Uncovering vulnerabilities in triple-negative breast cancer

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Chapter 3

A kinome screen for lapatinib drug resistance identifies FYN as a suppressor of EGFR/PI3K/AKT signaling axis dependency in triple-negative breast cancer

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Abstract

Triple-negative breast cancer (TNBC) is molecularly intricate, with limited therapeutic options. Although approximately 80% of TNBC overexpresses epidermal growth factor receptor (EGFR), TNBC is generally resistant to EGFR targeted therapies, including EGFR tyrosine kinase inhibitors lapatinib, gefitinib and erlotinib. Understanding compensatory signaling mechanisms that mediate the EGFR inhibitor resistance may facilitate to harness effective targeted therapies for TNBC. Here, kinome RNA-interference loss-of-function screen and pharmacological inhibition profiling have defined the vulnerability of AKT/mTOR signaling in EGFR inhibitor resistant TNBC cells. Contrarily, EGFR/MAPK signaling is vulnerable for TNBC cells resistant to mTOR inhibition. EGFR inhibitor lapatinib and kinome siRNA combinatorial screening identified numerous kinase targets that alleviate lapatinib resistance, including FYN, KIT, HK2, NME6 and DCK. Intriguingly, silencing of FYN, a Src family kinase, enhanced EGFR autophosphorylation and downstream AKT phosphorylation. Consequently, pharmacological inhibition of different components of the EGFR/PI3K/AKT axis synergized with FYN depletion. Our results indicate FYN as an EGFR signaling suppressor mediating TNBC resistance to EGFR targeted therapies in TNBC cells that do have increased expression of EGFR. We suggest FYN expression as a potential vulnerability for EGFR inhibitor drug resistance in TNBC.

Introduction

Triple-negative breast cancer (TNBC) does not express progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) that are vulnerable to ER, PR and/or HER2-targeted therapies for other breast cancer types [107]. Although comprehensive genomic profiling has characterized more than six molecular subtypes of TNBC [8], none of them has been benefited from molecularly targeted therapies. TNBC still relies on cytotoxic chemotherapies, yielding unfavorable prognoses, with high likelihood of distant recurrence and death during the first 3-5 years after diagnosis [4]. Treatment of TNBC remains a profound clinical challenge. The lack of treatment options for TNBC makes identifying alternative TNBC vulnerable targets paramount [6].

Many of protein kinases have been defined to play essential roles in cancer initiation and progression and drug resistance [146, 147]. Proto-oncogenic kinases include receptor tyrosine kinases (RTK), BCR-ABL, PIK3CA and MAPK kinases driving proliferation and angiogenesis in cancer [111]. The epidermal growth factor receptor (EGFR) has been defined as a key oncogenic RTK, which coordinates multiple pro-mitogenic signal transduction cascades, principally the Ras-Raf-MEK-ERK, PI3K-AKT-mTOR and Src-STAT3 signaling pathways, to promote cell proliferation, motility, and survival [148-150]. As frequent overexpression of EGFR is observed in various cancer types, such as lung and colorectal cancer and glioblastoma [148, 149, 151, 152], EGFR has been representing an attractive therapeutic target for cancer targeted therapies [153], eliciting high response rates improving patient clinical outcomes [25, 154]. Approximately >80% of TNBC tumors have increased expression of EGFR, hence EGFR targeted therapies have been clinically explored for treating TNBC patients [25, 155]. Also, therapies targeting MAPK/ERK and PI3K/AKT/mTOR kinases downstream of EGFR signaling have been trialed [156, 157]. Yet, the clinical benefits of these targeted therapies for TNBC have been disappointing, due to low response rates and resistance [150]. The mechanisms of TNBC resistance to targeted therapies are likely owing to mutations of the drug targets and compensation by interactive dysregulated signaling pathways [158-160], suggesting to explore alternative combinatorial targeted therapies blocking vulnerable kinase targets in compensatory signaling pathways.

More than 500 protein kinases are encoded in the human genome [161, 162]. Protein kinases are one of the most targeted groups of drug targets [146]. Our previous studies have shown that TNBC cells, regardless of molecular subtypes, are commonly resistant to EGFR inhibitors (EGFRi) [163] and differentially responsive to MEK inhibitors (MEKi) and AKT inhibitors (AKTi) (van de Noord et al, submitted), as well as mTOR inhibitors (mTORi) (He et al, submitted). In this study, by means of high-throughput screen of 720 siRNAs targeting the whole kinome and kinase-related components, in combination with EGFRi lapatinib, we aimed to define alternative signaling pathways and kinase targets vulnerable for therapy-refractory TNBC cells and synergistically targetable with EGFR targeted

therapies. Our kinome-wide siRNA screen and validation of targeted effects by kinase inhibitors revealed that alternatively targeting AKT/mTOR and EGFR/MAPK pathways succumbed resistant TNBC cells to EGFRi and mTORi, and blocking basal cell cycle machinery effectively overcame the differential EGFRi- and mTORi-resistant phenotypes of TNBC cells. Combined drug and kinome siRNA screen identified several novel kinase targets, including FYN, KIT, HK2, NME6 and DCK, whose silencing sensitized EGFRi-refractory TNBC cells to the clinically applied EGFR targeted therapies, such as lapatinib (Lap), gefitinib (Gef) and erlotinib (Erl). Importantly, the Src family kinase FYN [164] was defined to act as a negative regulator of EGFR activity in the EGFRi-resistance of TNBC cells. Targeting FYN enhanced activation of EGFR and AKT signaling, facilitating the effects of EGFR/PI3K/AKT directed inhibitors on signaling inhibition, thereby restoring TNBC cell responsiveness. Our work demonstrated unique kinase dependencies in therapy-resistant TNBC cells and revealed FYN as a potential kinase target vulnerable for EGFRi-refractory TNBC.

Materials and methods

Reagents and antibodies

All kinase inhibitors were purchased from Selleckchem® (Huissen, the Netherlands). The phospho(Ser473)-AKT (9271), phospho(Thr202/Tyr204)-p44/42 MAPK (ERK1/2, 9101), phospho(Ser2448)-mTOR (5536S), phospho((Tyr1148))-EGFR (4404) and phospho(Thr37/46)-4EBP1 (2855) antibodies were from Cell Signaling TECHNOLOGY® (Bioké, Leiden, Netherlands). EGFR (1005) antibody was from Santa Cruz (Heidelberg, Germany). FYN antibody [N1C2] (GTX109428) was from GeneTex (Irvine, US). The antibody against tubulin (T-9026) and human epidermal growth factor (EGF, E9644) were from Sigma Aldrich (Zwijndrecht, the Netherlands).

Cell culture

Cell line Hs578T (ATCC-HBT-126) and MDA-MB-231 (ATCC-HBT-26) were purchased from ATCC. Cell line HCC1806, SUM229PE and SUM52PE were kindly provided by Prof. John W. M. Martens (Erasmus Medical Centre, Rotterdam, the Netherlands). All cell lines were authenticated by short tandem repeat (STR) profiling as previously described [165], and subjected to Mycoplasma test using the Mycosensor PCR kit (#302108, Stratagene). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 25 U/mL penicillin and 25 µg/mL streptomycin in a humidified incubator at 37°C with 5% CO_2.

SRB proliferation assay

A sulforhodamine B (SRB) colorimetric assay was used to measure total amount of proteins indicative of cell proliferation, as previously described [125].

High-throughput human kinome siRNA screen

The primary screen was carried out by use of kinase SMARTpool siRNA library containing 720 siRNAs targeting human kinome-wide 720 kinases and kinase-related components (GE Dharmacon, Lafayette, CO, USA). In the validation screen, SMARTpool siRNA and single siRNA_1, _2, _3 and _4 that comprise the SMARTpool mix were used to target each candidate hit. To silence target genes, 50 nM siGENOME Human SMARTpool siRNA mix was transfected into cells, which were seeded overnight in 96-well plate with optimized densities, by transfection reagent INTERFERin (Polyplus-Transfection SA, Illkirch-Graffenstaden, France) according to the manufacturer's instructions. A pool of 720 kinase siRNAs at stock concentration of 1 µM, which has no effect on gene expression, was taken as non-targeting siRNA control (siCtrl). The medium was refreshed 24 h post-transfection and TNBC cells were transfected for 2 days and proliferated for 4 days under indicated condition. In drug and siRNA combination screen, drug solution was supplemented to cells 2 days post siRNA transfection at indicated concentration, followed by 4-day treatment. SRB colorimetric assay was used as read-out for cell proliferation. Primary kinome siRNA screen data were analyzed using an unbiased sample-based analysis as previously and presented in Z scores [166].

Western Blotting and gene expression data

Cells were seeded in 12-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 stimulated with 100 ng/ml EGF (Sigma; E9644) for 30 min in SFM. Cells were lysed with RIPA buffer containing 1% protease/phosphatase inhibitor cocktail (Sigma-Aldrich, P8340). Proteins were resolved by SDS-PAGE and transferred to polyvinylidine difluoride membranes. Membranes were blocked in 5% BSA in Tris-buffered saline with 0.05% Tween-20 (TBS-T), followed by overnight incubation with primary antibodies, washing, and 1 h incubation with HRP-conjugated secondary antibodies. Chemiluminescence was generated in the presence of HRP substrate and detected with an Amersham Imager 600 (GE Healthcare Life Sciences, Eindhoven, the Netherlands). Log2-based RNA-seq expression profile of TNBC cell lines was retrieved from our own established data (Koedoot et al, submitted).

Gene network and pathway analysis

The STRING database (version 10.5) (https://string-db.org) was applied to analyze and integrate direct and indirect protein-protein interactions (PPI) and functional associations of screened kinase hits. The interaction networks were further analyzed and visualized using Cytoscape v3.7.0. Metascape portal [167] (http://metascape.org) that combines over 40 independent knowledgebases, such as GO Biological Processes, KEGG pathway, Reactome gene sets, Canonical Pathways, CORUM complexes; DAVID functional

annotation tool was used to visualize the functionally enriched pathways and identify MCODE (molecular complex detection) complex of kinase targets, statistically significant ($p < 0.05$) with ≥ 3 targets per cluster.

Statistical analysis

Pearson correlation analysis was performed using GraphPad Prism 7. Statistical analysis of all experimental data was performed using two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All experiments were performed in at least three independent biological replicates. Data were expressed as mean ± SEM. Significance was set at $p < 0.05$. The hierarchical clustering in heatmap was performed using CRAN pheatmap package in RStudio (version 0.99.887).

Results

TNBC cells display differential phenotypic responses to EGFR and mTOR targeted therapies

TNBC patients have not been benefited from targeted therapies in the clinic, owing to the heterogeneous nature of TNBC [107]. Despite frequent overexpression of EGFR in ~80% TNBC tumors [150, 168, 169], targeting EGFR is ineffective for treating TNBC patients [170, 171]. To

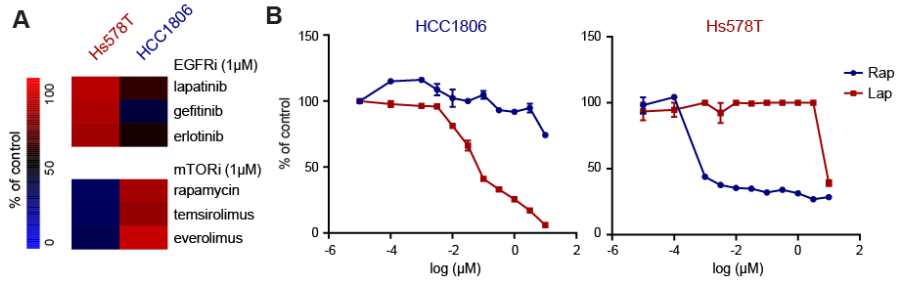

Figure 1. Differential responses of TNBC Hs578T and HCC1806 cells to EGFR inhibitors (EGFRi) and mTOR inhibitors (mTORi). (A) Resistance of Hs578T cells to EGFRi (lapatinib, gefitinib, erlotinib) and HCC1806 cells to mTORi (rapamycin, everolimus, temsirolimus), at 1 μM. **(B)** Dose responses of Hs578T and HCC1806 to lapatinib (Lap) and rapamycin (Rap). Cells were treated with inhibitors at indicated concentrations for 4 days, followed by SRB proliferation assay. Proliferation of cells was presented as % of control, normalized to non-treatment condition with DMSO.

understand the phenotypic responses of TNBC cells to targeted therapies, we have previously screened several clinically applied kinase-targeting small molecules across ~20 TNBC cell lines. Consistent to the clinical results, our previous study has demonstrated the common resistance in >85% of TNBC cell lines to various EGFRi, including Lap, gefitinib (Gef) and erlotinib (Erl) [163], as shown representatively for the TNBC cell line Hs578T (Figure 1A). Exceptionally, one TNBC cell line HCC1806 was found to display sensitivity to the EGFR targeted therapies. Intriguingly, the EGFRi-sensitive HCC1806 cells were strongly

resistant to different mTORi, such as rapamycin (Rap), temsirolimus and everolimus, whereas the EGFRi-refractory Hs578T cells were highly responsive to the mTOR-targeted therapies (Figure 1A). The EGFRi-refractory Hs578T cells and the mTORi-refractory HCC1806 cells were phenotypically responsive in proliferation inhibition to mTORi Rap and EGFRi Lap, respectively, in dose dependent manner (Figure 1B). These differential phenotypic responses of TNBC cells to EGFR and mTOR targeted therapies implicated alternative kinase dependencies in the refractory TNBC cells, rationalizing the discovery of vulnerable kinase targets bypassing inhibition of EGFR-related or mTOR-related signaling, to combat TNBC resistance.

Kinome siRNA screen reveals specific vulnerable kinase targets in EGFRi- and mTORi-resistant TNBC cells

To identify alternatively vulnerable kinase targets in the circumstance of EGFRi-resistance of TNBC, we next performed human kinome-wide siRNA screen targeting 720 protein kinases and kinase-related components in the EGFRi-refractory and mTORi-sensitive Hs578T cells, compared to the EGFRi-sensitive and mTORi-resistant HCC1806 cells. The

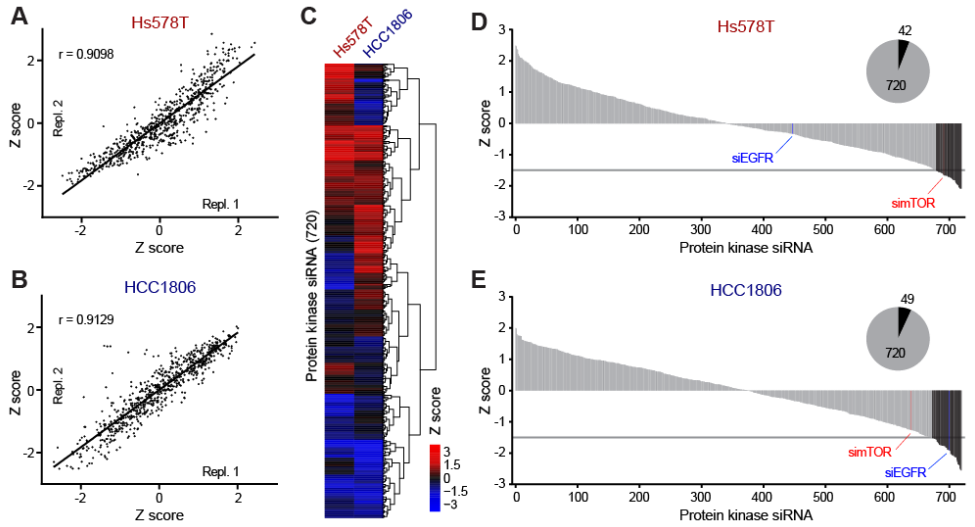

Figure 2. Kinome siRNA screen of EGFRi-resistant Hs578T and mTORi-resistant HCC1806 TNBC cells. (A-B) Effect of replicate kinome siRNA screens of Hs578T cells **(A)** and HCC1806 **(B)** cells was presented in Z scores**.** Cells were transfected with siRNAs for 2 days, and then refreshed and cultured for 4 days, followed by SRB proliferation assay. Pearson correlation coefficient r was representative for reproducibility of replicate screens in Hs578T (r = 0.9098) and HCC1806 (r = 0.9129) cells. **(C)** Heatmap clustering displaying differential targeting effects of kinome-wide siRNAs on Hs578T and HCC1806 cells. Color in blue indicated inhibitory effect on proliferation; in black, no effect; in red, enhanced effect. **(D)** Ranking of individual siRNA targeting effect on Hs578T cell proliferation. **(E)** Ranking of individual siRNA targeting effect on HCC1806 cell proliferation. The effect with Z score < -1.5 (black bars) was considered significant on proliferation inhibition. The significant hits were numbered in pie charts. The effects of siRNA targeting EGFR (siEGFR) and siRNA targeting mTOR (simTOR) were indicated.

targeting effect of each siRNA on cell proliferation was presented in Z score, displaying high reproducibility in the replicate screens of Hs578T (Pearson coefficient r = 0.9098) (Figure 2A) and HCC1806 (r = 0.9129) cells (Figure 2B). Hierarchical clustering revealed distinct vulnerabilities of Hs578T and HCC1806 cells to the kinome RNA interference (Figure 2C; Supplementary Table S1), indicating the different kinase dependencies within these TNBC cell lines. Of 720 kinases, 42 vulnerable kinase targets were screened showing significant targeted effects (Z score < -1.5) on proliferation inhibition of Hs578T cells (Figure 2D), and 49, on HCC1806 proliferative inhibition (Figure 2E).

Consistent to the phenotypic responses to EGFRi and mTORi kinase inhibitors (Figure 1), the EGFRi-resistant Hs578T cells were highly prone to mTOR siRNA (simTOR) silencing (Figure 2D), while the mTORi-resistant HCC1806 cells were strongly sensitized by EGFR siRNA (siEGFR) silencing (Figure 2E). Remarkably, among 42 kinase targets that were vulnerable for the EGFRi-refractory Hs578T cells, mTOR was centered in the protein-protein interaction (PPI) network, and the cell cycle and mTOR pathways were the most vulnerable (Figure 3A), involving the cell cycle kinases PLK1, CDKN2D, CHEK1 and WEE1 and the mTOR signaling components RPS6KA2, STRADA and mTOR (Supplementary Table S2). Within the PPI network of 49 vulnerable kinase targets for the mTORi-resistant HCC1806 cells, EGFR was tightly connected with other components, and the pathways downstream EGFR signaling, such as MAPK pathway, Ras pathway and PI3K/AKT, and the FoxO signaling pathways, were significantly enriched (Figure 3B; Supplementary Table S2). Commonly, 12 kinases were vulnerable for both cell lines, including, MAP3K9, CHEK1, WEE1, RPS6KA2, GUCY2D, RELA, PMVK, AURKA, CAMKIINALPHA, PCTK3, PLK1, CDC2L1, which are functionally involved in cell cycle pathway (Figure 3C).

Next, we reasoned if pharmacological inhibition of the enriched signaling clusters above could attenuate proliferation of the TNBC cells. To this end, we treated the cells with groups of kinase inhibitors pharmacologically targeting EGFR/MAPK, AKT/mTOR and cell cycle signaling, respectively. In line with the findings of kinome siRNA screen, the kinase inhibitor response profiling exhibited that the EGFRi-refractory Hs578T cells were responsive to inhibition of AKT/mTOR signaling by different AKT inhibitors (AKTi) and mTORi (Figure 3D), remaining resistant to diverse EGFRi and less responsive to various MEK inhibitors (MEKi) targeting MAPK signaling (Figure 3E). In contrast, the mTORi-resistant HCC1806 cells were sensitive to EGFR/MAPK inhibition by the EGFRi and MEKi and relatively resistant to AKT and mTOR inhibition. Consistently, both cell lines were sensitive to pharmacological inhibitors targeting cell cycle protein kinases, such as PLK, CDK and Chk (Figure 3F).

The results above indicated that alternative addictions to AKT/mTOR signaling and EGFR/MAPK signaling pathways might confer therapy-refractory phenotypes on TNBC cells. Obstruction of mTOR-centralized network may sensitize the EGFRi-refractory Hs578T cells, while blockage of EGFR/MAPK signaling dependency in the mTORi-resistant HCC1806

cells may restore responses to mTOR-targeted therapies. Targeting PLK, CDK and Chk signaling in the basal cell cycle machinery makes the TNBC cells responsive, suggesting the vulnerabilities of the cell cycle related kinase targets for the refractory TNBC cell types.

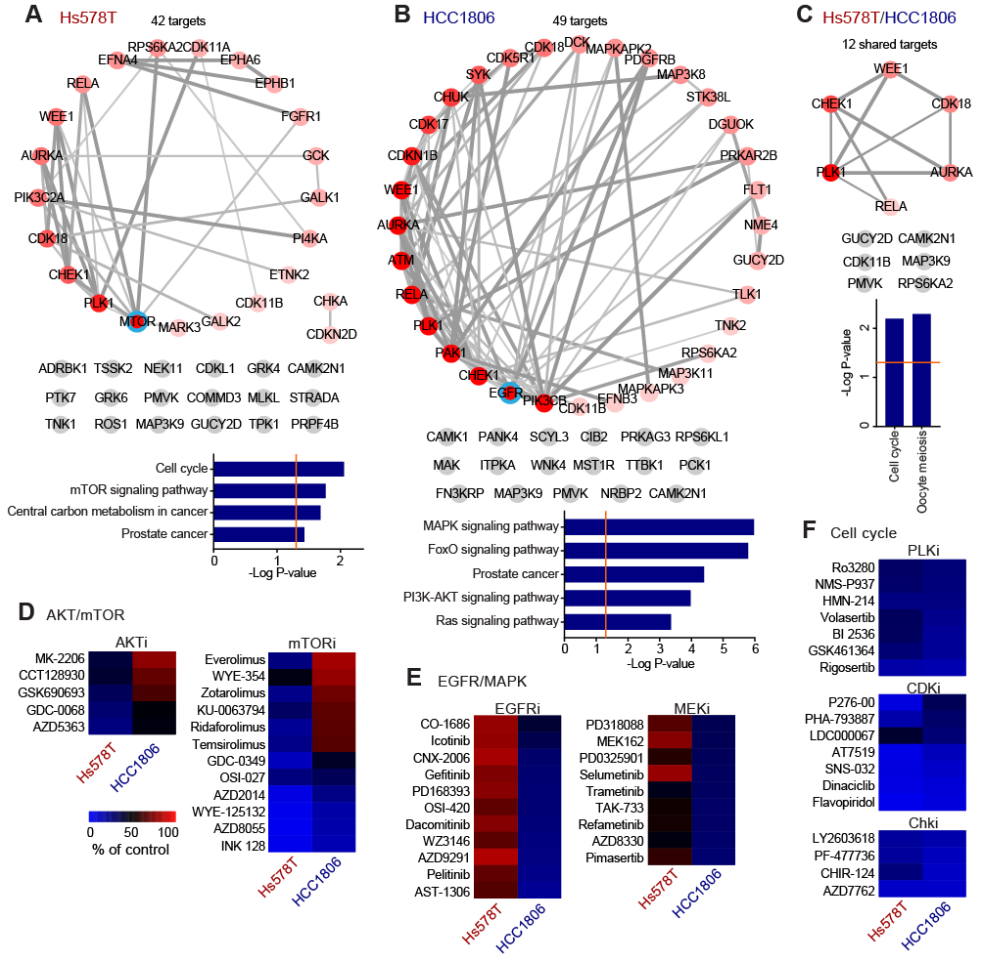

Figure 3. Distinct kinase dependencies of EGFRi-refractory Hs578T cells and mTORi-resistant HCC1806 cells. (A-C) STRING protein-protein interaction (PPI) network and KEGG pathway enrichment of 42 kinase targets vulnerable for EGFRi-resistant Hs578T **(A)**, 49 for mTORi-resistant HCC1806 **(B)**, and 12 for both Hs578T and HCC1806 cells **(C)**. The primary targets EGFR and mTOR were circled in blue. Edge thickness indicated the interaction score. Red color signified the connected kinases. Unconnected kinases were marked in grey. **(D-F)** Proliferative responses of Hs578T and HCC1806 cells to AKT inhibitors (AKTi) and mTORi **(D)**, to EGFRi and MEK inhibitors (MEKi) **(E)**, and to cell cycle related PLK1 inhibitors (PLKi), CDK inhibitors (CDKi) and Chk inhibitors (Chki) **(F)**. Cells were treated with inhibitors at 1 μM for 4 days, followed by SRB proliferation assay.

Lapatinib and kinome siRNA combination screen identifies vulnerable kinase targets sensitizing TNBC cells to EGFR inhibition

Cancer drug resistance is a multifaceted process involving different mechanisms, such as target alteration, alternative cell surface receptors, escape from apoptosis, and DNA damage repair, hence combination of targeted therapies has been applied to sensitize resistant cancer cells [75]. Resistance to EGFR targeted therapies is a common phenotype of TNBC, despite high levels of EGFR expression and clear inhibition of EGFR in TNBC tumor cells [170, 171], suggesting alternative survival signaling pathways bypassing EGFR inhibition,

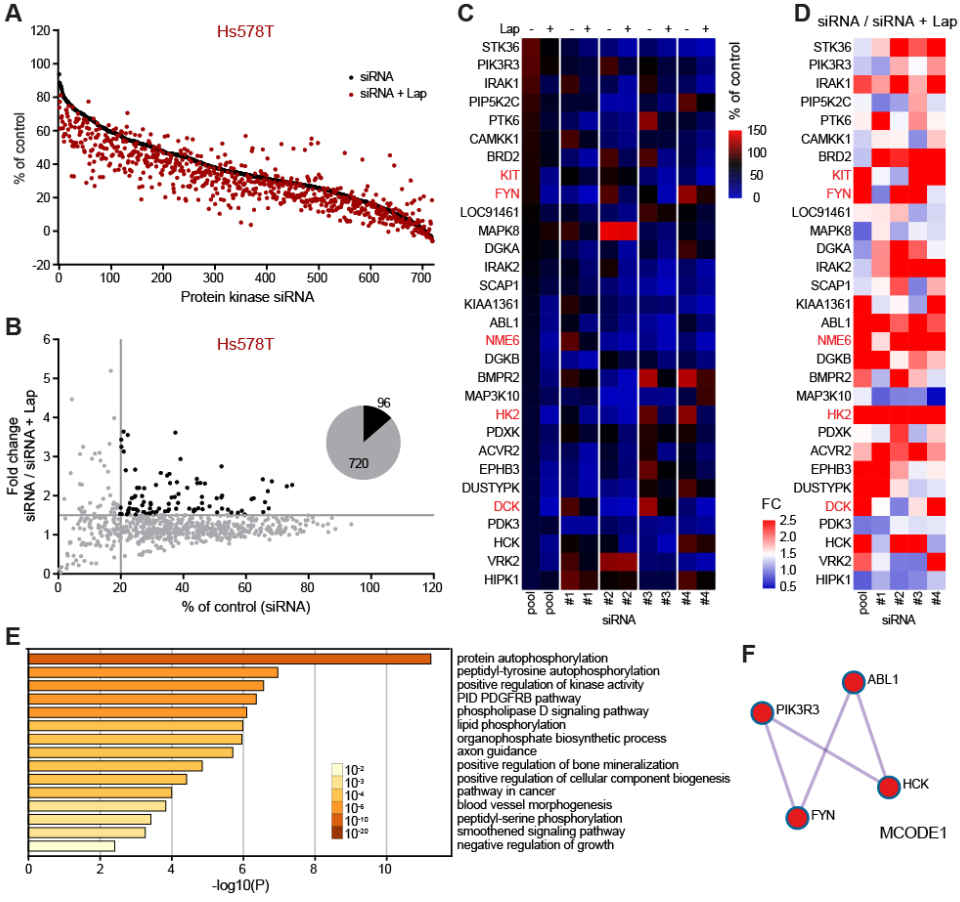

Figure 4. Combined lapatinib and kinome siRNA screen of EGFRi-refractory Hs578T cells. (A) Effect of siRNA and combined siRNA and lapatinib (siRNA + Lap) on Hs578T cell proliferation. Cells were transfected with siRNAs for 2 days, followed by 4-day treatment with Lap at 1 µM or DMSO as control. Percentage proliferation (% of control) was relative to siCtrl + DMSO control. **(B)** Fold change (FC) of proliferation in comparison of siRNA alone to siRNA + Lap combination (siRNA / siRNA + Lap). 96 primary hits with FC > 1.5 were selected, significantly synergizing with Lap to inhibit proliferation. **(C)** Deconvolution screen of Lap synergistic hits with SMARTpool siRNA and individual (1, 2, 3 and 4) siRNAs. Heatmap showed top 30 hits enabling > 50% of proliferation under SMARTpool siRNA silencing alone. **(D)** FC of proliferation (siRNA / siRNA + Lap) controlled by the 30 hits. Five Lap synergistic hits with significant effect by SMARTpool and ≥ 2/4 individual siRNAs (FC > 1.5) were marked in red. **(E)** Metascape bar graph viewing top enrichment clusters of the 30 hits. Color scale represented statistical significance by p-values in log base 10, Log10(P). **(F)** MCODE algorithm displaying the most densely connected hits.

such as AKT/mTOR as shown above. Therefore, to identify kinome-wide alternative kinase targets that are synergistically targetable with EGFR inhibition, thereby understanding the mechanisms underlying EGFRi resistance in TNBC cells, we performed kinome siRNA screen in combination with EGFRi Lap (at a clinically relevant dose, 3.16 μM [172]) in the EGFRi-refractory Hs578T cells. The results showed that the majority of kinases were vulnerable by siRNA silencing to enhance the inhibitory effect of Lap on Hs578T cell proliferation (Figure 4A). We selected kinases, whose silencing per se controlled >20% of proliferation and enhanced 1.5 fold change (FC) of inhibition when combined with Lap (siRNA / siRNA + Lap), as potential synergistic hits. Consequently, 96 hits were selected as potential targets vulnerable for Lap resistance in Hs578T cells (Figure 4B; Supplementary Table S3). These hits were further validated for their vulnerabilities by deconvolution screen with SMARTpool siRNA and 4 deconvoluted siRNAs. 30 hits were further ranked by their SMARTpool siRNA direct silencing effect on >50% of proliferation control (Figure 4C, Supplementary Table S4) and selected for their siRNA silencing effect significantly synergizing Lap with FC ratio (siRNA / siRNA + Lap) greater than 1.5 (Figure 4D), being considered the most vulnerable kinase targets sensitizing TNBC cells to EGFRi Lap.

Next, Metascape pathway enrichment analysis [167] identified 14 clusters where the 30 Lap synergistic kinase targets were significantly enriched (Figure 4E). The most statistically enriched cluster was related to protein autophosphorylation, involving 60% of the Lap synergistic kinase targets (18/30), including FYN, ABL1, EPHB3, HCK, IRAK1, KIT, MAP3K10, PTK6, VRK2, DSTYK, PKDCC, PIK3R3, PIP4K2C, CAMKK1, HK2, BMPR2, PDK3 and SKAP1 (Supplementary Table S5). Peptidyl-tyrosine autophosphorylation and positive regulation of kinase activity clusters were highly ranked, involving 12 (FYN, ABL1, HCK, PTK6, PIK3R3, MAPK8, EPHB3, IRAK1, SKAP1, KIT, IRAK2 and BMPR2) and 18 (FYN, ABL1, IRAK1, IRAK2, KIT, MAP3K10, DSTYK, CAMKK1, MAPK8, PIK3R3, HCK, STK36, SKAP1, PTK6, PDK3, VRK2, HIPK1 and DGKB), respectively. Remarkably, MCODE (Molecular Complex Detection) algorithm identified a tyrosine autophosphorylation network node where FYN, ABL1, HCK and PIK3R3 were densely connected (Figure 4F) and frequently enriched in the phosphorylation and kinase activity related clusters, suggesting their essential implications in TNBC resistance to EGFR targeted therapies.

Lapatinib synergistic kinase target FYN negatively regulates EGFR/AKT signaling

Among the 30 significant Lap synergistic targets, FYN, KIT, HK2, NME6 and DCK displayed on-target silencing effects by pooled and deconvoluted siRNAs (≥ 2/4) to synergize EGFRi Lap (Figure 4C-D). These five targets were further validated for their targeting effects in combination with Lap at 3.16 μM, compared to non-targeting siRNA control (siCtrl) (Figure 5A). Ligand EGF binding results in EGFR autophosphorylation and activation of downstream signaling [173]. As Lap synergistic targets were largely involved in regulation of autophosphorylation and kinase activity (Figure 4E, Supplementary Table S5), we then

further tested their targeting effects on EGFR signaling activation in EGF stimulation. Silencing of these Lap synergistic kinase targets, except for KIT, generally upregulated EGF-induced EGFR phosphorylation at Y1148, a major autophosphorylation site of EGFR [173], as detected by anti-phospho-EGFR (pY1148) antibody. Of them, the targeting effect of FYN on EGFR signaling upregulation was most significant, implying its strong negative regulation on EGFR activity (Figure 5B-C). Downstream of the upregulated EGFR signaling by FYN silencing upon EGF stimulation, phosphorylation of AKT, rather than ERK or mTOR, was increased (Figure 5B-C). Consistently, FYN silencing significantly enhanced the pharmacological inhibitory effect on cell proliferation by EGFRi Lap, Gef and Erl, PI3K inhibitors (PI3Ki) BEZ235 (BEZ) and AKTi AZD5363, not by MEKi selumetinib (Sel) and Src inhibitors (Srci) dasatinib (Das) (Figure 5D). Yet, while FYN knockdown strongly synergized the pharmacological inhibitory effect of EGFRi Lap on proliferation, simultaneous FYN and EGFR silencing did not lead to the similar synthetic lethal effect (Figure 5E), further suggesting the essential impact of FYN on EGFR signaling activation. However, co-silencing of FYN and AKT showed a synergistic effect, similarly to the effect of FYN silencing with EGFRi Lap (Figure 5E).

FYN and the PI3K regulatory subunit, PIK3R3, were tightly interactive in the tyrosine autophosphorylation MCODE network (Figure 4F). Together, the results above suggested that FYN negatively regulated EGFR signaling pathway and downstream AKT effector. Hence, targeting FYN released EGFR/PI3K/AKT signaling, thereby restoring responses of TNBC cells to EGFR/PI3K/AKT-directed treatments, independently of MAPK signaling.

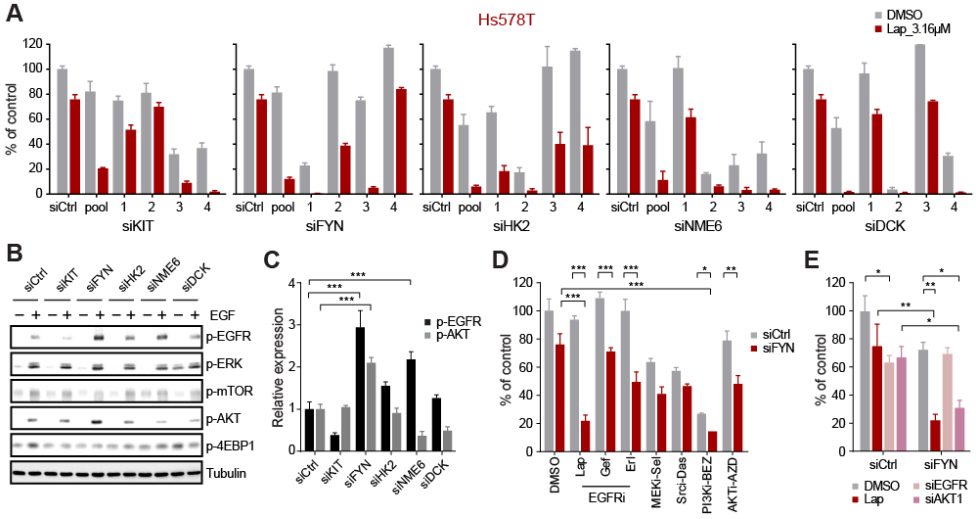

Figure 5. FYN silencing enhanced EGFR/PI3K/AKT signaling in EGFRi-refractory Hs578T cells. (A) Validation of Lap synergistic hits KIT, FYN, HK2, NME6 and DCK in EGFRi-refractory Hs578T cells. Cells were transfected with SMARTpool or 4 single siRNAs for 2 days prior to 4-day Lap treatment. **(B)** Silencing effects of Lap synergistic hits on EGF-induced EGFR signaling. Hs578T cells were transfected with SMARTpool siRNAs for 2 days, starved

overnight, and then exposed to EGF (100 ng/ml) for 30 min. **(C)** Quantification of EGFR and AKT phosphorylation level in response to EGF, relative to tubulin, data derived from **(B)**. **(D)** Effects of FYN silencing on Hs578T cellular response to EGFRi (Lap, Gef and Erl), MEKi selumetinib (Sel), Src inhibitor (Srci) dasatinib (Das), PI3Ki BEZ235 (BEZ), and AKTi AZD5363 (AZD). **(E)** Co-silencing Effects of FYN and EGFR or AKT on Hs578T cell proliferation (two-way ANOVA * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

FYN silencing-mediated lapatinib synergy associates with active EGFR/PI3K/AKT signaling

Next, we questioned whether FYN silencing-mediated Lap synergy was associated with expression and activation of EGFR and the downstream active AKT signaling in TNBC cells. We chose three TNBC cell lines, Hs578T, MDA-MB-231 and SUM229PE, with high EGFR expression, and one EGFR negative TNBC cell line SUM52PE as negative control (Figure 6A). All these TNBC cell lines expressed overall similar levels of FYN, with Hs578T cells showing the highest expression. Downstream active p-AKT was positively detected in SUM52PE and Hs578T cell lines, but were largely absent in MDA-MB-231 and SUM229PE cell lines (Figure 6B), consistent with our previous findings (van de Noord et al, submitted). Treatment with the EGFR inhibitors lapatinib, gefitinib and erlotinib did not affect SUM52PE cells, as they lack expression of EGFR. In contrast, Hs578T, MDA-MB-231 and SUM229PE were commonly resistant to the EGFR targeted therapies, despite the presence of EGFR (Figure 6B-C), recapitulating our previous findings [163]. FYN is a member of Src family [164]. Treatment with inhibitors for FYN (FYNi) PP1, PP2 and saracatinib (Sar), which also potently inhibit other Src family members (Selleckchem®), did not cause any cellular responses (Supplementary Figure S1A), exhibiting common resistance of these TNBC cell lines to single FYN inhibition. Moreover, the FYNi PP1, PP2 or Sar did also not synergize with EGFRi Lap to inhibit proliferation of Hs578T cells (Supplementary Figure S1B), suggesting that FYN kinase activity does not bypass EGFR signaling inhibition. Remarkably, siRNA silencing of FYN significantly synergized with EGFRi Lap, PI3Ki BEZ and AKTi AZD in the EGFR positive and p-AKT positive Hs578T cells, but not in the EGFR negative SUM52PE and the p-AKT negative MDA-MB-231 and SUM229PE cells (Figure 6E). FYN silencing enhanced upstream EGFR and downstream AKT phosphorylation (Figure 5B), and synergized with knockdown of AKT, but not EGFR, to inhibit proliferation (Figure 5E). Together, these results indicated that EGFR expression and phosphorylation and EGFR-activated downstream PI3K/AKT signaling were essential for FYN silencing-mediated sensitization of TNBC cells to EGFR targeted therapies, such as Lap, as illustrated (Figure 6E), further suggesting FYN as a potential target vulnerable for TNBC resistance to EGFR inhibition. This combinatorial effect of FYN and EGFR targeting seems highly TNBC context dependent, and warrants strategies for stratifying drug sensitivity for individual TNBC patients.

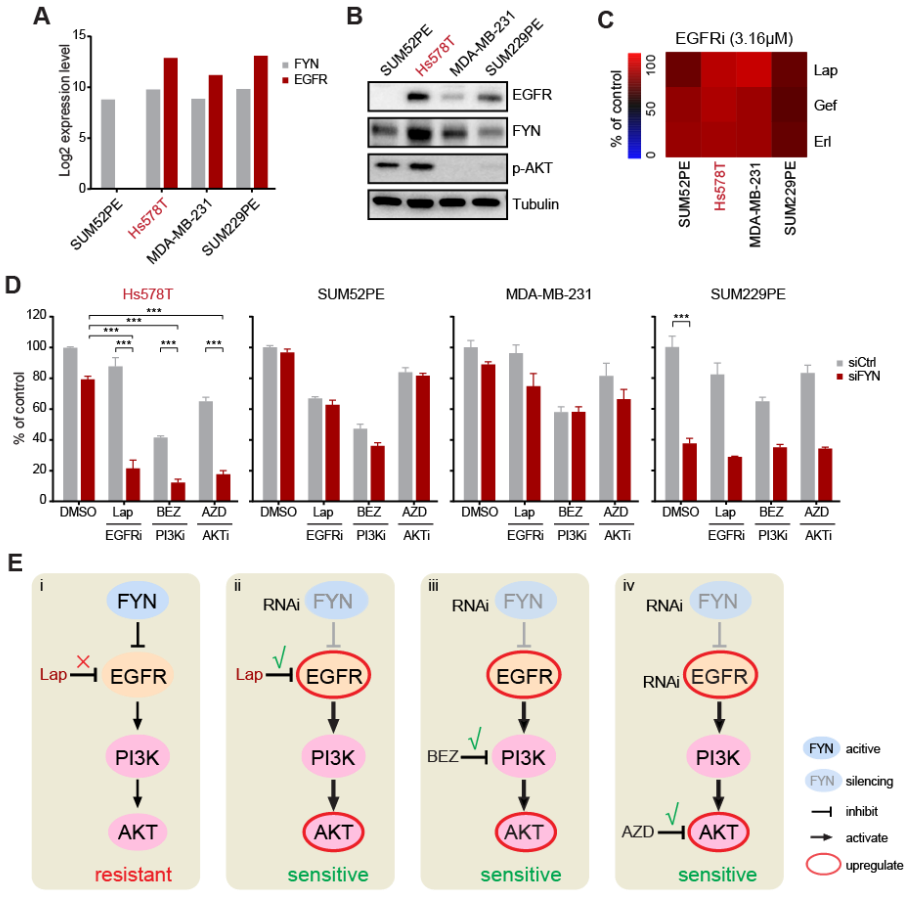

Figure 6. Association of FYN silencing-mediated lapatinib synergy with EGFR and p-AKT expression. (A) Log2-based RNA-seq gene expression levels of FYN and EGFR in SUM52PE, Hs578T, MDA-MB-231 and SUM229PE TNBC cell lines. **(B)** Expression of EGFR, FYN, and phosphorylated AKT (p-AKT) in the TNBC cell lines. **(C)** Proliferative responses of the TNBC cell lines to EGFRi Lap, Gef and Erl. Cells were treated with EGFRi at 1 μM for 4 days, followed by SRB proliferation assay. **(D)** Effects of FYN silencing on cellular responses of the TNBC cell lines to EGFRi Lap, PI3Ki BEZ and AKTi AZD (two-way ANOVA * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **(E)** Diagram illustrating mechanisms underlying FYN silencing-mediated Lap synergy. FYN negatively regulated EGFR activity, limiting downstream PI3K/AKT signaling, rendering TNBC cells refractory to EGFRi Lap (i). RNA interfering (RNAi) of FYN released the negative regulation of EGFR, amplifying EGFR signaling responsiveness to EGFRi Lap (ii). Release of EGFR activity from FYN RNAi enhanced downstream AKT signaling, leading sensitivity to PI3Ki BEZ (iii) and AKTi AZD (iv).

Discussion

TNBC commonly expresses high level of EGFR [150, 151]. However, in most cases the TNBC disease is poorly responsive to EGFR targeted therapies [25, 174]. Various resistance mechanisms can operate in cancer, such as increased drug efflux, mutations of the drug targets, DNA damage repair, and alternation of signaling pathways to evade cell death [24, 174, 175]. Activated downstream pathways, primarily the Ras-Raf-MEK-MAPK and PI3K-AKT

pathways, are causatively one of the major resistance mechanisms to EGFR tyrosine kinase inhibitors in lung cancer [24, 175]. The molecular mechanism of TNBC resistance to EGFR targeted therapies is yet uncertain. In this study, our siRNA-based kinome-wide loss of function screen revealed numerous vulnerable kinase targets mediating proliferative phenotypes of EGFRi-refractory TNBC cells. Among these kinase targets, mTOR was the most vulnerable and centralized in the interactive signaling network. These kinases fell mainly into cell cycle (such as PLK1, CHEK1 and WEE1) and mTOR signaling (such as RPS6KA2 and mTOR) pathways, and cancer-related metabolism process (including FGFR1 and GCK). Treatment with inhibitors targeting AKT/mTOR signaling attenuated proliferation of EGFRi-refractory TNBC cells. In addition, we defined addiction to EGFR/MAPK signaling in one mTORi-resistant TNBC cell line. These results indicated that TNBC cells develop alternative addictions, for instance, to AKT/mTOR signaling pathway, to gain resistant phenotypes against EGFR targeted therapies. Exploration of the kinase targets vulnerable for EGFRi-refractory phenotype of TNBC cells may further facilitate understanding the resistance mechanisms and identifying selective targeted therapies against the alternative kinase addictions in TNBC cells. Importantly, vulnerabilities of EGFRi- and mTORi-resistant TNBC cells to inhibitors targeting cell cycle kinases, such as PLK, CDK and Chk, reflected the dependency of TNBC cells on basal cell cycle machinery for maintaining cell proliferation.

To overcome EGFRi-refractory phenotypes of cancer cells, another option is to define potential combinatorial inhibition of alternative signaling activations to enhance cellular responsiveness to EGFR targeted therapies. Clinical data confirmed that PIK3CA oncogenic mutation resulted in dramatically suppressed sensitivity of lung adenocarcinomas to EGFRi [176]. A genome-wide loss-of-function screen identified PI3K hyperactivation was associated with lapatinib resistance of HER2-positive breast cancer cells and could be reversed by PI3Ki BEZ235 [177]. Loss of PTEN, which mediates activation of AKT, contributes to erlotinib resistance in colorectal cancer [178]. Our EGFRi lapatinib and kinome siRNA combination screen identified a group of kinases vulnerable for lapatinib resistance of TNBC cells. The majority of the kinases were functionally enriched in regulation of protein phosphorylation and kinase activity. Among them, the kinases ABL1, FYN, HCK, IRAK1, KIT, PIK3R3, PTK6 and SKAP1 were most commonly clustered and annotated to regulate tyrosine autophosphorylation, an essential process that RTKs and non-receptor tyrosine kinases undergo to be self-phosphorylated and activated. These results suggested that these signaling regulatory components might regulate autophosphorylation and activation of EGFR, leading to resistance of TNBC cells to EGFR targeted therapies. Therefore, targeting these vulnerable kinase targets may potentially unleash effective EGFR inhibition by EGFR tyrosine kinase inhibitors, such as gefitinib, erlotinib and lapatinib, for treating the refractory TNBC.

Our subsequent EGFRi lapatinib and siRNA deconvolution screen has functionally validated a few kinases, including FYN, KIT, HK2, NME6 and DCK, as potential vulnerable targets for lapatinib resistance of TNBC cells. Most importantly, silencing of these kinases, particularly FYN, a member of Src family kinases (SFKs), enhanced the ligand EGF binding-induced autophosphorylation of EGFR and activation of downstream PI3K/AKT signaling, thereby enabling EGFRi lapatinib to effectively inhibit EGFR/PI3K/AKT signaling and restoring the responsiveness of TNBC cells. SFKs are non-receptor tyrosine kinases, including Src, Fyn, Yes, Blk, Yrk, Frk, Fgr, Hck, Lck, Srm, and Lyn [179]. SFKs intensively integrate with transmembrane RTKs and transduce RTK signaling to downstream effectors, such as PI3K, AKT and STAT3, promoting cell proliferation, survival, migration, invasion and drug resistance [179, 180]. FYN is overexpressed in various cancers, including prostate cancer, head and neck carcinoma and melanoma [181, 182]. Yet, the role of FYN overexpression in cancer is to be well defined. One study presents that overexpression of FYN in prostate cancer supports FYN as a novel potential target for prostate cancer therapy [181], whereas another shows that loss of FYN expression in prostate cancer and high levels of FYN in benign prostatic hyperplasia propose FYN as a potential tumor suppressor [183]. Functionally, in breast cancer, while FYN knockdown does not attenuate tumor cell proliferation and tumor growth rate of basal type breast cancer MDA-MB-231 cells, depletion of FYN suppresses metastatic ability of MDA-MB-231 tumor cells [184]. FYN promotes mesenchymal phenotypes of basal type breast cancer cells through epithelial-mesenchymal transition via STAT5/NOTCH2 signaling node [184]. Increased FYN activity blocks EGFR mitogenic signaling to suppress growth of keratinocytes [164]. Phospho-proteomics profiling has linked FYN, as well as LYN and LCK, to EGFRi lapatinib resistance in a cohort of HER2 positive breast tumors following lapatinib treatment [185]. Here, our results have defined FYN as a negative regulator of EGFR signaling, specifically on autophosphorylation and kinase activity of EGFR, mediating lapatinib resistance in TNBC cells. Depletion of FYN enhanced EGF-induced EGFR autophosphorylation and downstream AKT phosphorylation. FYN silencing synergized with lapatinib in EGFR-positive but not in EGFR-negative TNBC cells, while co-silencing of FYN and EGFR in EGFR-positive TNBC cells did not support the synergistic effect, suggestive of the regulatory effect of FYN on EGFR phosphorylation level but not EGFR expression. Particularly, the synergistic effect of FYN silencing and EGFR pharmacological inhibition by lapatinib required active AKT signaling, indicating a prerequisite of an intact EGFR/PI3K/AKT signal route for the FYN silencing-mediated synergy. Our work reveals that FYN-mediated EGFR/PI3K/AKT signaling is one mechanism of TNBC resistance against EGFR targeted therapies, supporting FYN as a potential vulnerable target for EGFRi-refractory TNBC.

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Supplementary data

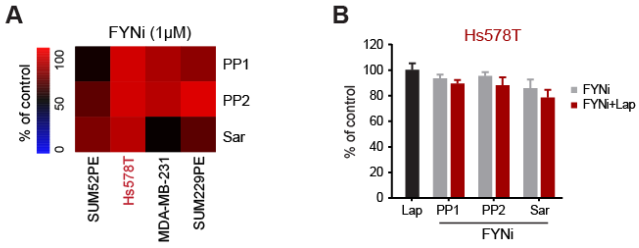

Figure S1. Proliferative responses of TNBC cells to FYN inhibitors (FYNi) PP1, PP2 and saracatinib (Sar) alone (A) and in combination with EGFRi Lap (B). Cells were treated with FYNi at 1 µM alone or in combination with Lap at 3.16 µM for 4 days, followed by SRB proliferation assay.

Table S1. Differential targeting effects of kinome-wide siRNAs on Hs578T and HCC1806 cells.

Table S2. KEGG pathway enrichment of direct targeting kinases in Hs578T and HCC1806 cells.

Pathway Enrichment of 42 kinase targets in Hs578T cells				
Category	**Term**	**-logP**	**PValue**	**Symbols**
KEGG_PATHWAY	hsa04110:Cell cycle	2.0567	0.008776	PLK1, CDKN2D, CHEK1, WEE1
KEGG_PATHWAY	hsa04150:mTOR signaling pathway	1.7665	0.017121	RPS6KA2, STRADA, MTOR
KEGG_PATHWAY	hsa05230:Central carbon metabolism in cancer	1.6858	0.020618	FGFR1, GCK, MTOR
KEGG_PATHWAY	hsa05215:Prostate cancer	1.4293	0.037217	FGFR1, RELA, MTOR
Pathway Enrichment of 49 kinase targets in HCC1806 cells				
Category	**Term**	**-logP**	**PValue**	**Symbols**
KEGG_PATHWAY	hsa04010:MAPK signaling pathway	5.9751	1.06E-06	EGFR, RPS6KA2, RELA, MAP3K8, MAPKAPK3, PDGFRB, PAK1, MAPKAPK2, CHUK, MAP3K11
KEGG_PATHWAY	hsa04068:FoxO signaling pathway	5.7906	1.62E-06	PRKAG3, EGFR, CDKN1B, PIK3CB, PLK1, ATM, CHUK, PCK1
KEGG_PATHWAY	hsa05215:Prostate cancer	4.3985	3.99E-05	EGFR, CDKN1B, PIK3CB, RELA, PDGFRB, CHUK
KEGG_PATHWAY	hsa04151:PI3K-Akt signaling pathway	3.9773	1.05E-04	EGFR, CDKN1B, FLT1, PIK3CB, RELA, PDGFRB, CHUK, SYK, PCK1
KEGG_PATHWAY	hsa04014:Ras signaling pathway	3.3618	4.35E-04	EGFR, FLT1, PIK3CB, RELA, PDGFRB, PAK1, CHUK
Pathway Enrichment of 12 kinase targets in both Hs578T and HCC1806 cells				
Category	**Term**	**-logP**	**PValue**	**Symbols**
KEGG_PATHWAY	hsa04114:Oocyte meiosis	2.2891	0.00514	RPS6KA2, PLK1, AURKA
KEGG_PATHWAY	hsa04110:Cell cycle	2.1952	0.00638	PLK1, CHEK1, WEE1

Table S3. 96 Lap synergistic kinase targets for EGFRi-refractory Hs578T cells.

Table S4. Deconvolution screen of 96 Lap synergistic kinase targets in Hs578T cells.

Table S5. Metascape gene annotation and pathway enrichment of 30 Lap synergistic kinase targets.

Pathway Enrichment								
GroupID	**Category**	**Term**	**Description**	**LogP**	**Log(q-value)**	**InTerm_InList**	**Genes**	**Symbols**
1_Summary	GO Biological Processes	GO:0046777	protein autophosphorylation	-11.2472622	-6.930	9/235	25,2049,2	IK3,SKAP1
2_Summary	GO Biological Processes	GO:0038083	peptidyl-tyrosine autophosphorylation	-6.96820337	-3.253	4/37	25,2534,3	IK2,BMPR2
3_Summary	GO Biological Processes	GO:0033674	positive regulation of kinase activity	-6.57270683	-3.126	8/574	25,2534,3	IPK1,DGKB
4_Summary	Canonical Pathways	M186	PID PDGFRB PATHWAY	-6.36614423	-3.003	5/129	25,2534,3	DCC,HIPK1
5_Summary	KEGG Pathway	hsa04072	Phospholipase D signaling pathway	-6.099446	-2.861	5/146	1606,1607	PHB3,HK2
6_Summary	GO Biological Processes	GO:0046834	lipid phosphorylation	-5.99472614	-2.823	4/64	1606,1607	MAPK8,KIT
7_Summary	GO Biological Processes	GO:0090407	organophosphate biosynthetic process	-5.96452684	-2.823	8/691	1606,1633	NK1,IRAK2
8_Summary	KEGG Pathway	hsa04360	Axon guidance	-5.71144786	-2.649	5/175	25,659,20	36,MAPK8
9_Summary	GO Biological Processes	GO:0030501	positive regulation of bone mineralization	-4.85987918	-2.048	3/39	92,659,91	IRAK1,KIT
10_Summary	GO Biological Processes	GO:0044089	positive regulation of cellular component biogenesis	-4.42485898	-1.789	6/541	25,2049,3	1,PIP4K2C
11_Summary	KEGG Pathway	hsa05200	Pathways in cancer	-4.00905126	-1.506	5/395	25,3815,5	K,ACVR2A
12_Summary	GO Biological Processes	GO:0048514	blood vessel morphogenesis	-3.84175921	-1.410	6/690	25,659,20	4K2C,HCK
13_Summary	GO Biological Processes	GO:0018105	peptidyl-serine phosphorylation	-3.42436367	-1.195	4/289	4294,5165	PK8,VRK2
14_Summary	GO Biological Processes	GO:0007224	smoothened signaling pathway	-3.27014482	-1.093	3/133	4294,2714	K36,HIPK1
15_Summary	GO Biological Processes	GO:0045926	negative regulation of growth	-2.4177288	-0.475	3/264	659,5753,	ME6,ABL1