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Targeted therapy for triple-negative breast cancer

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Chapter 3

Blockage of cdc7/CDK9 signalling sensitises triple-negative breast cancer to EGFR-targeted therapy

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ABSTRACT

Background

The effective treatment of triple-negative breast cancer (TNBC) remains a profound clinical challenge. Despite frequent EGFR overexpression and reliance on downstream signalling pathways in TNBC, resistance to EGFR-tyrosine kinase inhibitors (TKIs) remains endemic. Therefore, the identification of targeted agents, which synergise with current therapeutic options, is paramount.

Methods

Compound-based, high-throughput, proliferation screening was used to profile the response of TNBC cell lines to EGFR-TKIs; western blotting and siRNA transfection being used to examine the effect of inhibitors on EGFR-mediated signal transduction and cellular dependence on such pathways, respectively. A kinase inhibitor combination screen was used to identify compounds that synergised with EGFR-TKIs in TNBC, utilising sulphorhodamine B (SRB) assay as read-out for proliferation. The impact of drug combinations on cell cycle arrest, apoptosis and signal transduction was assessed using flow cytometry, automated live-cell imaging and western blotting, respectively. RNA-sequencing was employed to unravel transcriptomic changes elicited by this synergistic combination and to permit identification of the signalling networks most sensitive to co-inhibition.

Results

We demonstrate that dual Cdc7/CDK9 inhibitor PHA-767491 synergises with multiple EGFR-TKIs (lapatinib, erlotinib and gefitinib) to overcome resistance to EGFR-targeted therapy in various TNBC cell lines. Combined inhibition of EGFR and Cdc7/

CDK9 resulted in reduced cell proliferation, accompanied by induction of apoptosis, G2-M cell cycle-arrest, inhibition of DNA replication and abrogation of CDK9-mediated transcriptional elongation, in contrast to mono-inhibition. Moreover, high expression of *cdc7* and RNA polymerase II Subunit A (POLR2A), the direct target of CDK9, is significantly correlated with poor metastasis-free survival in a cohort of breast cancer patients. RNA-sequencing revealed marked down-regulation of pathways governing proliferation, transcription and cell survival in TNBC cells treated with the combination of an EGFR-TKI and a dual Cdc7/CDK9 inhibitor. A number of genes enriched in these down-regulated pathways are associated with poor metastasis-free survival in TNBC.

Conclusions

Our results highlight that dual inhibition of Cdc7 and CDK9 is a potential strategy for targeting TNBC resistant to EGFR-TKIs.

Key words: CDK9/Cdc7 inhibition, EGFR-targeted therapy, drug resistance, triple-negative breast cancer

BACKGROUND

Triple-negative breast cancer (TNBC) is a notoriously aggressive, heterogeneous disease defined as lacking expression of oestrogen receptor (ER) and/or progesterone receptor (PR) as well as amplification of human epidermal growth factor receptor 2 (HER2), respectively¹. Although TNBC only constitutes approximately 15-20% of breast cancer cases, it is disproportionately responsible for breast cancer-associated deaths and carries a dismal prognosis, compared with hormone receptor-positive (HR+) breast cancers²⁻⁴. For patients with HR+ breast cancer, endocrine therapy targeting ER is available in the form of aromatase inhibitors and selective oestrogen receptor modulators (e.g. tamoxifen) and other antagonists¹. Contrastingly, no effective targeted therapy which exploits the molecular properties of tumour cells exists for TNBC patients; clinical trials of targeted agents in TNBC have been disappointing^{5,6}. Consequently, aggressive chemotherapy, radiotherapy and surgery remain the mainstay treatments⁷. Furthermore, patients who develop resistance to treatment, or who do not respond to treatment whatsoever, follow an aggressive clinical course characterised by metastasis and a higher 5-year mortality post-diagnosis⁸. Further complicating the treatment of TNBC is the degree of genetic heterogeneity observed in this disease. By analysing the gene expression profiles of TNBC cases, *Lehmann et al* sub-classified TNBC into six different molecular subtypes: mesenchymal (M), mesenchymal stem-like (MSL), luminal androgen receptor-positive (LAR), immunomodulatory (IM), basal-like 1 (BL1) and basal-like 2 (BL2)⁹. Most importantly, these subtypes exhibit dissimilar drug-sensitivity profiles, resulting in varied clinical responses^{9,10}. The nature of TNBC clearly necessitates a more tailored approach to treatment, one which exploits the unique oncogenic addictions present. For

chemotherapy-resistant TNBC patients, the development of targeted therapeutics which synergise with current treatment options to overcome resistance is therefore paramount.

Epidermal growth factor receptor (EGFR; also known as ERBB1/HER1) is often expressed at higher levels in triple-negative tumours than in HR+ tumours¹¹, though expression levels vary, with up to ~80% of TNBC cases reported as being EGFR+¹². EGFR amplification has also been reported to occur in a substantial proportion of TNBC cases^{13–15} with EGFR overexpression associated with a much poorer prognosis in general⁸. Furthermore, EGF signalling is highly enriched in the basal and mesenchymal TNBC subtypes⁹. EGFR therefore represents a *bona fide* drug target in triple-negative tumours. Various EGFR inhibitors have been developed, most notably anti-EGFR monoclonal antibodies (e.g. cetuximab) and EGFR-tyrosine kinase inhibitors (EGFR-TKIs) (e.g. erlotinib, gefitinib and lapatinib)¹⁶. Despite these efforts, EGFR-TKI single treatment has performed poorly in clinical trials for TNBC patients with advanced or metastatic breast cancer, despite clear inhibition of EGFR^{17,18} suggesting bypass inhibition of EGFR-related signalling in TNBC tumors^{19,20}. Nonetheless, EGFR-TKIs have shown more promising results as combination therapies in TNBC²¹, perhaps indicating that monotherapeutic inhibition of EGFR is insufficient to shut down the myriad signalling pathways responsible for promoting aberrant proliferation and survival.

Here we demonstrate that multiple EGFR-TKIs synergise with the dual cdc7/CDK9 inhibitor PHA-767491 in a panel of TNBC cell lines resistant to EGFR-TKIs. This combination inhibited cell proliferation, induced apoptosis and G2-M arrest and down-regulated components critical to cell cycle progression, DNA-replication and transcription, thereby reversing resistance to EGFR-TKIs. Therefore, targeting

deficiencies in regulation of the cell cycle and DNA-replication in conjunction with transcriptional addiction downstream of growth factor pathways may constitute a powerful therapeutic opportunity for this difficult-to-treat breast cancer subtype.

MATERIALS & METHODS

Cell culture

All cell lines were maintained in RPMI-1640 medium (Gibco, ThermoFisher Scientific, Breda, The Netherlands) supplemented with 10% FBS (Thermo Fisher Scientific; 10270106) and 25 IU/ml Penicillin and 25 µg/ml Streptomycin (ThermoFisher Scientific; 15070-063). Cells were cultured in a humidified incubator at 37°C, 5% CO₂.

Antibodies and kinase inhibitors

The primary antibodies against pEGFR (Y1173, #4407), pERK1/2 (T202/Y204, #9101), ERK1/2 (#4695), pAKT (S473, #9271), AKT (#9272), MCM2 (#3619), pRNA-II (S2/5; #4735), RNA-II (#2629), p-pRb (S780, # 9307) were commercially supplied from Cell Signaling TECHNOLOGY®, EGFR (sc-03), pRb (sc-102), CDK4 (sc-601), Cyclin D1 (sc-20044) from Santa Cruz BIOTECHNOLOGY®, Cdc7 (ab10535) and pMCM2 (S40/41; ab70371) from Abcam®, and Tubulin (T-9026) from Sigma®. Secondary antibodies included Cy5-conjugated anti-mouse and horseradish peroxidase (HRP) anti-mouse or anti-rabbit (Jackson ImmunoResearch). Individual kinase inhibitors lapatinib (S2111), gefitinib (S1025), erlotinib (S7786) and PHA-767491 (S2742), plus the 273 kinase inhibitor library (L1200) were purchased from Selleckchem® and dissolved in DMSO solution at 10mM.

Kinase inhibitor treatment and drug combination screen

Cells were seeded into 96-well plates at the appropriate densities. The following day, cells were treated with individual kinase inhibitors in dose range as indicated. Vehicle DMSO (1:1000) was used as control. For the kinase inhibitor library screen, cells were screened in duplicate against the kinase inhibitor library containing 273 kinase inhibitors at concentration of 1 μM alone, or the 1 μM library inhibitors in combination with lapatinib at 3.16 μM , since this concentration effectively inhibited EGFR phosphorylation in all cell lines tested and since studies have shown that levels of lapatinib in patient tumours vary between 1-12 μM depending on dosing schedule²². After 4-day treatment, proliferation was evaluated by sulphorhodamine B (SRB) colorimetric assay²³, and analysed by % of control cell growth = (mean sample OD – mean 0-day OD) / (mean control OD – mean 0-day OD) \times 100. To assess synergistic interaction of combined drugs, combination index (CI) analysis^{24,25} was performed, using the formula 'CI = (D)1/(Dx)1 + (D)2/(Dx)2'. (D)1 and (D)2 are respective combination doses of two compounds that yield an effect of 50% of proliferation inhibition, with (Dx)1 and (Dx)2 being the corresponding single doses for either compound that results in the same effect, which is by definition the IC₅₀ of each compound. CI values less than 1 (CI < 1), equal to 1 (CI = 1) or greater than 1 (CI > 1), indicate synergy, additivity or antagonism, respectively.

Western blotting

Cells were seeded in 6-well plates at the appropriate density. For stimulation/starvation assays, medium was refreshed with serum-free medium (SFM) the following day and cells were starved overnight. Thereafter, cells were pre-treated with drug solutions for 4 hours, then stimulated with 100 ng/ml EGF (Sigma; E9644) for 5

minutes in SFM. For time-course exposures to drugs, cells were treated with drug solutions prepared in complete medium. Cell lysates were harvested at the indicated time points in RIPA lysis buffer with 1:100 Protease Inhibitor Cocktail (Sigma; P8340). Cellular proteins were denatured in sample buffer containing 10% β -mercaptoethanol, loaded with 30 μ g/lane into 7.5% polyacrylamide gels, resolved using SDS-PAGE and subsequently transferred to PVDF membranes (Merck Chemicals; IPVH00010) overnight. PVDF membranes were then blocked with 5% BSA-TBST (Tris-buffered saline 0.05 % Tween-20) and subsequently incubated at 4°C overnight with appropriate primary antibodies. The following day, membranes were incubated for 1 hour with HRP- or Cy5-conjugated secondary antibodies and chemiluminescence or fluorescence was detected using the Las4000 (GE Healthcare).

Annexin-V staining

Cells were seeded overnight in 96-well μ CLEAR plates (Corning) at appropriate densities, then treated with drug solutions at indicated concentrations. At 24, 48 or 72 hours post-treatment, cells were stained with Hoechst 33258 (1:10,000) and Annexin-V (1:1000) for 45 minutes at 37°C, 5% CO₂ before being imaged using BD Pathway 855 Microscope (BD Biosciences). Annexin-V staining was quantified using Cell Profiler software.

Cell cycle flow cytometry analysis

Cells were seeded in 6-well plates at the appropriate density. 24 or 48 hours post-treatment all cells were harvested, re-suspended in ice-cold 200 μ l 1 mM EDTA-PBS and 800 μ l 100% Ethanol, and stored at -20°C before being centrifuged at 1000 rpm

at 4°C. Cells were then re-suspended in 1 ml PBS and rehydrated for 15 minutes. After being spun at 1000rpm for 5 minutes at room temperature, the pellet was re-suspended in 250µl 3mM DAPI (Sigma, 10236276001) staining buffer (100 µM Tris pH7.4, 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂), incubated for 15 minutes at room temperature in the dark, followed by filtration through 70µm EASYstrainer filters and analysed using FACS Conto II (*BD Biosciences*). Data were analysed using FlowJo V10.

siRNA transfection

Cells were seeded in 96-well plates at the appropriate density. For each siRNA transfection, 50 nM siGENOME siRNAs (Dharmacon) were transfected into cells per 96-well using INTERFERin transfection reagent (Polyplus; 409-50). The following day medium was refreshed. 48 hours post-transfection, cells were either lysed for western blot to confirm knockdown or treated with drugs for the appropriate duration as described, then fixed for SRB proliferation assay.

Clinical evaluation of candidate target genes

The clinical relevance of CDC7, POLR2A and CDK9 was evaluated using in-house gene expression and metastasis-free survival data of 123 lymph node-negative, non-neoadjuvantly treated, oestrogen receptor-negative (ER-neg) primary breast cancer patients. The clinical relevance of synergy-related candidate genes was evaluated using in-house as well as publicly available gene expression and MFS data of lymph node-negative, non-neoadjuvantly treated primary breast cancer patients, leading to a cohort of 142 triple-negative patients. Data were gathered from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) entries GSE2034, GSE5327, GSE2990,

GE7390 and GSE11121, with all data available on Affymetrix U133A chip. Raw.cel files were processed using fRMA parameters (median polish)²⁶ after which batch effects were corrected using ComBat²⁷.

Transcriptome RNA-Sequencing and pathway integration analysis

Cells were seeded overnight in 6-well plates and treated in triplicate for 6 hours with individual or combined kinase inhibitors at indicated concentrations, or vehicle. RNA was isolated with RNeasy Plus Mini Kit as described by manufacturer (QIAGEN, Cat. 74136). Transcriptome RNA-Sequencing (RNA-Seq) was performed using Illumina high-throughput RNA sequencing. DNA libraries were prepared from the samples with the TruSeq Stranded mRNA Library Prep Kit. The DNA libraries were sequenced according to the Illumina TruSeq v3 protocol on an Illumina HiSeq2500 sequencer. Paired-end reads were generated of 100 base-pairs in length were generated. Alignment was performed against the human GRCh38 reference genome using the STAR aligner (version 2.4.2a). Marking duplicates, sorting and indexing were performed using Sambamba (version 0.6.6). Gene expression was quantified using the FeatureCounts software (version 1.4.6) based on the ENSEMBL gene annotation for GRCH38 (release 84). RNA-Seq data were normalised by TMM using EdgeR's normalisation-factor²⁸, followed by quantile normalisation and presented in Log₂ fold change (Log₂ FC) scales. Genes with significant down- or up-regulation (Log₂ FC ≥ |0.5|) under indicated conditions were analysed by web-based functional analysis tool Ingenuity Pathway Analysis (IPA, QIAGEN®) to visualise and annotate their biological functions and pathways.

Statistical Analyses

All statistical analyses, where appropriate, were performed in GraphPad Prism software version 7.0. One-way ANOVA multiple comparison test with Tukey's post-hoc test was applied with p-values less than 0.05 considered as statistically significant.

RESULTS

TNBC cells are resistant to EGFR-TKIs

EGFR is expressed at higher levels in TNBC tumours compared to ER-positive BC tumours (Figure 1a); also in human basal A and basal B TNBC cell lines there is higher EGFR expression than in human luminal cell lines (Figure 1b). Therefore, we sought to systematically elucidate the response of TNBC to a broad range of different EGFR kinase inhibitors. A panel of TNBC cell lines with varying EGFR expression (Figure 1c) was screened against 24 EGFR-TKIs (Figure 1d). 12 cell lines (> 57%) could be classified as refractory to almost all 24 EGFR-TKIs; only HCC1806 was highly sensitive to most EGFR-TKIs, the remainder having variable sensitivity to EGFR-TKIs (Figure 1d). Next, three TNBC cell lines highly resistant to EGFR-TKIs, Hs578T, BT549 and SKBR7, and one sensitive cell line, HCC1806, were selected for further evaluation. The proliferation of Hs578T, BT549 and SKBR7 cells was unaffected by dual EGFR-HER2 inhibitor lapatinib, up to the concentration of 10 μ M (Figure 1e). Concordantly, lapatinib failed to significantly induce apoptosis in these cell lines at 3.16 μ M (Figure S1a). In contrast, HCC1806 cells displayed enhanced growth inhibition in response to lapatinib (IC₅₀ ~100 nM; Figure 1e) with significantly increased Annexin-V apoptotic signal (Figure S1a). Regardless of their response to lapatinib, all these cell lines maintained functional EGFR-mediated signal

transduction, with prominent phosphorylation of EGFR (Y1173) and downstream components AKT (S473) and ERK1/2 (T202/Y204) in response to EGF stimulation (Figure 1f), indicating that resistance was not due to absence of a functionally intact EGFR pathway. In response to lapatinib, EGFR phosphorylation was completely inhibited in all cell lines (Figure 1f). However, EGF-induced ERK activation persisted in all lapatinib-resistant cell lines, with AKT phosphorylation also unaffected in Hs578T and BT549 cells. These data suggest that these resistant cells are capable of bypassing EGFR-kinase inhibition through the activation of downstream proliferative pathways. Despite the lack of impact of EGFR-TKIs on TNBC proliferation, siRNA-mediated silencing of EGFR and downstream components, including ERK2 and FRAP1 (mTOR), led to a significant reduction in cell proliferation, supporting the notion TNBC cells depend to a certain extent on EGFR-mediated signalling for their proliferation (Figure S1b).

Kinase inhibitor combination screening identifies a dual Cdc7/CDK9 inhibitor PHA-767491 which synergises with lapatinib in TNBC.

Next, we sought to identify compounds which synergise with lapatinib by performing a combinatorial kinase inhibitor screen in Hs578T cells treated with 273 kinase inhibitors at 1 μM with or without 3.16 μM lapatinib. Most notably, amongst a number of compounds which augmented the response of TNBC cells to EGFR inhibition, the dual Cdc7/CDK9 inhibitor PHA-767491 inhibited proliferation by >90% compared to control or either monotherapy (Figure 2a). Subsequent dose response experiments revealed that combinations of 1-3.16 μM PHA-767491 with 0.0316-3.16 μM lapatinib greatly enhanced inhibition of Hs578T growth compared to either monotherapy (Figure 2b), indicative of synergy. To confirm this interaction was not cell line-specific, 17 TNBC cell lines were screened with a dose range of lapatinib or PHA-767491 (0.0316-3.16 μM) or with a dose-range of lapatinib in combination with either 1 μM or 3.16 μM PHA-767491. These TNBC cell lines were generally resistant to both monotherapies at doses equal to or less than 1 μM , but lapatinib and PHA-767491 co-treatment strongly inhibited proliferation (Figure 2c). Combining lapatinib (3.16 μM) with PHA-767491 at either 1 μM or 3.16 μM led to strong synergistic responses in the vast majority of TNBC cell lines (with some additive responses also evident), yielding CI values well below 1 (Figure 2d), thus confirming the synergistic nature of this interaction.

Dual pharmacological inhibition of EGFR and Cdc7/CDK9 suppresses components of the transcriptional machinery and DNA-replicative program

Having confirmed the synergistic effect of PHA-767491 and lapatinib on TNBC proliferation, we elucidated the impact of co-treatment on EGFR, cdc7 and CDK9-mediated signalling transduction. 48 hours post-exposure, lapatinib in combination with PHA-767491 at synergistic concentrations led to depletion of Cdc7, accompanied by decreased phosphorylation of its downstream target MCM2 (Ser40/41), a critical component of DNA helicase, suggesting an inhibitory effect on initiation of DNA-replication in Hs578T, BT549 and SKBR7 cells (Figure 2e). The combination therapy also decreased CDK9-mediated phosphorylation of Ser2/5 residues in the C-terminal domain of RNA Polymerase II (RNAPII), implying impairment of CDK9 and/or CDK7-mediated productive transcriptional elongation (Figure 2e). These data indicate that co-treatment of TNBC cells with lapatinib and a dual Cdc7/CDK9 inhibitor blocks components essential for initiation of DNA replication and RNA polymerase II-mediated transcription.

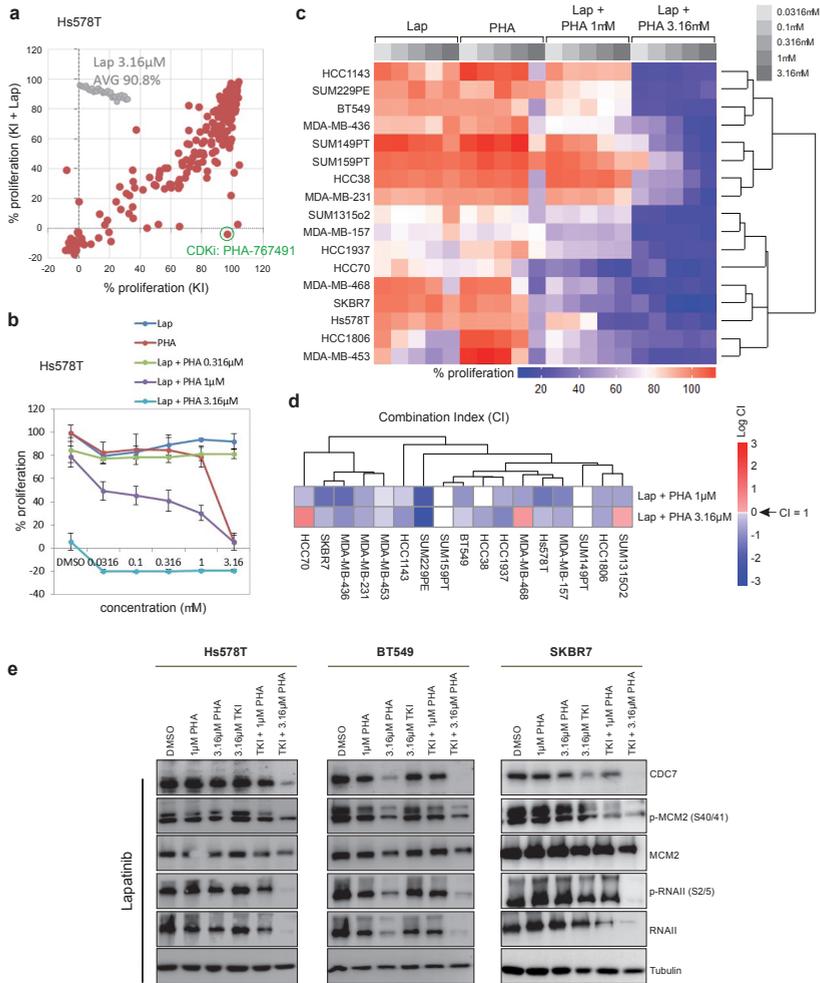


Figure 2: Kinase inhibitor combination screening identifies compounds which synergise with EGFR-TKIs in TNBC. **a.** EGFRi lapatinib (Lap) and kinase inhibitor (KI) combination screen in EGFRi-resistant Hs578T cells. Cells (8,000/well) were treated with 1 μ M of 273 individual KIs alone, or in combination with lapatinib at 3.16 μ M, for 4 days. The dual Cdc7/CDK9 inhibitor PHA-767491, one of the KIs which most strongly synergised with lapatinib is singled out, as indicated. **b.** Dose response curves for Hs578T cells. Cells were treated with a dose range of PHA-767491 or lapatinib (0.0316-3.16 μ M) or lapatinib combined with 0.316, 1 or 3.16 μ M PHA-767491. Data shown as mean \pm standard deviation for two independent experiments. **c.** Validation screen of lapatinib/PHA-767491 synergistic effect in a panel of 17 TNBC cell lines, under indicated combinations. Cells were treated with dose ranges of lapatinib or PHA-767491 (0.0316-3.16 μ M) or the indicated combinations. **d.** Combination Indices (CI) for Lapatinib (3.16 μ M) combined with either 1 μ M or 3.16 μ M PHA-767491. Log CI is shown. **e.** Initiation of DNA replication and productive transcriptional elongation in Hs578T, BT549 and SKBR7 cells treated with lapatinib and/or PHA-767491 as indicated, for 48h. TKI refers to lapatinib at 3.16 μ M.

Selective EGFR-TKIs erlotinib and gefitinib also synergise with PHA-767491 in TNBC

To verify whether synergy between lapatinib and PHA-767491 represents a general EGFR-TKI-related phenomenon, TNBC cells were treated with other selective EGFR-TKIs, erlotinib and gefitinib²⁹, in combination with PHA-767491. At a concentration of 3.16 μM , erlotinib and gefitinib inhibited EGF-mediated activation of EGFR phosphorylation and downstream phosphorylation of ERK1/2 and AKT in Hs578T and SKBR7 cells (Figure 3a), perhaps reflective of their increased potency compared to lapatinib in eliciting EGFR inhibition. However, proliferation of all cell lines tested was refractory to erlotinib and gefitinib monotherapy up to 3.16 μM (Figure 3b). Strikingly, at concentrations of 0.1 μM or above, erlotinib and gefitinib strongly synergised with 1 μM and 3.16 μM PHA-767491, almost completely inhibiting the proliferation of Hs578T and SKBR7 cells (Figure 3b-c). The effect of combining erlotinib or gefitinib with PHA-767491 on DNA-replication and transcription-related signal transduction largely resembled that of lapatinib combined with PHA-767491 (Figure 3d). Only combinations of erlotinib or gefitinib and PHA-767491 were capable of decreasing Cdc7 levels and consequently phosphorylation of MCM2 (Ser40/41). Similarly, inhibition of CDK9-mediated RNA-II (S2/S5) phosphorylation was most prominent after co-treatment. To validate the hypothesis that EGFR inhibition is critical for the synergistic interaction between EGFR-TKIs and PHA-767491, an EGFR-negative triple-negative cell line, SUM185PE (Figure S2a), was treated with this combination. Neither AKT nor ERK1/2 was activated in response to stimulation with EGF, confirming EGF-mediated signalling is redundant in SUM185PE (Figure S2b). As expected, lapatinib and PHA-767491 did not synergise in EGFR-negative SUM185PE cells (Figure S2c).

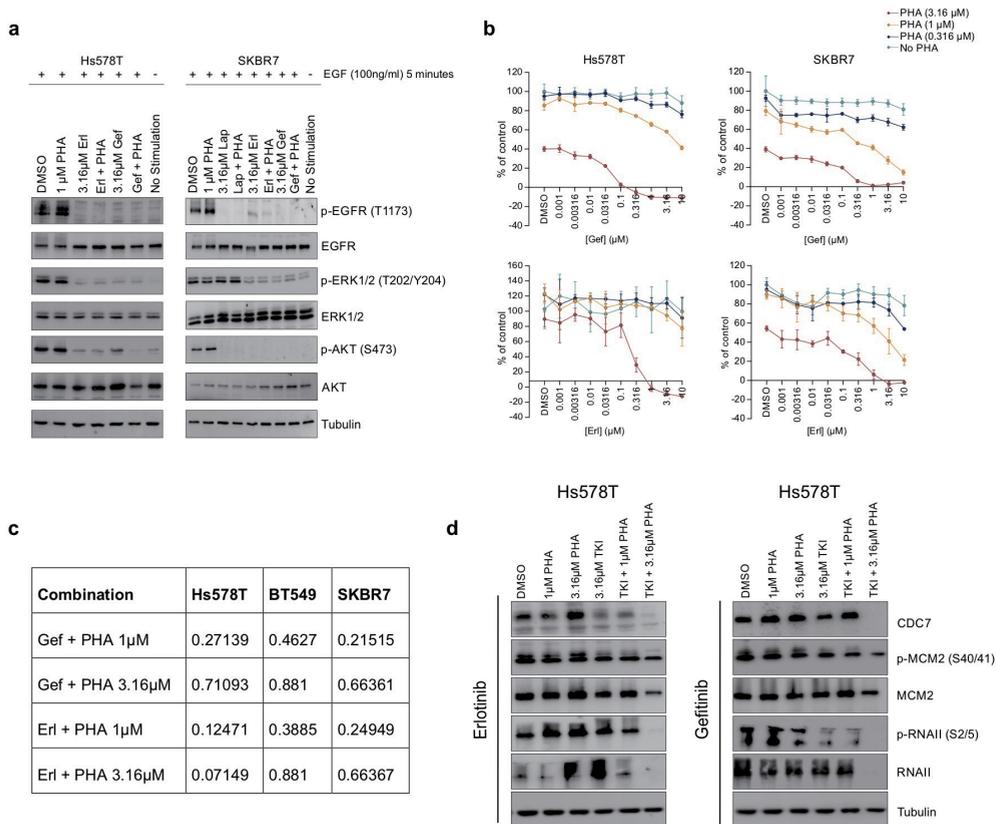


Figure 3: Selective EGFR-TKIs Erlotinib and Gefitinib also synergise with PHA-767491 in TNBC. a. Inhibitory impact of erlotinib and gefitinib on EGFR-mediated signal transduction in Hs578T and SKBR7 cells. **b.** Dose response of Hs578T and SKBR7 cells to gefitinib or erlotinib (0.0316-3.16 μ M) combined with 0.316, 1 or 3.16 μ M PHA-767491. Data shown as mean \pm standard deviation. Each graph shows one representative of two independent experiments performed in triplicate. Combination Indices (CI) of gefitinib (3.16 μ M) or erlotinib (3.16 μ M) with PHA-767491 (1 μ M or 3.16 μ M) in Hs578T, BT549 and SKBR7 cells. **d.** Initiation of DNA replication and productive transcriptional elongation in Hs578T cells treated with erlotinib or gefitinib and/or PHA-767491 as indicated, for 48h. TKI refers to either erlotinib or gefitinib at 3.16 μ M.

Dual treatment of TNBC cells with EGFR-TKIs and PHA-767491 induces G2/M cell cycle arrest and apoptosis

Whilst PHA-767491 at 3.16 μ M inhibits cdc7 and phosphorylation of MCM2 and RNA polymerase II, parallel inhibition of EGFR was necessary for eliciting a similar effect on cell cycle components. Decreased levels of CDK4, Cyclin D1 and phosphorylated-pRb were observed 48 hours post-exposure to co-treatment (Figure 4a), suggesting concomitant inhibition of EGFR, Cdc7 and CDK9 obstructs cell cycle progression in TNBC cells. Flow cytometric analysis confirmed that G2-M cell cycle arrest occurred only when lapatinib, erlotinib or gefitinib were combined with 3.16 μ M PHA-767491 in Hs578T and SKBR7 cells (Figure 4b; Figure S3a). Accordingly, Annexin-V staining indicated that monotherapies failed to induce appreciable levels of apoptosis in lapatinib-resistant TNBC cells, whilst combination treatments enhanced apoptosis (Figure 4c; Figure S3b).

Expression of CDC7 and RNAI1 (POLR2A) is linked to poor prognosis in breast cancer

Next, in relation to an in-house cohort of lymph node-negative, non-neoadjuvantly treated 123 oestrogen receptor-negative (ER-neg) breast cancer patients, high expression of CDC7 was significantly associated with a poor metastasis-free survival (MFS) (Figure 5a). Interestingly, high expression of POLR2A (RNA-II), the essential downstream target of CDK9, was also significantly associated with a poor metastasis-free survival in this patient group (Figure 5b), though CDK9 expression level was not.

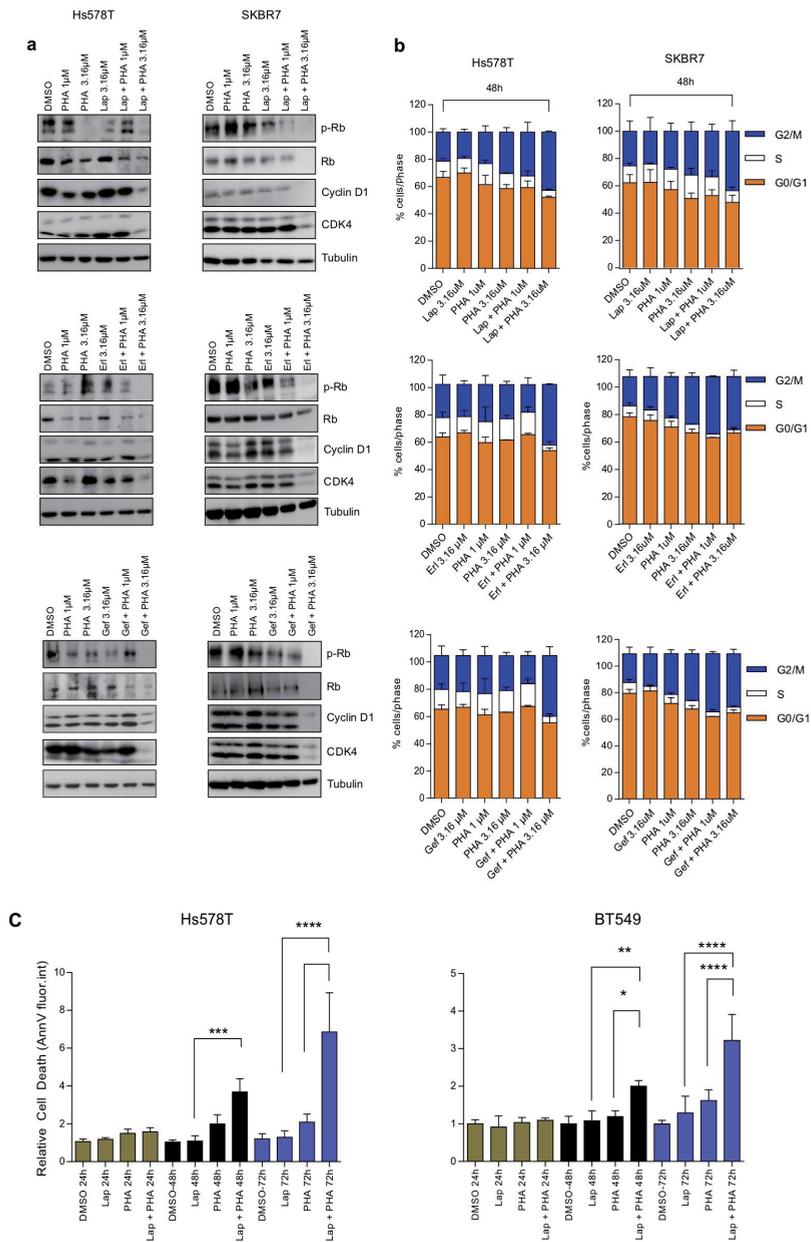


Figure 4: EGFR-TKIs and PHA-767491 synergise to induce G2/M cell cycle arrest and apoptosis. **a.** Inhibition of cell cycle components by co-treatment with EGFR-TKIs (lapatinib, erlotinib or gefitinib) and PHA-767491. Hs578T and SKBR7 cells were treated with EGFR-TKIs alone (3.16 μ M) or combined with PHA-767491 (1 μ M or 3.16 μ M) for 48 hours as indicated. **b.** Cell cycle distribution of Hs578T and SKBR7 cells after 48h treatment with EGFR-TKIs (3.16 μ M) alone or combined with PHA-767491 (1 μ M or 3.16 μ M), as indicated. Data shown as mean of two independent experiments \pm standard deviation. **c.** Induction of apoptosis by EGFR-TKI and PHA-767491 co-treatment. Hs578T and BT549 cells were treated with lapatinib (3.16 μ M), PHA-767491 (3.16 μ M), alone or combined, as indicated, for 24h, 48h or 72h, respectively, and then stained with Annexin-V and Hoechst, followed by imaging and image quantification. Data shown as mean \pm standard deviation of two independent experiments. Relative cell death was quantified by normalising the intensity of Annexin-V signal to that of DMSO control. One-way ANOVA **** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$.

Co-treatment of TNBC cells with lapatinib and PHA-767491 inhibits crucial signalling networks and the expression of genes linked to poor survival in TNBC

To investigate how combined EGFR-TKI and PHA-767491 treatment distorts the global transcriptional signature of TNBC cells, we performed RNA sequencing in Hs578T and SKBR7 cells treated with monotherapies or combination therapy (Figure 6a). We identified 2614 genes up-regulated by co-treatment in Hs578T cells, with 243 genes being up-regulated in SKBR7 cells under the same conditions (Figure 6b). 1387 and 2747 genes were down-regulated in co-treated Hs578T and SKBR7 cells, respectively (Figure 6b). Amongst these genes, 141 up- and 704 down-regulated genes (845 transcripts) were commonly responsive to co-treatment in both Hs578T and SKBR7 cells (Figure 6c). Integrated pathway analysis of these 845 synergy-related genes revealed striking disturbance of pro-proliferative pathways mediated by growth factors such as TGF- β , EGF and insulin, as well as pathways governing angiogenesis, stem cell pluripotency and metastatic signalling (Figure 6d). More specifically, an enrichment of genes related to biological networks governing cell survival, viability, migration, RNA transcription, cytokinesis, mitosis, and cell cycle progression, was found amongst the genes commonly down-regulated by co-treatment (Figure 6e). 34 (2 up- and 32 down-regulated) genes out of the 845 lapatinib and PHA-767491 co-targeted genes were significantly correlated with metastasis-free survival (MFS) in 142 lymph node-negative, non-neoadjuvantly treated TNBC patients, including LMBR1L, APAF1, DDX3X and GCNT3, with hazard ratios >2 (Figure 7a and Supplementary Table 1). These 34 clinically relevant genes were present in the main biological networks of apoptosis, transcription and proliferation (Figure 7b), in which MITF, HOXC6 and ROCK2 were all involved. MITF

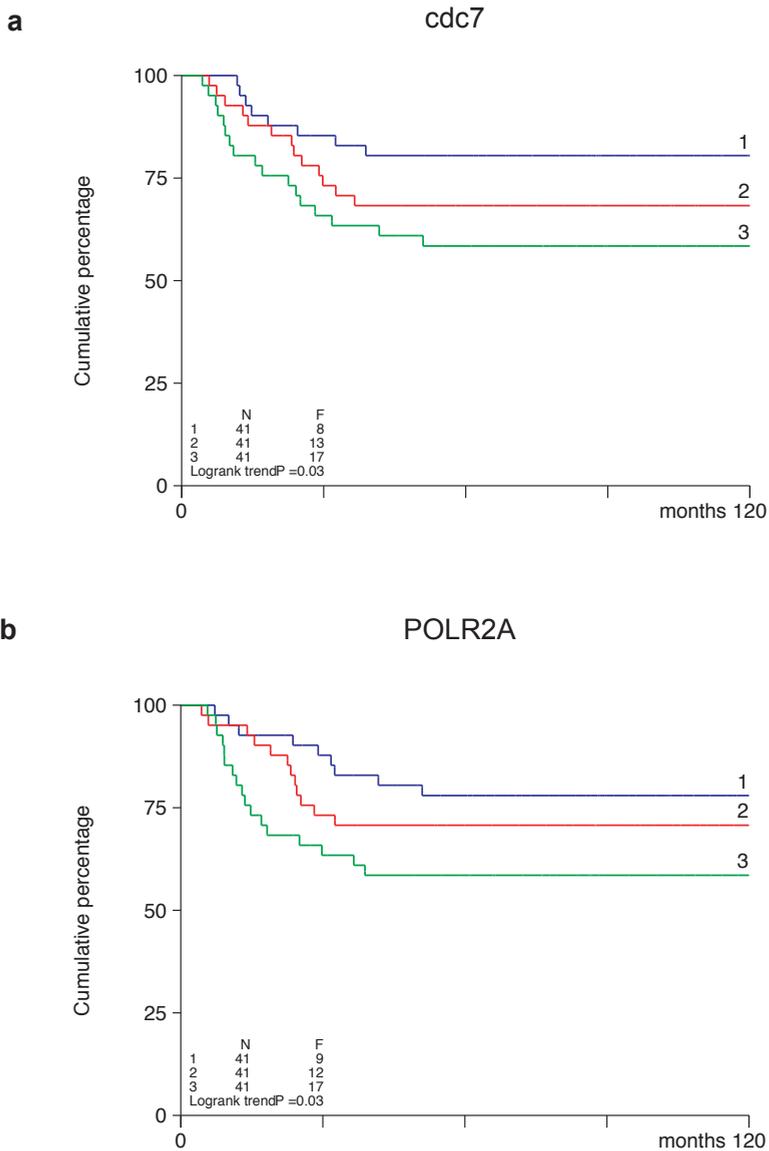


Figure 5. Expression of *cdc7* and RNAII (*POLR2A*) is linked to poor prognosis in breast cancer patients. Metastasis-free survival curves illustrating the relationship between expression of *cdc7* or RNAII (*POLR2A*) and prognosis in ER-negative breast cancer patients. Curves derived from gene expression and available survival data for 123 lymph node-negative, non-neoadjuvantly treated, ER-negative breast cancer patients at Erasmus MC Rotterdam.

was defined as a regulator known to influence the levels of anti-apoptotic protein BCL-2 as well as transcriptional regulators (ZNF114), G2/M regulators (CDC25B), mitotic regulators (DSN1) and DNA replication/repair proteins (TDG and PIF-1) these being down-regulated in response to MITF inhibition (Figure 7c).

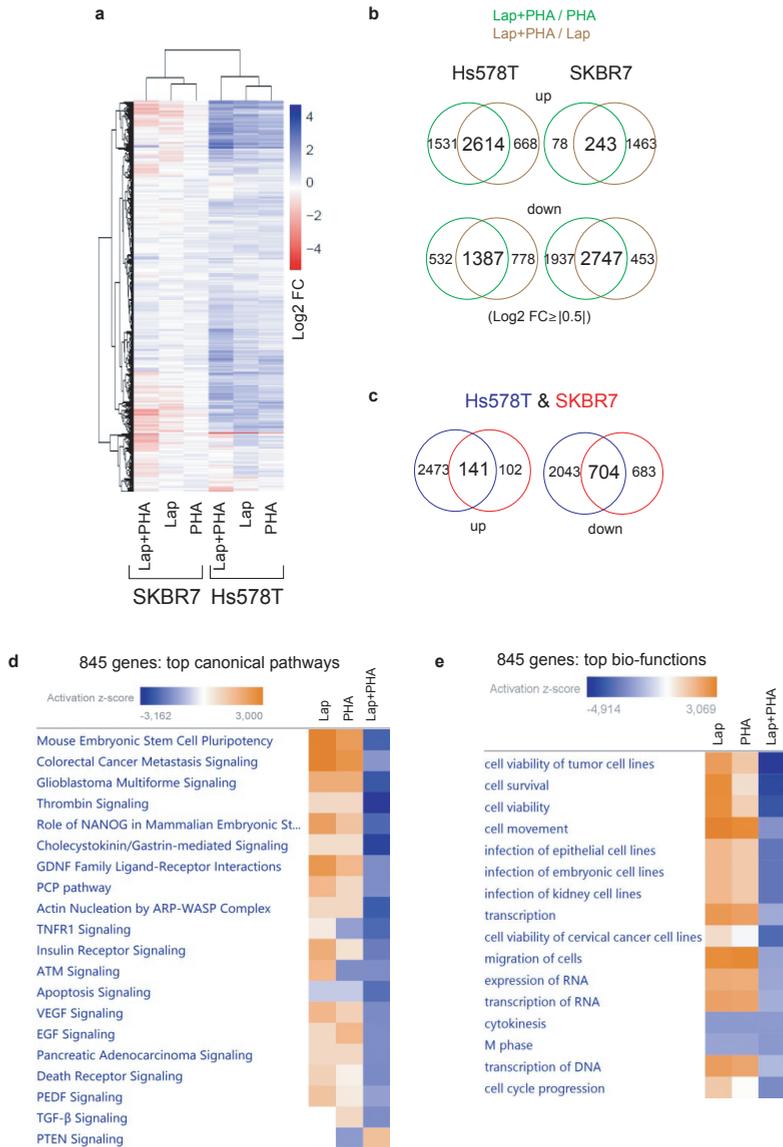


Figure 6: Co-treatment of TNBC cells with lapatinib and PHA-767491 inhibits crucial signalling networks in TNBC. **a.** Global transcriptomic signature in SKBR7 and Hs578T cells after 6h of treatment with lapatinib (3.16 μ M), PHA-767491 (1 μ M) or a combination of these two doses. Log₂ Fold Change (FC) normalised to DMSO control shown. **b.** Venn diagrams showing the number of genes significantly (Log₂ FC \geq 0.5) up- or down-regulated after monotherapy and combination therapy in Hs578T and SKBR7 cells. **c.** Venn diagrams showing the number of genes commonly and significantly (Log₂ FC \geq 0.5) up- or down-regulated after combination therapy in both Hs578T and SKBR7 cells. **d.** Top canonical pathways enriched in 845 significantly differentially expressed genes in combination therapy as predicted by IPA online analysis. Z-score indicates activation (orange) or inhibition (blue) of the pathway under indicated conditions, respectively. **e.** Top bio-functions enriched in 845 significantly differentially expressed genes in combination therapy conditions as predicted by IPA software.

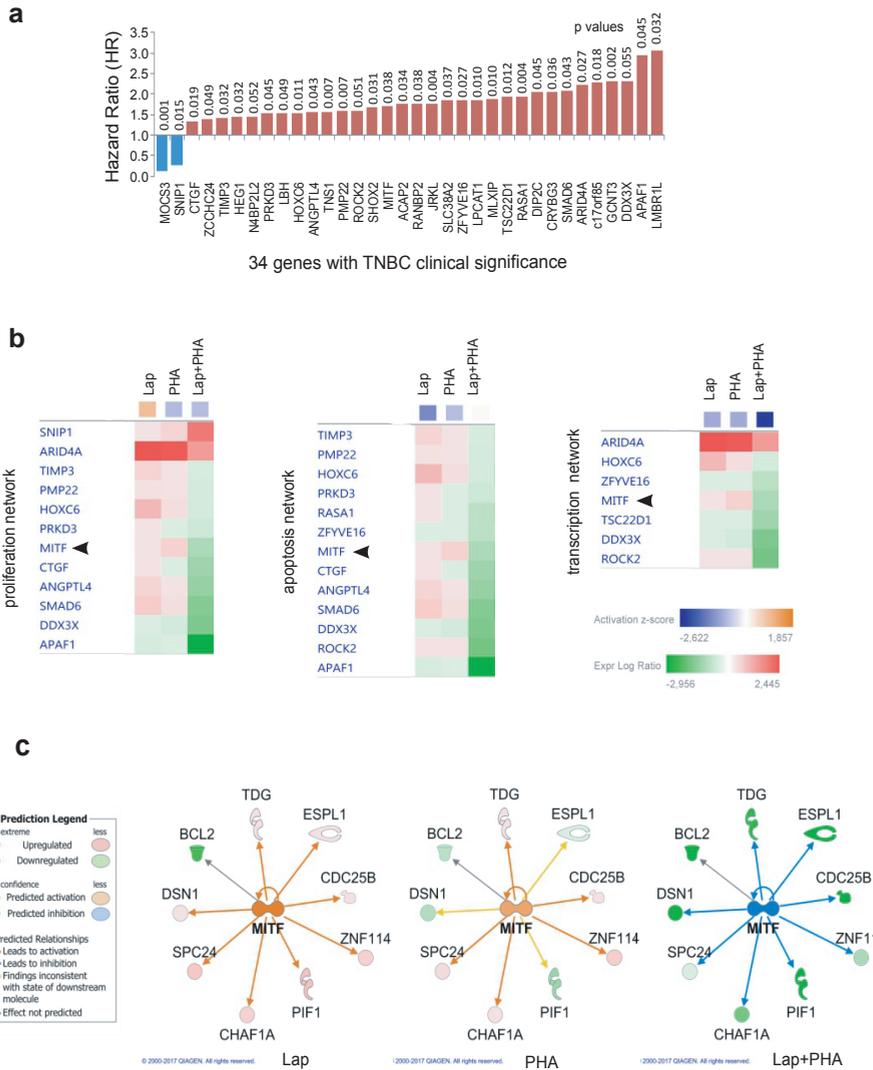


Figure 7: RNA-Seq reveals genes linked to poor survival in TNBC which are involved in regulation of transcription, apoptosis, and proliferation. a. 34 genes specifically down-regulated by combination therapy whose expression is significantly correlated with metastasis-free survival in 142 lymph node-negative, non-neoadjuvantly treated, TNBC patients (data derived from in-house cohort as well as from publicly available datasets). HR: Hazard Ratio. **b.** Presence of aforementioned clinically relevant, down-regulated genes in the top biological networks inhibited by combination therapy. **c.** MITF, an upstream regulator of genes which are down-regulated in response to inhibition of MITF under combination therapy.

DISCUSSION

EGFR is highly expressed in both TNBC tumours and cell lines, supporting a role for EGFR as an oncogenic driver in TNBC. However, clinical trials suggest single inhibition of EGFR signalling is incapable of eliminating TNBC cells^{17,18,21,30}. Consistently, our results demonstrated that targeting EGFR kinase activity by EGFR-TKIs, including lapatinib, erlotinib and gefitinib, insufficiently inhibits TNBC cell proliferation, despite inhibition of EGFR phosphorylation. Our kinase inhibitor combination screen demonstrated that targeting Cdc7/CDK9 signalling pathways in tandem enables EGFR-TKIs to inhibit proliferation, induce G2-M cell cycle arrest and promote apoptosis in various TNBC cell lines expressing high levels of EGFR. This synergistic drug interaction down-regulates the activity of components of the transcription apparatus and the DNA replication programme, including Cdc7, CDK9, pMCM2 (S40/41), p-RNAII (S2/5), CDK4, cyclin D1 and Rb, making the combination of EGFR and Cdc7/CDK9 molecular-targeted therapies promising for this subgroup of breast cancer.

CDK9 is a member of positive elongation factor P-TEFb, and together with CDK7 is vital for gene transcription since CDK7 and CDK9 sequentially phosphorylate the C-terminal domain (CTD) of RNA Polymerase II (RNA II) at Ser5/Ser7 and Ser2, respectively, allowing dissociation of negative elongation factors and subsequent elongation of mRNA transcripts^{31–33}. CDK9 is essential for growth of both HR+ and TNBC cell lines, whilst EGFR is one of many “Achilles’cluster” genes sensitive to CDK7 inhibition and vital for TNBC survival³⁴. Consistently, we showed that despite being resistant to inhibition of EGFR kinase activity by various EGFR-TKIs, complete silencing of EGFR is detrimental to TNBC cell growth.. In addition, EGFR is capable of acting as a transcription factor^{35,36}. The nuclear translocation of EGFR is

associated with resistance to chemotherapeutics in TNBC^{35,37-39} and shields the RTK from the effects of TKIs limited to the cell membrane, permitting EGFR to enhance transcription of genes which govern cell cycle progression, such as Cyclin D1 and Aurora Kinase^{36,40}. Cdc7 kinase is itself indispensable for correct regulation of cell cycle progression, exerting control over both initiation of DNA replication and the DNA damage response^{41,42}. By phosphorylating mini-chromosome maintenance proteins (MCM2-7) present in pre-replicative complexes formed during G1 phase, Cdc7 activates the helicase activity of these proteins, leading to unwinding of DNA strands and thereby initiating DNA replication at the G1-S phase checkpoint^{43,44}. It also has been reported that EGFR indirectly influences the initiation of DNA replication by eliciting phosphorylation of MCM7 in a Lyn kinase-dependent fashion, thereby delineating possible functional overlap between Cdc7 and EGFR⁴⁵. TNBC cells often possess p53-inactivating mutations which abolish the DNA replication origin activation checkpoint, rendering them susceptible to the induction of replicative-stress⁴⁶. Induction of G2-M arrest in our TNBC cell lines after combined inhibition of EGFR and Cdc7/CDK9 is consistent with data from other studies which demonstrated that a p53-dependent checkpoint is critical for mitigating aberrant cell cycle progression after cdc7 depletion^{47,48}.

The CDK4/Cyclin D1 complex phosphorylates Rb at Ser780/795 thereby inactivating Rb in G1/S checkpoint regulation^{49,50}. Consistently, co-inhibition of Cdc7/CDK9 and EGFR signalling in our TNBC cell lines reduces CDK4 and Cyclin D1 levels accompanied by reduced phosphorylation of Rb, thereby resulting in G2-M arrest and ultimately apoptosis. Whether the down-regulation of Cyclin D1 and CDK4 by EGFR-TKIs and PHA-767491 represents a global decrease in the transcription of rapidly expressed, immediate response genes due to inhibition of CDK9-mediated

transcriptional elongation, a decrease in the transcriptional activity of EGFR, or a by-product of the cell cycle arrest induced by Cdc7 depletion, merits further investigation. Taken together, these results identify possible functional links between signalling downstream of EGFR and the function of both Cdc7 and CDK9, which may to some extent explain the observed synergy between EGFR-TKIs and PHA-767491. Additionally, silencing of cell cycle-regulatory or transcriptional CDKs in combination with a Cdc7-specific inhibitor (XL413) in breast cancer cells has been shown to mimic the cell cycle disruption caused by PHA-767491⁵¹. Silencing of CDK9 led to negligible impact on progression of MCF10A cells through S-phase, whilst CDK9-depleted cells treated with XL413 accumulated in late S-phase, suggesting that the profound cell cycle arrest in TNBC cells caused by PHA-767491 or cdc7 depletion may be somewhat dependent on CDK9 or can at least be augmented by inhibiting CDK9.

RNA-seq transcriptomics identified genes specifically down-regulated by co-treatment with EGFR-TKIs and PHA-767491, which were involved in pathways regulating survival, transcription and cell cycle progression. The decreased expression of these genes (a number of them associated with poorer MFS in TNBC) by co-inhibition of EGFR and Cdc7/CDK9 leads to apoptosis and down-regulation of transcription and proliferation. Interestingly, the transcription factor MITF (microphthalmia-associated transcription factor), a major upstream regulator of pathways governing apoptosis, proliferation and transcription, was decreased together with its downstream targets pro-survival BCL-2 and cell cycle-regulatory CDC25B as a result of combining lapatinib and PHA-767491. With regards to TNBC, little is known about MITF's contribution to EGFRi-resistant phenotypes. Further research is therefore required to validate whether targeting of MITF function

constitutes a logical therapeutic avenue in TNBC, or whether MITF inhibition is sufficient to reverse the resistance of TNBC cells to EGFR targeted therapies.

CONCLUSIONS

In summary, we show that multiple EGFR-TKIs synergise with the dual Cdc7/CDK9 inhibitor PHA-767491 in various EGFR-TKI-resistant TNBC cell lines resulting in decreased proliferation, induction of apoptosis and G2/M cell cycle arrest. Combination therapy leads to inhibition of proteins crucial for accurate DNA-replication and CDK9/RNAIL-mediated gene transcription. This combination also leads to inhibition of crucial pro-oncogenic networks and reduces the expression of genes linked to ERK/mTOR signalling and poor progression-free survival in TNBC patients, perhaps identifying possible candidate genes for further research into the mechanism of this synergy and as therapeutic targets.

LIST OF ABBREVIATIONS

AKT: v-akt murine thymoma viral oncogene homologue

BL1: Basal-like 1 subtype

BL2: Basal-like 2 subtype

Cdc7: cell division cycle 7-related protein kinase

CDK4: Cyclin-dependent kinase 4

CDK7: Cyclin-dependent kinase 7

CDK9: Cyclin-dependent kinase 9

CI: Combination index

CTD: C-terminal domain

EGFR: Epidermal growth factor receptor

EGFR-TKI: Epidermal growth factor receptor tyrosine kinase inhibitor

ERK1/2: Extracellular signal-regulated kinase 1/2

HER2: human epidermal growth factor receptor 2
HR+/-: Hormone receptor-positive/negative
IM: Immunomodulatory subtype
LAR: Luminal androgen receptor-positive subtype
M: Mesenchymal subtype
MCM2: Mini-chromosome maintenance protein 2
MEK: Mitogen-activated protein kinase kinase
MITF: Microphthalmia-associated transcription factor
MSL: Mesenchymal stem-like subtype
POLR2A: RNA Polymerase II subunit 2A
P-TEFb: Positive transcription elongation factor b
Rb: Retinoblastoma protein
RNA II: RNA Polymerase II
S2/5: Serine 2/5 of RNA polymerase II subunit 2A's C-terminal domain
SRB: Sulphorhodamine B
TNBC: triple-negative breast cancer

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REFERENCES

1. Foulkes, W.D. *et al*, Triple-Negative Breast Cancer. *N Engl J Med*, **363**:1938-48 (2010).
2. Cheang, M. C. U. *et al*. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin. Cancer Res.* **14**, 1368–76 (2008).

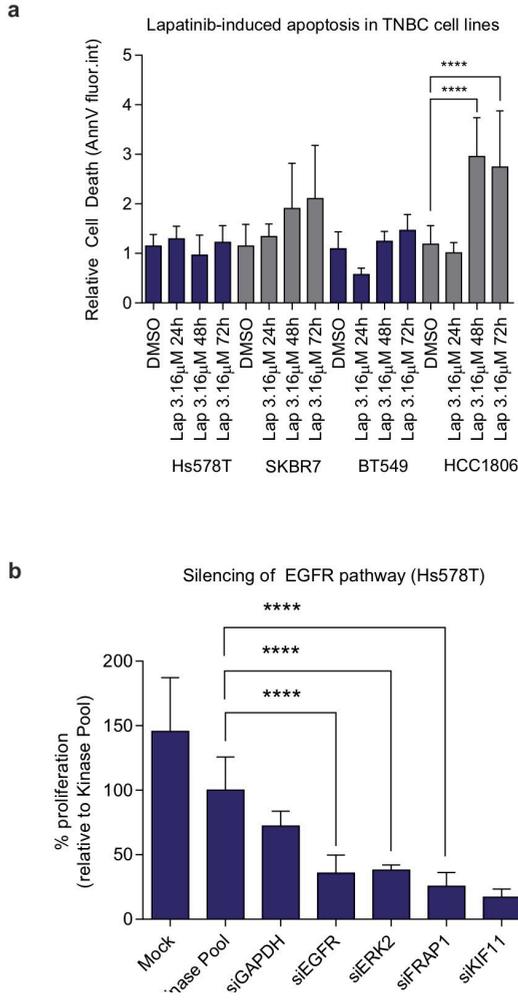
3. Perou, C. M. *et al.* Molecular portraits of human breast tumours. *Nature*. **406 (6797)**: 747–752 (2000).
4. Boyle, P. Triple-negative breast cancer: epidemiological considerations and recommendations. *Ann. Oncol.* **23 Suppl 6**, vi7-12 (2012).
5. Finn, R. S. *et al.* Estrogen Receptor , Progesterone Receptor , Human Epidermal Growth Factor Receptor 2 (HER2), and Epidermal Growth Factor Receptor Expression and Benefit From Lapatinib in a Randomized Trial of Paclitaxel With Lapatinib or Placebo As First-Line Treatment. *J Clin Oncol.* **27 (24)**: 3908-3915 (2009).
6. Schuler, M. *et al.* A phase II trial to assess efficacy and safety of afatinib in extensively pretreated patients with HER2-negative metastatic breast cancer. *Breast Cancer Res. Treat.* **134**, 1149–1159 (2012).
7. Mohamed, A., Krajewski, K., Cakar, B. & Ma, C. X. Targeted therapy for breast cancer. *Am. J. Pathol.* **183**, 1096–112 (2013).
8. Dent, R. *et al.* Pattern of metastatic spread in triple-negative breast cancer. *Breast Cancer Res. Treat.* **115**, 423–8 (2009).
9. Lehmann, B. D. *et al.* Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J. Clin. Invest.* **121 (7)**: 2750-67 (2011).
10. Masuda, H. *et al.* Differential response to neoadjuvant chemotherapy among 7 triple-negative breast cancer molecular subtypes. *Clin. Cancer Res.* **19**, 5533–40 (2013).
11. Rakha, E. a *et al.* Prognostic markers in triple-negative breast cancer. *Cancer* **109**, 25–32 (2007).
12. Park, H. S. *et al.* High EGFR gene copy number predicts poor outcome in triple-negative breast cancer. *Mod. Pathol.* **27**, 1212–22 (2014).
13. Secq, V. *et al.* Triple negative breast carcinoma EGFR amplification is not associated with EGFR, Kras or ALK mutations. *Br. J. Cancer* **110**, 1045–52 (2014).
14. Reis-Filho, J. S. *et al.* EGFR amplification and lack of activating mutations in metaplastic breast carcinomas. *J. Pathol.* **209**, 445–53 (2006).
15. Gumuskaya, B. *et al.* EGFR expression and gene copy number in triple-negative breast carcinoma. *Cancer Genet. Cytogenet.* **203**, 222–9 (2010).
16. Ciardiello, F. & Tortora, G. EGFR Antagonists in Cancer Treatment. *N. Engl. J. Med.* **358**, 1160–1174 (2008).
17. Baselga, J. *et al.* Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. *J. Clin. Oncol.* **23**, 5323–33 (2005).

18. Dickler, M. N., Cobleigh, M. a, Miller, K. D., Klein, P. M. & Winer, E. P. Efficacy and safety of erlotinib in patients with locally advanced or metastatic breast cancer. *Breast Cancer Res. Treat.* **115**, 115–21 (2009).
19. Linardou, H. *et al.* Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. *Lancet. Oncol.* **9**, 962–72 (2008).
20. Kurtze, I., Sonnemann, J. & Beck, J. F. KRAS-mutated non-small cell lung cancer cells are responsive to either co-treatment with erlotinib or gefitinib and histone deacetylase inhibitors or single treatment with lapatinib. *Oncol. Rep.* **25**, 1021–9 (2011).
21. Baselga, J. *et al.* Randomized phase II study of the anti-epidermal growth factor receptor monoclonal antibody cetuximab with cisplatin versus cisplatin alone in patients with metastatic triple-negative breast cancer. *J. Clin. Oncol.* **31**, 2586–92 (2013).
22. Spector, N. L. *et al.* Lapatinib Plasma and Tumor Concentrations and Effects on HER Receptor Phosphorylation in Tumor. *PLoS One* **10**, 142845 (2015).
23. Vichai, V. & Kirtikara, K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat. Protoc.* **1**, 1112–1116 (2006).
24. Huang, L., Jiang, Y. & Chen, Y. Predicting Drug Combination Index and Simulating the Network-Regulation Dynamics by Mathematical Modeling of Drug-Targeted EGFR-ERK Signaling Pathway. *Sci. Rep.* **7**, (2017).
25. Foucquier, J. & Guedj, M. Analysis of drug combinations: current methodological landscape. *Pharmacology Research and Perspectives* **3**, (2015).
26. McCall, M. N., Bolstad, B. M. & Irizarry, R. A. Frozen robust multiarray analysis (fRMA). *Biostatistics* **11**, 242–253 (2010).
27. Johnson, W. E., Li, C. & Rabinovic, A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* **8**, 118–127 (2007).
28. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–40 (2010).
29. Schettino, C., Bareschino, M. A., Ricci, V. & Ciardiello, F. Erlotinib: an EGF receptor tyrosine kinase inhibitor in non-small-cell lung cancer treatment. *Expert Rev. Respir. Med.* **2**, 167–178 (2008).
30. Nakai, K., Hung, M. C. & Yamaguchi, H. A perspective on anti-EGFR therapies targeting triple-negative breast cancer. *American Journal of Cancer Research*

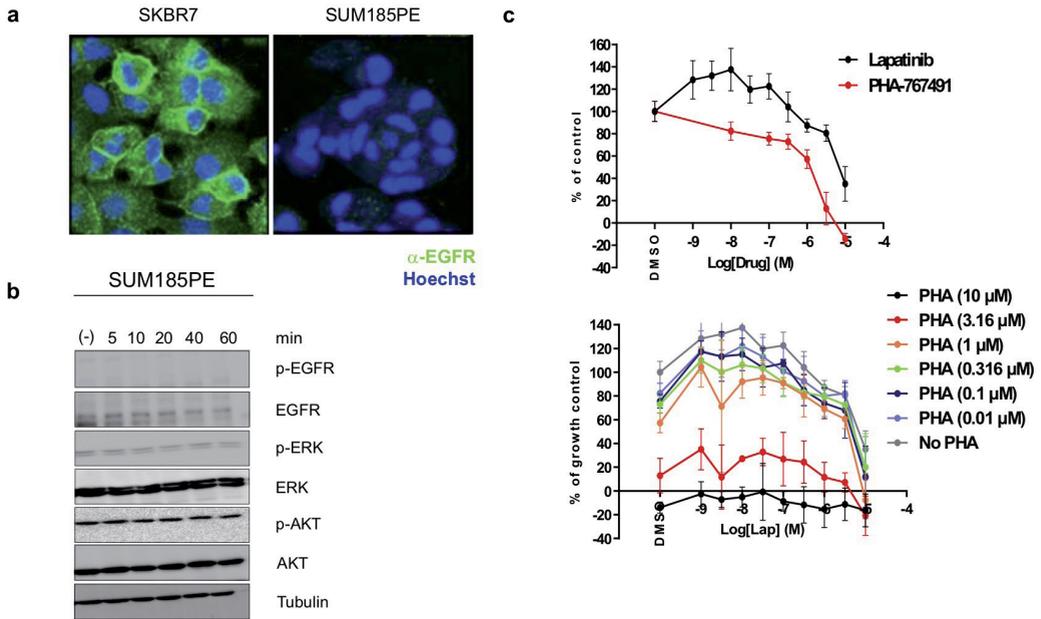
- 6**, 1609–1623 (2016).
31. Viladevall, L. *et al.* TFIIF and P-TEFb Coordinate Transcription with Capping Enzyme Recruitment at Specific Genes in Fission Yeast. *Mol Cell* **33**, 738–751 (2009).
 32. Yang, Z. *et al.* Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. *Mol Cell* **19**, 535–545 (2005).
 33. Ahn, S. H., Kim, M. & Buratowski, S. Phosphorylation of serine 2 within the RNA polymerase II C-terminal domain couples transcription and 3' end processing. *Mol Cell* **13**, 67–76 (2004).
 34. Wang, Y. *et al.* CDK7-dependent transcriptional addiction in triple-negative breast cancer. *Cell* **163**, 174–86 (2015).
 35. Lo, H.-W. & Hung, M.-C. Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival. *Br. J. Cancer* **94**, 184–8 (2006).
 36. Lin, S. Y. *et al.* Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat. Cell Biol.* **3**, 802–808 (2001).
 37. Lo, H.-W. *et al.* Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell* **7**, 575–89 (2005).
 38. Brand, T. M. *et al.* Nuclear epidermal growth factor receptor is a functional molecular target in triple-negative breast cancer. *Mol. Cancer Ther.* **13**, 1356–68 (2014).
 39. Huang, W.-C. *et al.* Nuclear translocation of epidermal growth factor receptor by Akt-dependent phosphorylation enhances breast cancer-resistant protein expression in gefitinib-resistant cells. *J. Biol. Chem.* **286**, 20558–68 (2011).
 40. Hung, L. Y. *et al.* Nuclear epidermal growth factor receptor (EGFR) interacts with signal transducer and activator of transcription 5 (STAT5) in activating Aurora-A gene expression. *Nucleic Acids Res.* **36**, 4337–4351 (2008).
 41. Swords, R. *et al.* Cdc7 kinase - a new target for drug development. *Eur. J. Cancer* **46**, 33–40 (2010).
 42. Yamada, M., Masai, H. & Bartek, J. Regulation and roles of Cdc7 kinase under replication stress. *Cell Cycle* **13**, 1859–66 (2014).
 43. Tudzarova, S. *et al.* Molecular architecture of the DNA replication origin activation checkpoint. *EMBO J.* **29**, 3381–94 (2010).
 44. Tudzarova, S. *et al.* p53 controls CDC7 levels to reinforce G1 cell cycle arrest upon genotoxic stress. *Cell Cycle* **15**, 2958–2972 (2016).
 45. Huang, T.-H. *et al.* Epidermal growth factor receptor potentiates MCM7-mediated DNA replication through tyrosine phosphorylation of Lyn kinase in

- human cancers. *Cancer Cell* **23**, 796–810 (2013).
46. Network, T. C. G. A. Comprehensive molecular portraits of human breast tumors. *Nature* **490**, 61–70 (2012).
 47. Montagnoli, A. *et al.* A Cdc7 kinase inhibitor restricts initiation of DNA replication and has antitumor activity. *Nat. Chem. Biol.* **4**, 357–65 (2008).
 48. Montagnoli, A. *et al.* Cdc7 Inhibition Reveals a p53-Dependent Replication Checkpoint That Is Defective in Cancer Cells. *Cancer Res.* **64 (19)**: 7110–7116 (2004).
 49. Connell-Crowley, L., Harper, J. W. & Goodrich, D. W. Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. *Mol. Biol. Cell* **8**, 287–301 (1997).
 50. Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E. & Sherr, C. J. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev.* **7**, 331–342 (1993).
 51. Rainey, M. D., Quachthithu, H., Gaboriau, D. & Santocanale, C. DNA Replication Dynamics and Cellular Responses to ATP Competitive CDC7 Kinase Inhibitors. *ACS Chem. Biol.* **12**, 1893–1902 (2017).

ADDITIONAL FILES

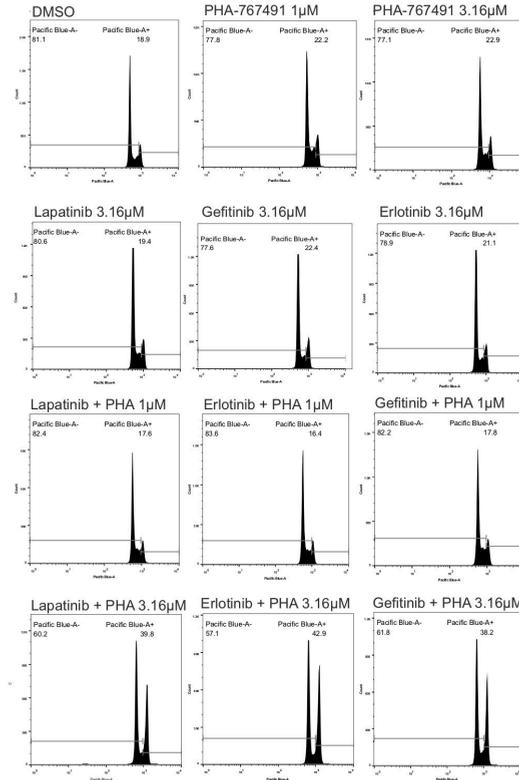


Additional File 1. Figure S1. Resistance of TNBC cells to EGFR-TKIs. a. Effect of Lapatinib on induction of apoptosis in selected lapatinib-resistant and sensitive TNBC cell lines. Hs578T, BT549, SKBR7 and HCC1806 cells were treated with lapatinib (3.16 μM) as indicated, for 24, 48 or 72 hrs, respectively, stained with Annexin-V and Hoechst, followed by imaging and image quantification. Relative cell death was quantified by normalising the intensity of Annexin-V signal to that of DMSO control. One-way ANOVA **** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. **b.** Impact of silencing EGFR and downstream components of EGFR signalling pathway on the proliferation of Hs578T cells. Hs578T cells were transfected with siRNAs targeting EGFR, ERK2 and FRAP1 as well as positive (siKIF11) and negative controls (siGAPDH and siKinase Pool) as described and grown for 4 days. Proliferation was then assessed using sulphorhodamine B assay. Results were normalised to Kinase Pool using the % control method as described in materials and methods.

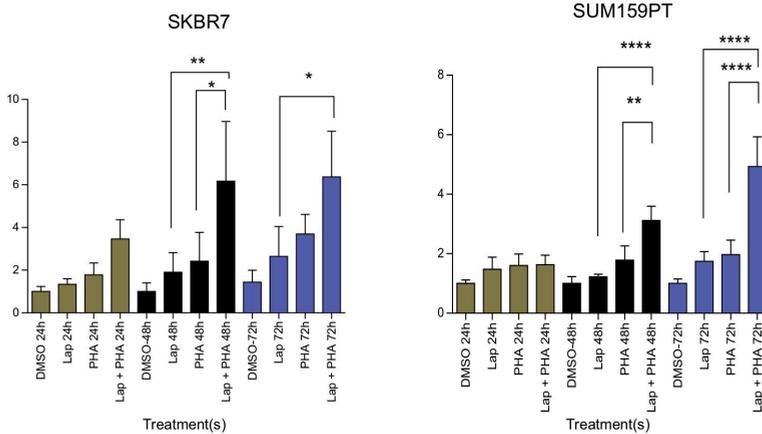


Additional File 2. Figure S2: EGFR-negative TNBC cell line SUM185PE is insensitive to co-treatment with lapatinib and PHA-767491. **a.** Immunofluorescence imaging of EGFR-positive (SKBR7) and EGFR-negative (SUM185PE) TNBC cell lines. Cells were fixed using 1% paraformaldehyde and 0.1% Triton-X for 15 minutes before being washed thrice with 1x PBS and blocked with 0.5% bovine serum albumin in PBS for 30 minutes. EGFR antibody (sc-03; Santa Cruz Biotechnology®) was used to stain EGFR overnight at 4°C. Fixed cells were then incubated with anti-rabbit Alexa 488-conjugated secondary antibody (A11008; Molecular Probes®) or Hoechst (nuclear stain; 1:10,000) for 1 hour at room temperature in the dark before being imaged at 20x magnification. **b.** Impact of EGF-stimulation on EGFR-mediated signal transduction in EGFR-negative cell line SUM185PE. Cells were starved overnight in serum-free medium before being treated with lapatinib (3.16 μM) for 4 hours and subsequently stimulated with EGF (100 ng/ml) for 5 minutes. Cells were then lysed and protein samples subjected to SDS-PAGE and immunoblotting with indicated antibodies. **c.** Dose-response experiment combining lapatinib and PHA-767491 in SUM185PE cells. Upper graph shows response of SUM185PE cells to lapatinib and PHA-767491 monotherapies. Lower graph displays proliferation of SUM185PE cells after combining lapatinib (3.16 μM) with dose range (0.01-10 μM) of PHA-767491. Proliferation was assessed using sulphorhodamine B assay and results were normalised to DMSO using the % control method as outlined in materials and methods.

a



b



Additional File 3. Figure S3. EGFR-TKIs and PHA-767491 synergise to induce G2/M cell cycle arrest and apoptosis. **a.** Cell cycle distribution histograms of Hs578T and SKBR7 cells after 48h treatment with EGFR-TKIs (lapatinib, erlotinib or gefitinib at 3.16 µM) alone or combined with PHA-767491 (1µM or 3.16µM), as indicated. **b.** Induction of apoptosis by EGFR-TKI and PHA-767491 co-treatment. SKBR7 and SUM159PT cells were treated with lapatinib (3.16 µM), PHA-767491 (3.16 µM), alone or combined, as indicated, for 24h, 48h or 72h, respectively, and then stained with Annexin-V and Hoechst, followed by imaging and image quantification. Relative cell death was quantified by normalising the intensity of Annexin-V signal to that of DMSO control. One-way ANOVA **** P ≤ 0.0001, *** P ≤ 0.001, ** P ≤ 0.01, * P ≤ 0.05.

Table S1 (Related to Figure 6)				
Genes	HR	p-value	95% CI low	95% CI high
MOCS3	0.11	0.00	0.031	0.415
SNIP1	0.28	0.01	0.099	0.778
CTGF	1.33	0.02	1.047	1.683
ZCCHC24	1.40	0.05	1.001	1.963
TIMP3	1.43	0.03	1.031	1.989
HEG1	1.44	0.03	1.032	2.013
N4BP2L2	1.44	0.05	0.997	2.092
PRKD3	1.53	0.05	1.009	2.328
LBH	1.54	0.05	1.002	2.353
HOXC6	1.54	0.01	1.106	2.157
ANGPTL4	1.55	0.04	1.015	2.378
TNS1	1.56	0.01	1.128	2.163
PMP22	1.58	0.01	1.132	2.211
ROCK2	1.61	0.05	0.998	2.583
SHOX2	1.69	0.03	1.049	2.715
MITF	1.71	0.04	1.031	2.821
ACAP2	1.75	0.03	1.044	2.950
RANBP2	1.76	0.04	1.032	3.000
JRKL	1.76	0.00	1.200	2.583
SLC38A2	1.84	0.04	1.038	3.254
ZFYVE16	1.84	0.03	1.071	3.154
LPCAT1	1.84	0.01	1.154	2.931
MLXIP	1.89	0.01	1.164	3.076
TSC22D1	1.93	0.01	1.158	3.231
RASA1	1.95	0.00	1.236	3.074
DIP2C	2.04	0.05	1.015	4.095
CRYBG3	2.04	0.04	1.046	3.994
SMAD6	2.07	0.04	1.023	4.177
ARID4A	2.23	0.03	1.096	4.530
C17orf85	2.27	0.02	1.152	4.471
GCNT3	2.31	0.00	1.361	3.926
DDX3X	2.32	0.05	0.982	5.492
APAF1	2.94	0.04	1.026	8.414
LMBR1L	3.06	0.03	1.099	8.491

Additional File 5. Supplementary Table 1. 34 clinically relevant genes specifically down-regulated after co-treatment with Lapatinib and PHA-767491 in Hs578T and SKBR7 cells.