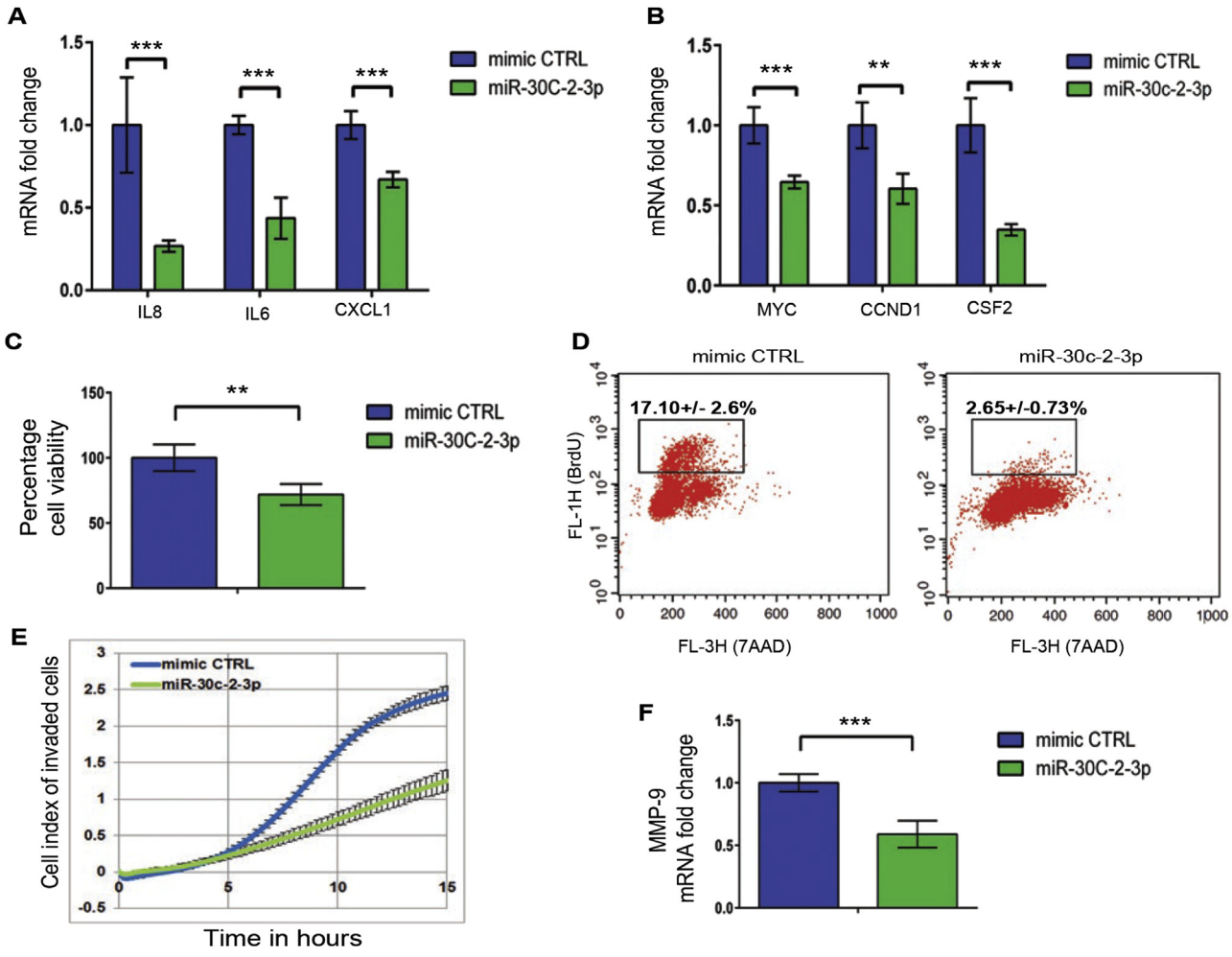

Figure 2 – Effect of miR-30c-2-3p overexpression on expression of cytokines, cell proliferation and cell invasion in MDA-MB-231 breast cancer cell line. (A) mRNA expression of inflammatory cytokines *IL8*, *IL6* and *CXCL1* is reduced upon miR-30c-2-3p overexpression. To activate NF-κB signaling, cells were treated with TNF-α (20 ng/ml) for 5 h and later RNA was isolated (*P ≤ 0.001 compared to stimulated control, *t* test). (B) In the same experimental setup, reduced mRNA levels of proliferation related genes *MYC*, *CCND1* and *CSF2* was observed upon miR-30c-2-3p overexpression (***P ≤ 0.001, **P ≤ 0.01 compared to stimulated control, *t* test). (C) miR-30c-2-3p reduced cell viability compared to mimic CTRL measured 72 h post-transfection. (**P ≤ 0.01 compared to control, *t* test). (D) A reduced S-phase population of cell cycle (gated for BrdU and 7-AAD positive cells) was observed upon miR-30c-2-3p overexpression, measured 72 h post-transfection with miRNAs (***P ≤ 0.001 compared to control, *t* test). (E) miR-30c-2-3p overexpression reduced the invasive capability of MDA-MB-231 cells. Cells were seeded for invasion assay 48 h post-transfection with miRNAs, allowed to invade through matrigel for 15 h and measured in Real-Time Cell Analyzer (RTCA). Significance was tested for 15 h time point. (***P ≤ 0.001 compared to control, *t* test). (F) Concordantly, mRNAs level of *MMP-9* was reduced upon miR-30c-2-3p overexpression in MDA-MB-231 after 48 h of transfection. Cells were treated with TNF-α for 5 h at a concentration of 20 ng/ml and RNA was isolated. (***P ≤ 0.001 compared to stimulated control, *t* test). Data are shown as mean ± s.d. of two biological and three technical replicates.**

3.3. *miR-30c-2-3p directly targets* TRADD

Next, we wanted to elucidate the molecular mechanism of regulation by miR-30c-2-3p. To this end we performed a genome-wide mRNA expression analysis of MDA-MB-231 cells that had been transfected with miR-30c-2-3p compared to cells transfected with mimic control (gene expression data is available in the ArrayExpress database, www.ebi.ac.uk/arrayexpress under the accession number E-MTAB-3083). Gene expression analysis identified 367 genes (Supplementary Table 4) which were down-regulated at the mRNA level with a corrected P value ≤0.05. We performed signaling pathway and GO process enrichment analyses on downregulated mRNAs using MetaCore™ (http://thomsonreuters.com/metacore/) to identify the most deregulated signaling pathways (Supplementary Table 5). Cell cycle related pathways were significantly downregulated by miR-30c-2-3p. Since the MDA-MB-231 cells had not been stimulated with TNF-α prior to performing gene expression analysis, the NF-κB signaling pathway was not enriched among the downregulated pathways (Supplementary Table 5). However, we were interested in finding potential targets that could explain the downregulation of NF-κB signaling in response to TNF-α by miR-30c-2-3p (Figure 1B and C). To identify putative target

genes of miR-30c-2-3p within the NF-κB signaling pathway, we intersected the 367 differentially expressed genes with a list of genes involved in NF-κB signaling obtained from KEGG pathway database (Kanehisa et al., 2014), (http://www.genome.jp/kegg/pathway.html) and identified *TRADD*, the TNF Receptor Superfamily Member 5 (*CD40*), and inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon (*IKBKE*) to be downregulated at the mRNA level (Supplementary Table 4). We followed up on *TRADD* as a potential target gene because of its role in TNF-α induced NF-κB activation through canonical signaling (Hayden and Ghosh, 2014), while both CD40 and IKK-epsilon (encoded by *IKBKE*) are involved in non-canonical NF-κB activation (Coope et al., 2002; Shen and Hahn, 2011) that had not been tested in the screen (Keklikoglou et al., 2012). To investigate if *TRADD* is indeed a target of miR-30c-2-3p, we first verified that in line with microarray data, the mRNA level of *TRADD* was significantly reduced upon miR-30c-2-3p overexpression compared to mimic control transfected in MDA-MB-231 cells (Figure 3A). *In silico* analysis using miRWalk (Dweep et al., 2011) (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) revealed that *TRADD* was a predicted target with one target site mapping to the 3′UTR (Figure 3B). Immunoblotting confirmed the miRNA effect on TRADD also at the protein level (Figure 3C). To prove direct targeting of *TRADD* by miR-30c-2-3p, we cloned the wild type 3′UTR of *TRADD*, or the mutant containing 4 point mutations within the miRNA target site seed region, downstream of a *Renilla* luciferase reporter. We observed a significant downregulation of the *Renilla* luciferase signal in the presence of miR-30c-2-3p compared to mimic control when using reporter construct containing wild type 3′UTR of *TRADD* in MDA-MB-231 cells (Figure 3D). In contrast, this effect was abrogated when we tested the mutated *TRADD* 3′UTR (Figure 3D). These findings suggested that *TRADD* is indeed a direct target of miR-30c-2-3p.

3.4. *miR-30c-2-3p regulates NF-κB signaling by targeting* TRADD *in breast cancer cells*

To assess the impact of *TRADD* on NF-κB signaling, we employed RNAi to knockdown TRADD expression in MDA-MB-231 cells (Supplementary Figure 6) and then repeated the NF-κB reporter assay. Similar to the effects upon miR-30c-2-3p overexpression (Figure 1B), knockdown of TRADD abrogated NF-κB signaling despite TNF-α stimulation (Figure 4A). In another experimental setup we used lipopolysaccharide (LPS) instead of TNF-α to stimulate NF-κB signaling and found NF-κB activity to be reduced in miR-30c-2-3p overexpressing and TRADD knockdown cells (Supplementary Figure 7A). This emphasized the role of *TRADD* and miR-30c-2-3p in the regulation of NF-κB signaling. This observation is in line with previous findings where TRADD had been shown to play an important role in NF-κB activation also within the toll-like receptor (TLR) pathway (Chen et al., 2008). Similar to effects we had observed upon overexpression of miR-30c-2-3p (Figure 2A), the expression of cytokines *IL8, IL6* and *CXCL1* was downregulated upon TRADD inhibition in MDA-MB-231 cells after stimulation with TNF-α (Figure 4B). To investigate the effect of ectopic expression of miR-30c-2-3p and TRADD knockdown on NF-κB signaling activation by TNF-α, we analyzed the phosphorylation level of p105. Indeed in both cases, a decrease in phosphorylation was observed compared

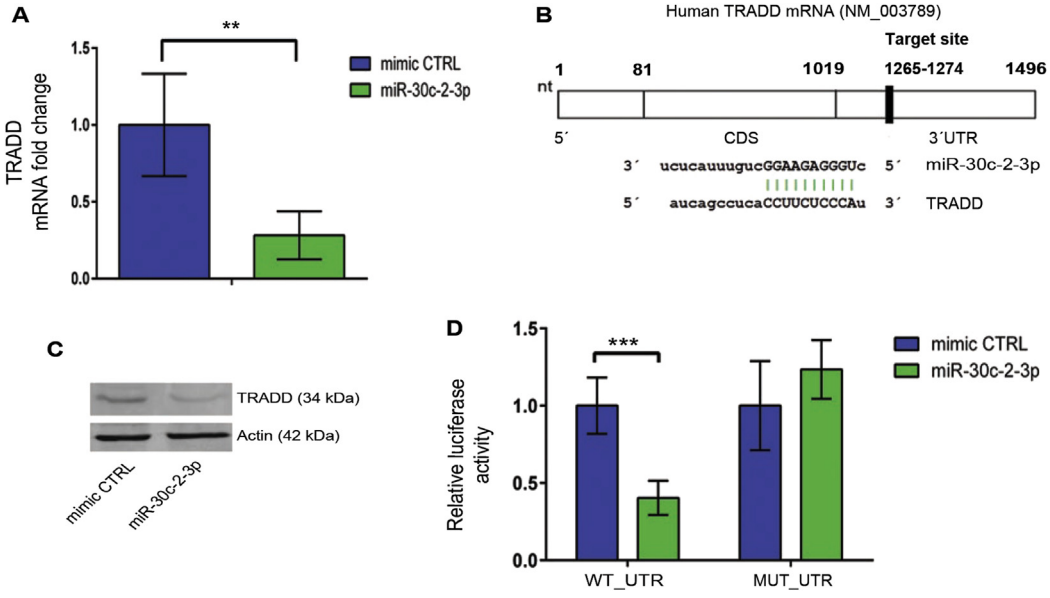

Figure 3 – *TRADD* is a direct target of miR-30c-2-3p. (A) Downregulation of *TRADD* at mRNA level upon miR-30c-2-3p overexpression seen 48 h post-transfection with miRNAs in MDA-MB-231 cells (P ≤ 0.01 compared to control, *t* test). (B) Alignment of miR-30c-2-3p with target site within *TRADD* 3′UTR. (C) Downregulation of TRADD protein level upon overexpression of miR-30c-2-3p compared to mimic CTRL observed 48 h post-transfection with miRNAs in MDA-MB-231 cells. Actin was used as a loading control. (D) Reduced luciferase signal in MDA-MB-231 cells transfected with wild type (WT) 3′UTR of *TRADD* and miR-30c-2-3p compared to mimic CTRL measured 48 h post-transfection with miRNAs (**P ≤ 0.01 compared to control, *t* test). This effect was lost when the binding site in 3′UTR was mutated (MUT). Data are shown as mean ± s.d. of two biological and three technical replicates.**

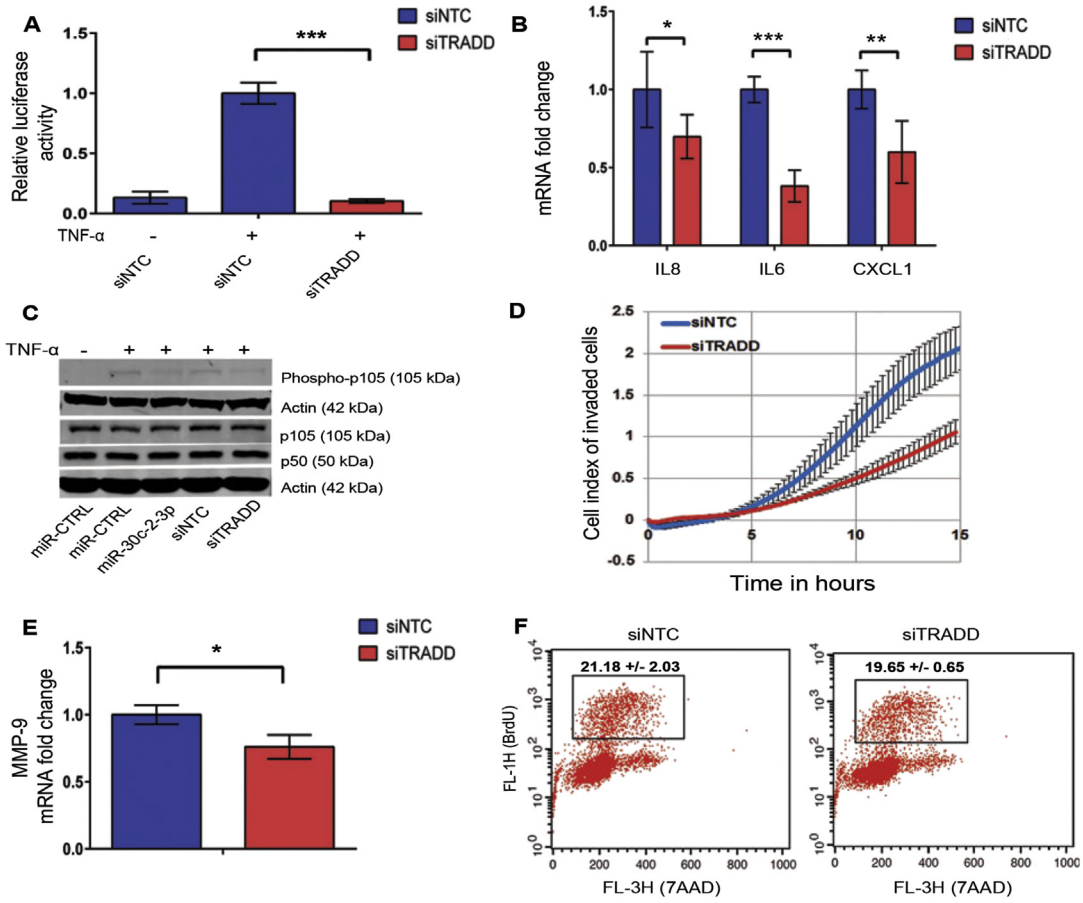

Figure 4 – miR-30c-2-3p regulates NF-κB signaling by targeting *TRADD*. (A) Downregulation of TRADD significantly decreased the luciferase signal measuring NF-κB activity in HEK293FT cells. 48 h post-transfection, cells were stimulated with TNF-α (20 ng/ml) for 5 h and harvested (*P ≤ 0.001 compared to stimulated control, *t* test). (B) mRNA expression of inflammatory cytokines *IL8*, *IL6* and *CXCL1* was reduced upon TRADD knockdown in MDA-MB-231 cells. 48 h post-transfection, cells were stimulated with TNF-α (20 ng/ml) for 5 h and RNA was isolated (***P ≤ 0.001, **P ≤ 0.01,*P ≤ 0.05 compared to stimulated control, *t* test). (C) Reduced phospho-p105 protein level were observed in MDA-MB-231 cells upon treatment with TNF-α (20 ng/ml) for 10 min, 48 h post-transfection with miR-30c-2-3p or siTRADD compared to controls. No change in total protein levels (p105 and p50) was seen. Actin was used as a loading control. (D) Reduced the invasive capability of MDA-MB-231 cells through matrigel was seen upon TRADD knockdown. Significance was tested at the 15 h time point measured in Real-Time Cell Analyzer (RTCA), which was 48 h post-transfection with siRNAs (***P ≤ 0.001 compared to control, *t* test). (E) In addition, mRNA level of *MMP-9* was reduced upon TRADD knockdown in MDA-MB-231 cells, 48 h post-transfection. Cells were treated with TNF-α for 5 h at a concentration of 20 ng/ml and RNA was isolated (*P ≤ 0.05 compared to stimulated control, *t* test). (F) Knockdown of TRADD did not have a significant effect on S-phase population of the cell cycle (gated for BrdU and 7-AAD positive cells) compared to siNTC, 72 h post-transfection in MDA-MB-231 cells. Data are shown as mean ± s.d. of two biological and three technical replicates.**

to stimulated non-targeting control transfected cells (Figure 4C). Furthermore, either overexpressing miR-30c-2-3p or inhibiting TRADD reduced phosphorylation of IKKα/IKKβ upon stimulation with TNF-α (Supplementary Figure 7B), and expression of *CSF2* was significantly reduced upon TRADD knockdown similar to our observations with miR-30c-2-3p overexpression (Supplementary Figures 8 and 2B). Next we checked the role of TRADD knockdown on the invasive capability of MDA-MB-231 cells. Indeed, similar to miR-30c-2-3p overexpression (Figure 2E), TRADD knockdown significantly blocked cell invasion (Figure 4D) and reduced mRNA levels of *MMP-9* upon TNF-α stimulation (Figure 4E). However, unlike miR-30c-2-3p, TRADD knockdown only mildly reduced S-phase of the cell cycle (Figure 4F) and did not reduce the expression of proliferation genes *MYC* and *CCND1* in MDA-MB-231 cells after stimulation with TNF-α (Supplementary Figure 8). These results suggested that TRADD knockdown was able to only partially phenocopy the effects of miR-30c-2-3p, indicating that there are additional targets of this miRNA.

3.5. *miR-30c-2-3p regulates cell cycle progression by directly targeting* CCNE1

GO-term enrichment analysis upon overexpression of the miRNA in MDA-MB-231 cells had identified cell cycle related processes to be among the most significantly regulated by miR-30c-2-3p (Supplementary Table 5). To uncover potential

target genes responsible for the observed reduction in S-phase of the cell cycle, we intersected the list of downregulated mRNAs with cell cycle related proteins obtained from KEGG pathway database (Kanehisa et al., 2014). This analysis identified cell cycle related proteins CCNE1, MCM6, MCM7, CDK4, SMAD3 and TFDP1 to be downregulated at mRNA level (Supplementary Table 4). We focused on *CCNE1*, encoding cyclin E1 which is involved in G1 to S transition (Rosenberg et al., 2001), because of its strong anti-correlation with miR-30c2-3p (Supplementary Table 6) in breast cancer patients from the METABRIC study (Curtis et al., 2012; Dvinge et al., 2013) Moreover, CCNE1 is frequently overexpressed in breast cancer (Spruck et al., 1999). We confirmed the effect on *CCNE1* at the mRNA level by miR-30c-2-3p (Figure 5A) and found a predicted target site within its 3′UTR (Figure 5B). Next, we checked CCNE1 at the protein level upon miR-30c-2-3p overexpression and indeed found it to be downregulated (Figure 5C). Knockdown of CCNE1 (Supplementary Figure 9) indeed phenocopied the reduction in S-phase of the cell cycle (Figure 5D) that we had observed upon overexpression of miR-30c-2-3p (Figure 2D). Using reporter gene constructs, direct targeting of *CCNE1* by miR-30c-2-3p was proven (Figure 5E).

3.6. *Expression of miR-30c-2-3p significantly correlates with clinicopathological features in breast cancer patients*

Since miR-30c-2-3p significantly associated with ER status in breast cancer, we hypothesized that its expression might also correlate with other clinicopathological parameters in breast cancer; namely tumor grade, tumor size, age at diagnosis, lymph node invasion, HER2 expression, progesterone receptor (PR) status and p53 mutation status. To investigate this we categorized miRNA expression levels from METABRIC study (Dvinge et al., 2013) based on distribution quartiles into low (Q1), medium (Q2–Q3) and high (Q4) expression groups.

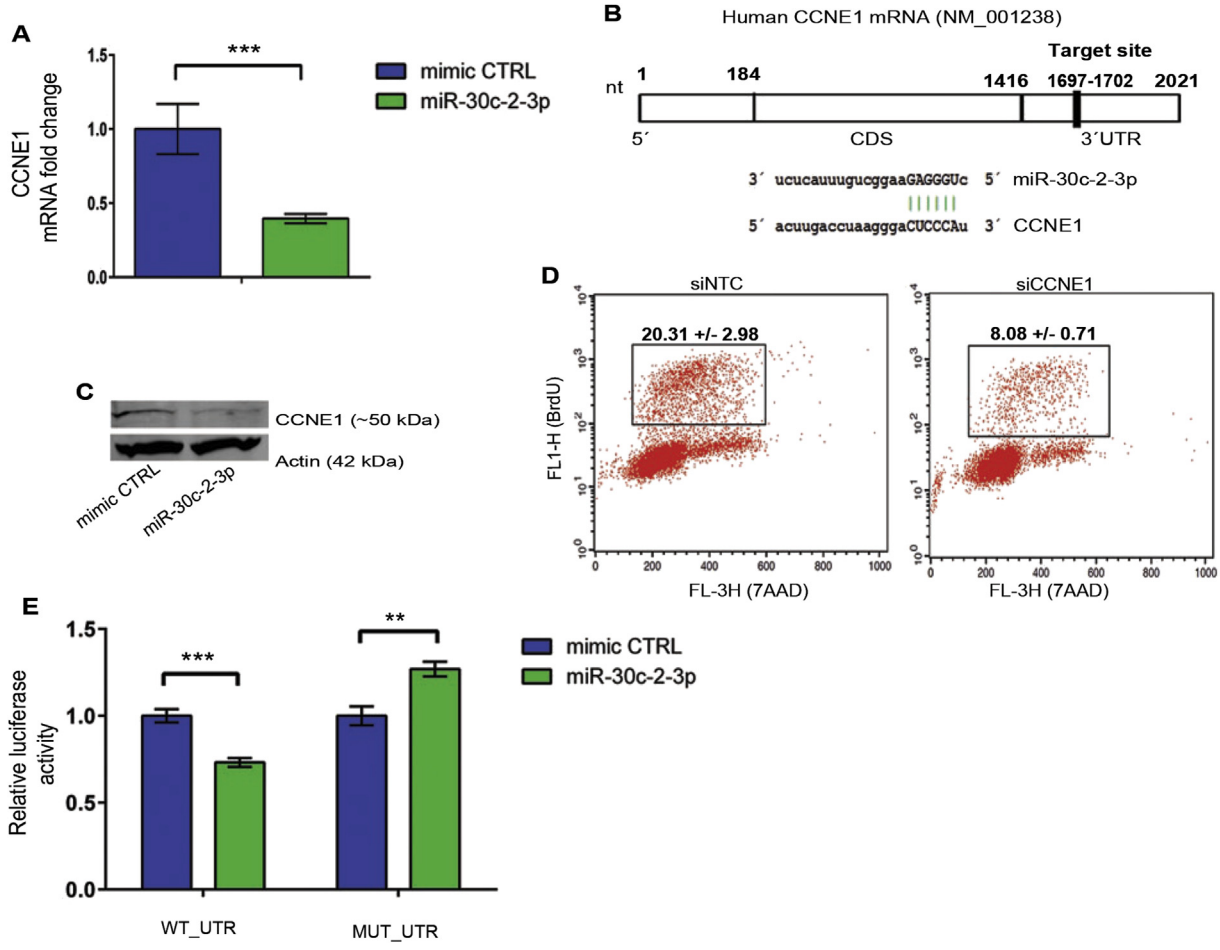

Figure 5 – miR-30c-2-3p regulates cell cycle progression through *CCNE1* targeting. (A) Downregulation of *CCNE1* at the mRNA level upon miR-30c-2-3p overexpression was measured 48 h after transfection with miRNAs in MDA-MB-231 cells (*P ≤ 0.001 compared to control, *t* test). (B) Alignment of miR-30c-2-3p with target site within *CCNE1* 3′UTR. (C) Downregulation of CCNE1 protein level upon overexpression of miR-30c-2-3p compared to mimic CTRL was observed 48 h after transfection with miRNAs in MDA-MB-231 cells. Actin was used as a loading control. (D) Reduced S-phase population of the cell cycle (gated for BrdU and 7-AAD positive cells) was observed upon CCNE1 knockdown compared to siNTC, 72 h after transfection in MDA-MB-231 cells. (***P ≤ 0.001 compared to control, *t* test). (E) Reduced luciferase signal in MDA-MB-231 cells transfected with wild type (WT) 3′UTR of *CCNE1* and miR-30c-2-3p compared to mimic CTRL was measured 48 h after transfection with miRNAs. This effect was lost when the binding site in 3′UTR was mutated (MUT) (***P ≤ 0.001, **P ≤ 0.01 compared to control, *t* test). Data are shown as mean ± s.d. of two biological and three technical replicates.**

Table 1 – Univariate and multivariate disease specific survival Cox analyses in breast cancer patients for miR-30c-2-3p expression in METABRIC study (Dvinge et al., 2013).

	Covariate	Level[a]	Ref[b]	Univariate analysis				Multivariate analysis			
				HR[c]	LCL[d]	UCL[e]	P value	HR	LCL	UCL	P value
1	miR-30c-2-3p expression in 3 groups	mid	low	0.64	0.46	0.87	0.00505	0.97	0.65	1.45	0.89594
		high	low	0.43	0.28	0.66	<1e-04	0.84	0.49	1.45	0.53023
2	Tumor grade[f]	2	1	1.10	0.53	2.32	0.79486	0.99	0.42	2.35	0.97807
		3	1	2.44	1.19	4.98	0.01451	1.57	0.66	3.71	0.30951
3	Tumor size			1.02	1.02	1.03	<1e-04	1.01	1.00	1.02	0.00805
4	Age at diagnosis			1.00	0.98	1.01	0.42995	1.00	0.99	1.02	0.74225
5	Lymph nodes positive			1.11	1.08	1.14	<1e-04	1.07	1.04	1.11	<1e-04
6	ER	+	–	0.44	0.32	0.60	<1e-04	0.75	0.47	1.21	0.24080
7	HER2	+	–	2.50	1.74	3.58	<1e-04	1.80	1.18	2.76	0.00632
8	PR	+	–	0.49	0.37	0.65	<1e-04	0.87	0.58	1.30	0.49510
9	P53 mutation status	WT	MUT	0.35	0.24	0.51	<1e-04	0.50	0.33	0.75	0.00098

a Low, mid and high levels refers to the expression level of miR-30c-2-3p based on distribution quartile.
b Reference.
c Hazard ratio.
d Lower confidence limit.
e Upper confidence limit.
f Tumor grade 1, 2, 3 refers to differentiation status, +/– indicate presence or absence of receptor.

These groups were then tested for association between miR-30c-2-3p and categorical clinicopathological factors. Indeed, we found a significant correlation between low miR-30c-2-3p expression levels and the presence of adverse clinicopathological feature (Supplementary Table 7). Disease-specific survival was significantly better in patients with medium (HR = 0.64, P = 0.005) and high (HR = 0.43, P < 0.0001) miR-30c-2-3p expression levels in univariate analysis (Table 1). However, in multivariate analysis miR-30c-2-3p expression did not remain an independent prognostic factor of disease specific survival in breast cancer patients (Table 1).

3.7. TRADD *and* CCNE1 *are upregulated in ER negative breast cancer patients*

Next, we wanted to further ascertain the relationship between *TRADD, CCNE1* and miR-30c-2-3p in breast cancer patients. Again using the METABRIC gene expression data (Curtis et al., 2012), we found *TRADD* and *CCNE1* expression to be significantly upregulated in ER-patients compared to ER+ breast cancer patients (Figure 6A and B), which corroborated with our *in vitro* findings. Subsequently, we analyzed the expression of miR-30c-2-3p among the different subtypes of breast cancer in METABRIC study (Dvinge et al., 2013). We found the miRNA to be significantly lower expressed in the basal and HER2 positive subtypes which have a poorer outcome (Engstrom et al., 2013) compared to the luminal subtypes with a more favorable outcome (Figure 6C). Finally, we assessed the association of miR-30-c-2-3p expression with survival of breast cancer patients (Dvinge et al., 2013). We found elevated miR-30c-2-3p correlated with better survival based on miRNA expression level distribution quartiles into low (<Q1), medium (Q1–Q3) and high (>Q3) expression groups (Figure 6D), further supporting a tumor suppressive role for this miRNA.

4. Discussion

In this study we present miR-30c-2-3p as a novel regulator of NF-κB signaling and cell cycle progression. The role of NF-κB signaling in cancer development and progression, specifically in breast cancer is well studied (DiDonato et al., 2012; Karin et al., 2002; Mantovani et al., 2008; Piao et al., 2014; Rivas et al., 2008; Singh et al., 2007). Here we have elucidated the molecular mechanism behind NF-κB regulation by miR-30c-2-3p and its implications in breast cancer. Moreover, miR-30c-2-3p negatively regulates cell cycle progression which is frequently aberrant in cancer cells. As a result, miR-30c-2-3p mediates effects on important hallmarks of cancer cells (Hanahan and Weinberg, 2011) like proliferation, invasion, and inflammation.

Our results are in line with a previous study on the tumor suppressive role of miR-30c-2-3p in ovarian cancer, where Jia and co-workers had identified BCL9 as a direct target gene of the miRNA and outlined its anti-proliferative effect (Jia et al., 2011). Another study in clear cell renal cell carcinoma found that lower expression of miR-30c-2-3p is associated with increased tumor growth through enhanced activity of hypoxia inducible factor (*HIF2α*), another direct target of miR-30c-2-3p (Mathew et al., 2013). Both of these targets of miR-30c-2-3p were not significantly downregulated in our gene expression data set generated in MDA-MB-231 cells, underlining tissue specific regulation. The precursor pre-miR-30c-2 is processed to form mature hsa-miR-30c-5p (miR-30c) and hsa-miR-30c-2-3p (mir-30c-2*) from the 5′ and 3′ arms of the precursor miRNA, respectively. miR-30c-5p has been shown to reduce chemoresistance in breast tumors through direct targeting of the cytoskeleton gene *TWF1* and indirect regulation of *IL-11* (Bockhorn et al., 2013). This is an interesting finding as the two mature forms of the same precursor miRNA have different seed sequences and thus different target genes.

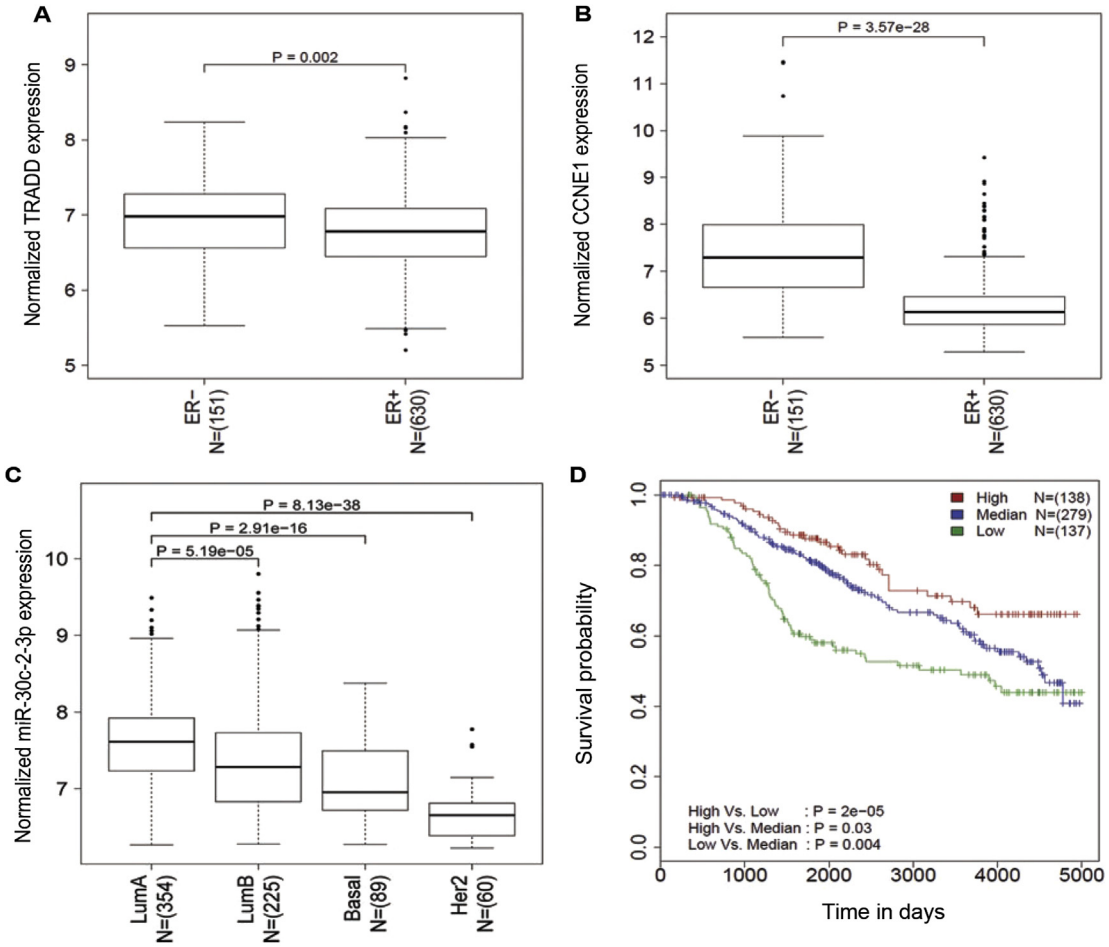

Figure 6 – Expression of *TRADD*, *CCNE1* and miR-30c-2-3p in breast cancer patients. (A & B) The mRNA expression of both *TRADD* and *CCNE1* is significantly higher in ER-breast cancer patients compared to ER+ breast cancer patients (Curtis et al., 2012). (C) Expression levels of miR-30c-2-3p across different molecular subtypes of breast cancer patients (Dvinge et al., 2013). (D) Higher expression (red) of miR-30c-2-3p is associated with better long term disease specific survival in breast cancer patients with a follow up of over 13 years (Dvinge et al., 2013).

However, both miR-30c-5p and miR-30c-2-3p coordinately function as important regulators of gene expression and show a positive correlation in breast cancer patients (Supplementary Figure 10) in METABRIC study (Dvinge et al., 2013).

We identified *TRADD* as a direct target gene of miR-30c-2-3p. TRADD, on the one hand plays an important role in the TNF-α induced pro-inflammatory response by interacting with TNFR1 (Wilson et al., 2009). On the other hand, TNF-α can also initiate apoptosis and necrosis, through recruitment of the FADD protein (Hsu et al., 1996). However, TNF-α induced NF-κB activation is attenuated in TRADD deficient cells as has been shown by somatic knockout of *TRADD* in a human B cell line (Schneider et al., 2008), demonstrating the importance of TRADD in TNFR1 signaling. Furthermore, cytoplasmic TRADD has been shown to confer poor prognosis in glioblastoma through NF-κB activation (Chakraborty et al., 2013). We confirmed that TRADD is required for TNF-α induced activation of NF-κB signaling as well as its effect on known NF-κB induced proinflammatory cytokines *IL8*, *IL6* and *CXCL1*. These cytokines contribute to the shaping of the tumor microenvironment by promoting inflammation in most solid tumors, including breast cancer (Erez et al., 2013; Mantovani et al., 2008). miR-30c-2-3p potently inhibited the expression of these inflammatory cytokines in MDA-MB-231 cells suggesting that this miRNA is a central factor in the regulation of tumor inflammatory processes that is executed via its direct target *TRADD*. Furthermore, TRADD is known to play a role in regulating TLR signaling by formation of a complex with TLR4 upon LPS stimulation (Chen et al., 2008). We could show that miR-30c-2-3p can inhibit LPS induced NF-κB signaling by direct targeting of *TRADD*. TLR signaling has been shown to support tumor growth by activating inflammatory response and supporting oncogenesis in the breast (Ahmed et al., 2013). Matrix metalloproteinases (MMPs), and specifically MMP-9, have been shown to have potent basement degrading capability (Kessenbrock et al., 2010). We observed expression of *MMP-9* was indeed downregulated upon miR-30c-2-3p overexpression as well as by knockdown of TRADD upon TNF-α stimulation. Hence, the observed block of invasion by miR-30c-2-3p could be partially explained through the direct targeting of *TRADD*.

We found *CCNE1* as another direct target of miR-30c-2-3p which could potentially explain the reduced proliferation

capacity we observed. CCNE1 is a cell cycle regulatory protein that, along with CDK2, drives the cell cycle from G1 to S phase (Rosenberg et al., 2001). CCNE1 is frequently deregulated in breast cancer and is associated with poor overall survival (Keyomarsi et al., 2002). Using the dataset from the METABRIC study (Curtis et al., 2012) we found both *CCNE1* and *TRADD* to be upregulated in ER-breast cancer patients compared to ER+ patients. This is in line with the finding that miR-30c-2-3p is lower expressed in the ER-patients.

In summary, we have shown that miR-30c-2-3p cooperatively regulates cytokine expression, invasion and cell proliferation in breast cancer cells by modulating *TRADD* and *CCNE1* gene expression. The synergistic regulation of NF-κB signaling and cell cycle regulation could potentially be exploited used for therapeutic intervention by upregulating miR-30c-2-3p in breast tumors.

Conflicts of interests

The authors have no conflicts of interests to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2015.01.008.

REFERENCES

Ahmed, A., Redmond, H.P., Wang, J.H., 2013. Links between toll-like receptor 4 and breast cancer. Oncoimmunology 2, e22945.

Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.

Bera, A., Zhao, S., Cao, L., Chiao, P.J., Freeman, J.W., 2013. Oncogenic K-Ras and loss of Smad4 mediate invasion by activating an EGFR/NF-kappaB Axis that induces expression of MMP9 and uPA in human pancreas progenitor cells. PloS One 8, e82282.

Biswas, D.K., Shi, Q., Baily, S., Strickland, I., Ghosh, S., Pardee, A.B., Iglehart, J.D., 2004. NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. Proc. Natl. Acad. Sci. U S A 101, 10137–10142.

Bockhorn, J., Dalton, R., Nwachukwu, C., Huang, S., Prat, A., Yee, K., Chang, Y.F., Huo, D., Wen, Y., Swanson, K.E., Qiu, T., Lu, J., Park, S.Y., Dolan, M.E., Perou, C.M., Olopade, O.I., Clarke, M.F., Greene, G.L., Liu, H., 2013. MicroRNA-30c inhibits human breast tumour chemotherapy resistance by regulating TWF1 and IL-11. Nat. Commun. 4, 1393.

Cancer Genome Atlas, N, 2012. Comprehensive molecular portraits of human breast tumours. Nature 490, 61–70.

Cao, Y., Karin, M., 2003. NF-kappaB in mammary gland development and breast cancer. J. Mammary Gland Biol. Neoplasia 8, 215–223.

Chakraborty, S., Li, L., Tang, H., Xie, Y., Puliyappadamba, V.T., Raisanen, J., Burma, S., Boothman, D.A., Cochran, B., Wu, J., Habib, A.A., 2013. Cytoplasmic TRADD confers a worse prognosis in glioblastoma. Neoplasia 15, 888–897.

Chen, N.J., Chio, I.I., Lin, W.J., Duncan, G., Chau, H., Katz, D., Huang, H.L., Pike, K.A., Hao, Z., Su, Y.W., Yamamoto, K., de Pooter, R.F., Zuniga-Pflucker, J.C., Wakeham, A., Yeh, W.C., Mak, T.W., 2008. Beyond tumor necrosis factor receptor: TRADD signaling in toll-like receptors. Proc. Natl. Acad. Sci. U S A 105, 12429–12434.

Coope, H.J., Atkinson, P.G., Huhse, B., Belich, M., Janzen, J., Holman, M.J., Klaus, G.G., Johnston, L.H., Ley, S.C., 2002. CD40 regulates the processing of NF-kappaB2 p100 to p52. EMBO J. 21, 5375–5385.

Cullen, B.R., 2004. Transcription and processing of human microRNA precursors. Mol. Cell 16, 861–865.

Curtis, C., Shah, S.P., Chin, S.F., Turashvili, G., Rueda, O.M., Dunning, M.J., Speed, D., Lynch, A.G., Samarajiwa, S., Yuan, Y., Graf, S., Ha, G., Haffari, G., Bashashati, A., Russell, R., McKinney, S., Group, M., Langerod, A., Green, A., Provenzano, E., Wishart, G., Pinder, S., Watson, P., Markowetz, F., Murphy, L., Ellis, I., Purushotham, A., Borresen-Dale, A.L., Brenton, J.D., Tavare, S., Caldas, C., Aparicio, S., 2012. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 486, 346–352.

DiDonato, J.A., Mercurio, F., Karin, M., 2012. NF-kappa B and the link between inflammation and cancer. Immunological Rev. 246, 379–400.

Dvinge, H., Git, A., Graf, S., Salmon-Divon, M., Curtis, C., Sottoriva, A., Zhao, Y., Hirst, M., Armisen, J., Miska, E.A., Chin, S.F., Provenzano, E., Turashvili, G., Green, A., Ellis, I., Aparicio, S., Caldas, C., 2013. The shaping and functional consequences of the microRNA landscape in breast cancer. Nature 497, 378–382.

Dweep, H., Sticht, C., Pandey, P., Gretz, N., 2011. miRWalk–database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J. Biomed. Inform. 44, 839–847.

Engstrom, M.J., Opdahl, S., Hagen, A.I., Romundstad, P.R., Akslen, L.A., Haugen, O.A., Vatten, L.J., Bofin, A.M., 2013. Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients. Breast Cancer Res. Treat. 140, 463–473.

Erez, N., Glanz, S., Raz, Y., Avivi, C., Barshack, I., 2013. Cancer associated fibroblasts express pro-inflammatory factors in human breast and ovarian tumors. Biochem. Biophys. Res. Commun. 437, 397–402.

Filipowicz, W., Jaskiewicz, L., Kolb, F.A., Pillai, R.S., 2005. Post-transcriptional gene silencing by siRNAs and miRNAs. Curr. Opin. Struct. Biol. 15, 331–341.

Friedman, R.C., Farh, K.K., Burge, C.B., Bartel, D.P., 2009. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 19, 92–105.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.
Hayden, M.S., Ghosh, S., 2004. Signaling to NF-kappa B. Genes Dev. 18, 2195–2224.
Hayden, M.S., Ghosh, S., 2014. Regulation of NF-kappa B by TNF family cytokines. Semin. Immunol. 26, 253–266.
Hsu, H., Shu, H.B., Pan, M.G., Goeddel, D.V., 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84, 299–308.
Hynes, N.E., Stoelzle, T., 2009. Key signalling nodes in mammary gland development and cancer: Myc. Breast Cancer Res. 11, 210.
Jaattela, M., Pinola, M., Saksela, E., 1991. Heat shock inhibits the cytotoxic action of TNF-alpha in tumor cells but does not alter its noncytotoxic actions in endothelial and adrenal cells. Lymphokine Cytokine Res. 10, 119–125.
Jia, W., Eneh, J.O., Ratnaparkhe, S., Altman, M.K., Murph, M.M., 2011. MicroRNA-30c-2* expressed in ovarian cancer cells suppresses growth factor-induced cellular proliferation and downregulates the oncogene BCL9. Mol. Cancer Res. 9, 1732–1745.
Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., 2014. Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res. 42, D199–D205.
Karin, M., Ben-Neriah, Y., 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa] B activity. Annu. Rev. Immunol. 18, 621–663.
Karin, M., Cao, Y., Greten, F.R., Li, Z.W., 2002. NF-kappaB in cancer: from innocent bystander to major culprit. Nat. Rev. Cancer 2, 301–310.
Keklikoglou, I., Koerner, C., Schmidt, C., Zhang, J.D., Heckmann, D., Shavinskaya, A., Allgayer, H., Guckel, B., Fehm, T., Schneeweiss, A., Sahin, O., Wiemann, S., Tschulena, U., 2012. MicroRNA-520/373 family functions as a tumor suppressor in estrogen receptor negative breast cancer by targeting NF-kappaB and TGF-beta signaling pathways. Oncogene 31, 4150–4163.
Kessenbrock, K., Plaks, V., Werb, Z., 2010. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141, 52–67.
Keyomarsi, K., Tucker, S.L., Buchholz, T.A., Callister, M., Ding, Y., Hortobagyi, G.N., Bedrosian, I., Knickerbocker, C., Toyofuku, W., Lowe, M., Herliczek, T.W., Bacus, S.S., 2002. Cyclin E and survival in patients with breast cancer. N. Engl. J. Med. 347, 1566–1575.
Korner, C., Keklikoglou, I., Bender, C., Worner, A., Munstermann, E., Wiemann, S., 2013. MicroRNA-31 sensitizes human breast cells to apoptosis by direct targeting of protein kinase C epsilon (PKCepsilon). J. Biol. Chem. 288, 8750–8761.
Li, Y., Ohms, S.J., Sun, C., Fan, J., 2013. NF-kappaB controls Il2 and Csf2 expression during T cell development and activation process. Mol. Biol. Rep. 40, 1685–1692.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402–408.
Mantovani, A., Allavena, P., Sica, A., Balkwill, F., 2008. Cancer-related inflammation. Nature 454, 436–444.
Mathew, L.K., Lee, S.S., Skuli, N., Rao, S., Keith, B., Nathanson, K.L., Lal, P., Simon, M.C., 2013. Restricted expression of miR-30c-2-3p and miR-30a-3p in clear cell renal cell carcinomas enhances HIF2 alpha activity. Cancer Discov., 53–60.
Nakshatri, H., Bhat-Nakshatri, P., Martin, D.A., Goulet Jr., R.J., Sledge Jr., G.W., 1997. Constitutive activation of NF-kappa B during progression of breast cancer to hormone-independent growth. Mol. Cell Biol. 17, 3629–3639.
Oida, K., Matsuda, A., Jung, K., Xia, Y., Jang, H., Amagai, Y., Ahn, G., Nishikawa, S., Ishizaka, S., Jensen-Jarolim, E., Matsuda, H., Tanaka, A., 2014. Nuclear factor-k B plays a critical role in both intrinsic and acquired resistance against endocrine therapy in human breast cancer cells. Scientific Rep. 4, 4057.
Park, B.K., Zhang, H., Zeng, Q., Dai, J., Keller, E.T., Giordano, T., Gu, K., Shah, V., Pei, L., Zarbo, R.J., McCauley, L., Shi, S., Chen, S., Wang, C.Y., 2007. NF-kappa B in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF. Nat. Med. 13, 62–69.
Parker, J.S., Mullins, M., Cheang, M.C., Leung, S., Voduc, D., Vickery, T., Davies, S., Fauron, C., He, X., Hu, Z., Quackenbush, J.F., Stijleman, I.J., Palazzo, J., Marron, J.S., Nobel, A.B., Mardis, E., Nielsen, T.O., Ellis, M.J., Perou, C.M., Bernard, P.S., 2009. Supervised risk predictor of breast cancer based on intrinsic subtypes. J. Clin. Oncol. 27, 1160–1167.
Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S.X., Lonning, P.E., Borresen-Dale, A.L., Brown, P.O., Botstein, D., 2000. Molecular portraits of human breast tumours. Nature 406, 747–752.
Piao, H.L., Yuan, Y., Wang, M., Sun, Y., Liang, H., Ma, L., 2014. Alpha-catenin acts as a tumour suppressor in E-cadherin-negative basal-like breast cancer by inhibiting NF-kappa B signalling. Nat. Cell Biol. 16, 245–254.
Remouchamps, C., Boutaffala, L., Ganeff, C., Dejardin, E., 2011. Biology and signal transduction pathways of the Lymphotoxin-alphabeta/LTbetaR system. Cytokine Growth Factor Rev. 22, 301–310.
Rivas, M.A., Carnevale, R.P., Proietti, C.J., Rosemblit, C., Beguelin, W., Salatino, M., Charreau, E.H., Frahm, I., Sapia, S., Brouckaert, P., Elizalde, P.V., Schillaci, R., 2008. TNF alpha acting on TNFR1 promotes breast cancer growth via p42/P44 MAPK, JNK, Akt and NF-kappa B-dependent pathways. Exp. Cell Res. 314, 509–529.
Rosenberg, E., Demopoulos, R.I., Zeleniuch-Jacquotte, A., Yee, H., Sorich, J., Speyer, J.L., Newcomb, E.W., 2001. Expression of cell cycle regulators p57(KIP2), cyclin D1, and cyclin E in epithelial ovarian tumors and survival. Hum. Pathol. 32, 808–813.
Schneider, F., Neugebauer, J., Griese, J., Liefold, N., Kutz, H., Briseno, C., Kieser, A., 2008. The viral oncoprotein LMP1 exploits TRADD for signaling by masking its apoptotic activity. PLoS Biol. 6, e8.
Shen, R.R., Hahn, W.C., 2011. Emerging roles for the non-canonical IKKs in cancer. Oncogene 30, 631–641.
Siegel, R., Naishadham, D., Jemal, A., 2012. Cancer statistics, 2012. CA Cancer J. Clin. 62, 10–29.
Singh, S., Shi, Q., Bailey, S.T., Palczewski, M.J., Pardee, A.B., Iglehart, J.D., Biswas, D.K., 2007. Nuclear factor-kappaB activation: a molecular therapeutic target for estrogen receptor-negative and epidermal growth factor receptor family receptor-positive human breast cancer. Mol. Cancer Ther. 6, 1973–1982.
Sorlie, T., 2004. Molecular portraits of breast cancer: tumour subtypes as distinct disease entities. Eur. J. Cancer 40, 2667–2675.
Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Lonning, P.E., Borresen-Dale, A.L., 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. U S A 98, 10869–10874.
Spruck, C.H., Won, K.A., Reed, S.I., 1999. Deregulated cyclin E induces chromosome instability. Nature 401, 297–300.

Takada, Y., Gillenwater, A., Ichikawa, H., Aggarwal, B.B., 2006. Suberoylanilide hydroxamic acid potentiates apoptosis, inhibits invasion, and abolishes osteoclastogenesis by suppressing nuclear factor-kappaB activation. J. Biol. Chem. 281, 5612–5622.

Uhlmann, S., Mannsperger, H., Zhang, J.D., Horvat, E.A., Schmidt, C., Kublbeck, M., Henjes, F., Ward, A., Tschulena, U., Zweig, K., Korf, U., Wiemann, S., Sahin, O., 2012. Global microRNA level regulation of EGFR-driven cell-cycle protein network in breast cancer. Mol. Syst. Biol. 8, 570.

Valencia-Sanchez, M.A., Liu, J., Hannon, G.J., Parker, R., 2006. Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev. 20, 515–524.

Walczak, H., 2011. TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. Immunol. Rev. 244, 9–28.

Ward, A., Shukla, K., Balwierz, A., Soons, Z., Konig, R., Sahin, O., Wiemann, S., 2014. MicroRNA-519a is a novel oncomir conferring tamoxifen resistance by targeting a network of tumour-suppressor genes in ER+ breast cancer. J. Pathol. 233 (4), 368–379.

Wilson, N.S., Dixit, V., Ashkenazi, A., 2009. Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat. Immunol. 10, 348–355.