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Combinatorial CRISPR screen reveals *FYN* and *KDM4* as targets for synergistic drug combination for treating triple negative breast cancer

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eLife Assessment

This study presents a **valuable** finding that synthetically lethal kinase genes FYN and KDM4 may play a role in drug resistance to kinase inhibitors in TNBC. The evidence supporting the claims of the authors is **solid**, although the exploration of the upstream mechanisms regulating KDM4A or the downstream pathways through which FYN upregulation confers drug resistance would have strengthened the study. The work will be of interest to medical biologists working in the field of breast cancer.

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Abstract

Tyrosine kinases play a crucial role in cell proliferation and survival and are extensively investigated as targets for cancer treatment. However, the efficacy of most tyrosine kinase inhibitors (TKIs) in cancer therapy is limited due to resistance. In this study, we identify a synergistic combination therapy involving TKIs for the treatment of triple negative breast cancer. By employing pairwise tyrosine kinase knockout CRISPR screens, we identify *FYN* and *KDM4* as critical targets whose inhibition enhances the effectiveness of TKIs, such as NVP-ADW742 (IGF-1R inhibitor), gefitinib (EGFR inhibitor), and imatinib (ABL inhibitor) both *in vitro* and *in vivo*. Mechanistically, treatment with TKIs upregulates the transcription of *KDM4*, which in turn demethylates H3K9me3 at *FYN* enhancer for *FYN* transcription. This compensatory activation of *FYN* and *KDM4* contributes to the resistance against TKIs. *FYN* expression is associated with therapy resistance and persistence by demonstrating its

upregulation in various experimental models of drug-tolerant persisters and residual disease following targeted therapy, chemotherapy, and radiotherapy. Collectively, our study provides novel targets and mechanistic insights that can guide the development of effective combinatorial targeted therapies, thus maximizing the therapeutic benefits of TKIs.

Introduction

Tyrosine kinases have emerged as important drug targets in cancer therapy due to their druggability and pivotal roles in cell proliferation and survival(1). They are implicated in various aspects of cancer development(2), such as cell survival, proliferation, angiogenesis, and invasion, making them attractive targets for drug intervention. Consequently, tyrosine kinase inhibitors (TKIs) have gained considerable attention as primary agents for cancer treatment.

Triple negative breast cancer (TNBC) treatment has limited options for targeted therapy. TNBC, characterized by the absence of estrogen receptor, progesterone receptor, and HER2 expression, exhibits elevated activity of tyrosine kinases, including EGFR and IGF1R(3, 4). However, several clinical trials investigating TKIs, such as VEGFR inhibitors, EGFR inhibitors, and FGFR inhibitors, in TNBC treatment have yielded disappointing results due to inadequate efficacy. Therefore, it is crucial to comprehend the mechanisms underlying TNBC's suboptimal response to TKIs to enable the development of more effective targeted therapies against TNBC.

The therapeutic efficacy of TKIs is compromised by intrinsic and acquired resistance(5). For instance, EGFR inhibitor gefitinib extended the median progression-free survival by only five months compared to conventional chemotherapy in non-small cell lung cancer (NSCLC) patients with EGFR mutation(6). Significant subset of drug resistance is driven by gene interactions that enable compensatory changes in signal transduction upon drug treatment. Compensatory activation of mitogenic signals, such as MET, PIK3CA amplification, and MAPK/ERK signaling activation, counterbalances the inhibition of EGFR by TKI osimertinib in a significant portion of NSCLC patients(7). Simultaneous inhibition of multiple signaling molecules that compensate for each other's loss is proposed as an effective strategy to overcome resistance to kinase inhibitor therapy, emphasizing the importance of combinatorial therapy(8).

Until recently, a highly scalable method for screening combinatorial therapy has been lacking. Combinatorial CRISPR screens have emerged as efficient tools to identify synergistic targets for combinatorial therapy. We and others recently developed combinatorial CRISPR screens to elucidate pairwise gene interactions(9–12). Our combinatorial genetic screen platform, combinatorial genetics *en Masse* (combiGEM), was successfully implemented to identify combinations of epigenetic regulators causing synthetic lethality in ovarian cancer cells (9).

In this study, we utilize CombiGEM-CRISPR technology to identify tyrosine kinase inhibitor combinations with synergistic effect in TNBC cell line and xenograft models for potential combinatorial therapy against TNBC. We highlight FYN as a key therapeutic target that, when inhibited, enhances the cytotoxic effect of inhibition of other tyrosine kinases (IGF1R, EGFR, and ABL2). Mechanistic studies reveal KDM4 as a crucial epigenetic regulator that demethylates H3K9me3 and transcriptionally upregulates *FYN* upon TKI treatment. *In vitro* and *in vivo* validation demonstrates the synergistic TNBC-shrinking effects of combining PP2, saracatinib (Src family kinase / FYN inhibitor) or QC6352 (KDM4 inhibitor) with TKIs. Additionally, we demonstrate the clinical significance of our findings by observing upregulation of *FYN* in various models of drug tolerant persisters and residual tumors after chemo-, radio-, or targeted therapy. Therefore, simultaneous targeting of *FYN-KDM4* and tyrosine kinase pathways through combinatorial therapy holds promise for effective therapy against TNBC.

Results

Pairwise tyrosine kinase knockout CRISPR screen reveals synergistic tyrosine kinase inhibition combination

For efficient translation of CRISPR screening data to drug combination, we selected 76 tyrosine kinases that could be inhibited by at least one drug from the drug repurposing hub database (table S1) (13). For pairwise CombiGEM library construction, we chose three guide RNAs from the optimized Brunello single guide RNA (sgRNA) list(14) employing the iterative cloning method as previously described(9, 15). The resulting library enabled screens of pairwise knockouts of the 76 tyrosine kinase genes, encompassing 54,289 sgRNA pairs representing 3,003 pairwise gene disruptions (**Fig. 1A**). To validate our library, we performed next-generation sequencing and confirmed that 99.5% (2,989/3,003) of gene pairs were represented by at least 6 pairs of sgRNAs, with the $\log_{10}$ reads per million of 0.5 (Fig. S1A).

Triple negative breast cancer cell line MDA-MB-231 cells stably expressing Cas9 were transduced with the lentiviral library at low multiplicity of infection (MOI) of 0.3. Genomic DNA was harvested 3 days after transduction (designated as day 0[D0]), and 23 days after transduction (D20) (**Fig. 1A**) to perform PCR amplification of sgRNA pairs for subsequent next-generation sequencing (NGS) analysis. sgRNAs, instead of barcodes in our previous CombiGEM screens, were directly sequenced using paired-end sequencing to rule out uncoupling of barcodes of sgRNAs and barcodes (Fig. S1B-C). We counted the occurrences of each sgRNA pair in the NGS data and calculated the normalized log2 fold change in counts between the day 20 and day 0 samples as the growth phenotype score Z (see Methods). The Z scores for the two permutations of an sgRNA pair (r=0.50 between sgRNA-A + sgRNA-B and sgRNA-B + sgRNA-A pairs), the biological replicates, (r = 0.74 between replicates #2 and #3), and independent sgRNA pairs targeting the same set of genes were positively correlated (r=0.3-0.72) (Fig. S1D-G).

Gene pairs that synergistically kill cells were identified by calculating gene interaction scores (GI). The GI scores were derived by comparing the growth phenotype score Z resulting from the disruption of a gene pair ($Z_{A+B,}$ observed Z score) to the sum of Z scores obtained from the disruption of each gene individually within the pair (Z_{A+Con} + $Z_{B+Con,}$ expected Z score) (**Fig. 1B**). The expected and observed Z scores for each gene (or sgRNA) pair exhibited a strong positive correlation (r=0.97 gene level, r=0.88 sgRNA level), suggesting that most random pairwise combinations of tyrosine kinase perturbations show additive effects (**Fig. 1B** and S1H). GI scores were calculated by quantifying each gene pair's normalized deviation from the quadratic fit of the expected-observed Z score plot(10) (**Fig.1C** and Fig. S1I, see methods).

We selected thirty synthetic lethal gene pairs using cutoffs for gene level GI score <-2 and p<0.01 for GI scores determined by RIGER analysis(16) and Z<-5 (**Fig. 1D** and dataset S1). Among these, the SRC-YES pair was one of the strongest synthetic lethal gene pairings (GI= −3.95). Notably, SRC-YES belong to the same tyrosine kinase family and are known to be functionally redundant and are expected to be synthetic lethal(17). These findings provide evidence for the effectiveness of our screening approach in identifying synthetic lethal gene pairs.

Synthetic lethal gene pairs are next validated by expressing the pair of sgRNAs targeting them. To achieve this, we introduced lentiviral vectors carrying two distinct sgRNAs targeting the candidate synthetic lethal pairs, each tagged with a different fluorescent protein (GFP and mCherry). MDA-MB-231 Cas9 cells were transduced with the lentivirus at a low titer (MOI ~0.5), resulting in a mixed population of cells expressing either one or both sgRNAs along with their respective fluorescent proteins (**Figs. 2A** and S2A). We monitored the decrease in the number of GFP/mCherry double-positive cells to evludate synthetic lethality (see methods). We validated the

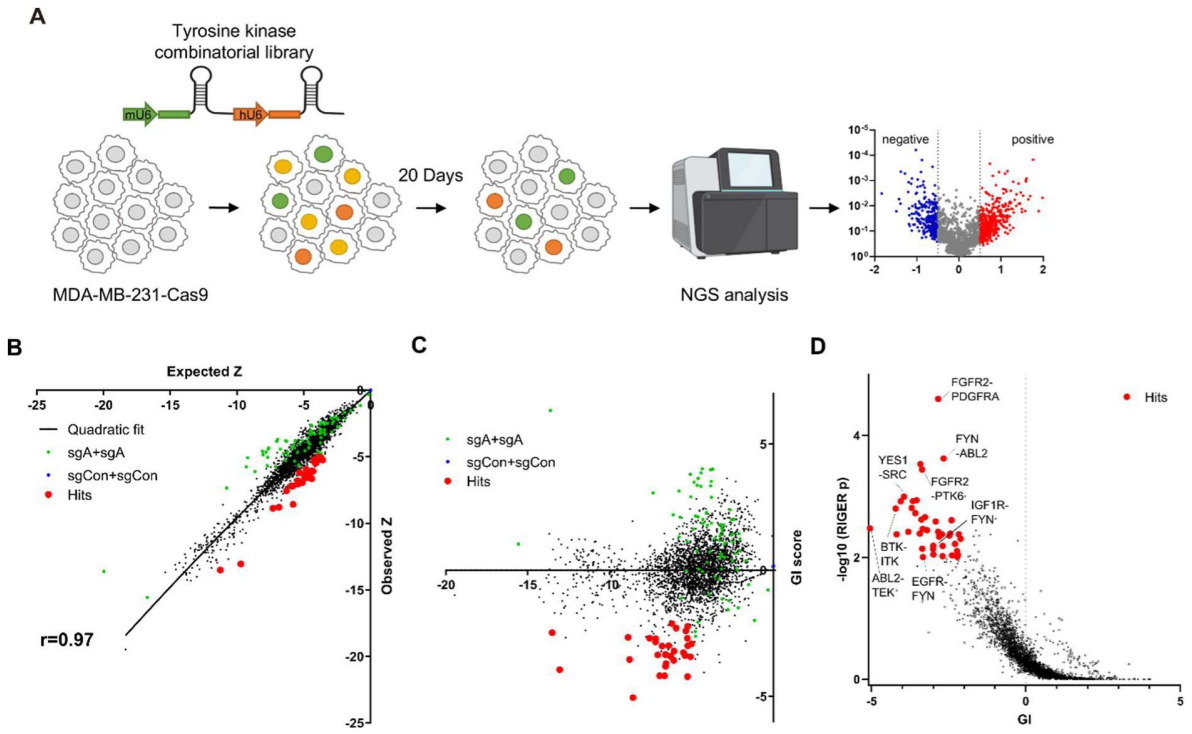

Figure 1.

Pairwise CRISPR screen reveals combinations of synthetic lethal tyrosine kinase ablations.

(A) Schematic diagram of combinatorial screens performed in TNBC cell line MDA-MB-231. (B) Scatter plot of expected growth phenotype Z score and observed growth phenotype Z score of each gene combination. Green dots indicate gene combinations where the identical gene is targeted by the two sgRNAs. Red dots indicate candidate synthetic lethal gene pairs listed in table S1. (C) Scatter plot of growth phenotype Z score and normalized GI score of each gene combination. (D) Scatter plot of gene level GI score and RIGER p value calculated with GI scores of each sgRNA pairs that target the given gene pair.

efficacy of the sgRNAs used in **Figure 2A** through the T7 endonuclease assay, which confirmed efficient gene editing (Fig. S2B). Consistent with our CRISPR screening results, we observed that the disruption of six out of eight synergistic target gene combinations led to a reduction in cell viability beyond what was predicted by the Bliss independence model (**Fig. 2B**). Moreover, the relative viability of double knockout cells and the rate of synergistic killing demonstrated a strong correlation with our screening data (r = 0.65 for both viability and synergistic effect; Fig. S2C). Collectively, our findings provide compelling evidence that our screening approach successfully identified synthetic lethal gene pairs with a high level of confidence.

FYN inhibition synergizes with IGF1R, EGFR, ABL2 inhibitions in cell killing

We noticed that several validated synergistic target gene pairs included *FYN* (e.g. *FYN*+IGF1R, *FYN*+EGFR, and *FYN*+ABL2). Notably, network analysis of the 30 candidate synergistic tyrosine kinase pairs revealed that *FYN* is one of the key nodes participating in synergistic interactions with multiple genes (**Fig. 2C**). Expression of FYN, a member of Src family kinase, has been implicated in cancer malignancy including drug resistance(18–21). Particularly, recent studies highlighted significant contribution of FYN in TNBC malignancy by promoting epithelial-to-mesenchymal transition (EMT)(22, 23). Interestingly, we found that *FYN*, but not *SRC*, exhibited significant upregulation in triple-negative breast cancer (TNBC) compared to other subtypes, as evidenced by microarray data from primary tumor samples (24) and the cancer cell line encyclopedia (CCLE)(25) (**Figs. 2D-E**). In contrast, other key nodes in **figure 2C**, including FGFR2, FRK and TEK were not expressed at appreciable levels in MDA-MB-231 (log2(TPM+1) for TEK: 0.0704, FRK:0.124, FGFR2:0.227), and their expressions were not significantly upregulated in TNBCs compared to other breast cancer subtypes (Fig. S3). Therefore, we proceeded further in focusing on validating FYN as key candidate synthetic lethal gene. These findings suggest that FYN could represent an attractive drug target for TNBC treatment. To investigate this further, we assessed whether simultaneous inhibition of FYN by PP2, which selectively targets the SRC family kinase inhibitor with the highest potency against FYN, in combination with other kinase inhibitors (TKIs), could inhibit cancer cell growth(26). PP2 as a single agent significantly downregulated MDA-MB-231 cell viability (Fig. S4A). Therefore, we focused on synergistic cell death by TKI combinations above additive effects by each TKI. To this end, analysis using SynergyFinder plus(27)revealed that all TKI combinations involving PP2 and NVP-ADW742 (IGF1R inhibitor), gefitinib (EGFR inhibitor) or imatinib (ABL inhibitor) synergistically induced cell death in MDA-MB-231 cells (**Fig. 2F**). Dose-response curves demonstrated that co-treatment with PP2 reduced the IC50 of the tested TKIs by 34-61%, indicating that PP2 sensitized cancer cells to TKI treatment (**Fig. 2G**). Similar synergy was observed when TKI combinations included saracatinib, a SRC family kinase inhibitor(28), in place of PP2 (Fig. S4B). Moreover, specific ablation of *FYN*, but not *SRC*, sensitized cells to TKIs, highlighting the critical role of FYN as a member of SRC kinase family responsible for TKI resistance (**Figs. 2H** and S4C). Ablation of FYN itself did not significantly decrease cell viability (Fig. S4D). Importantly, we observed similar synergy between the same drug combinations in other TNBC cell lines, including Hs578T, HCC1143, HCC1395, and HCC1937 cells (Figs. S4E-H). Further assessment using live-dead and BrdU assays revealed that both the PP2+NVP-ADW742 and PP2+gefitinib combinations synergistically induced cell death while inhibiting cell growth (**Fig. 2I**).

Persistent activation of MAPK pathway and PI3K-AKT pathway has been associated with TKI resistance in various cancers(5). Therefore, we investigated which downstream pathways were involved in sensitizing cells to TKI treatment. Notably, the p38 MAPK was significantly attenuated following treatment with either PP2 or saracatinib treatment (**Fig. 2J**). Previous studies with imatinib resistant CML cells identified ERK signaling as critical downstream of FYN activation(19, 20). However, FYN inhibition failed to significantly downregulate phosphorylated ERK level upon imatinib treatment, indicating downstream signals of FYN leading

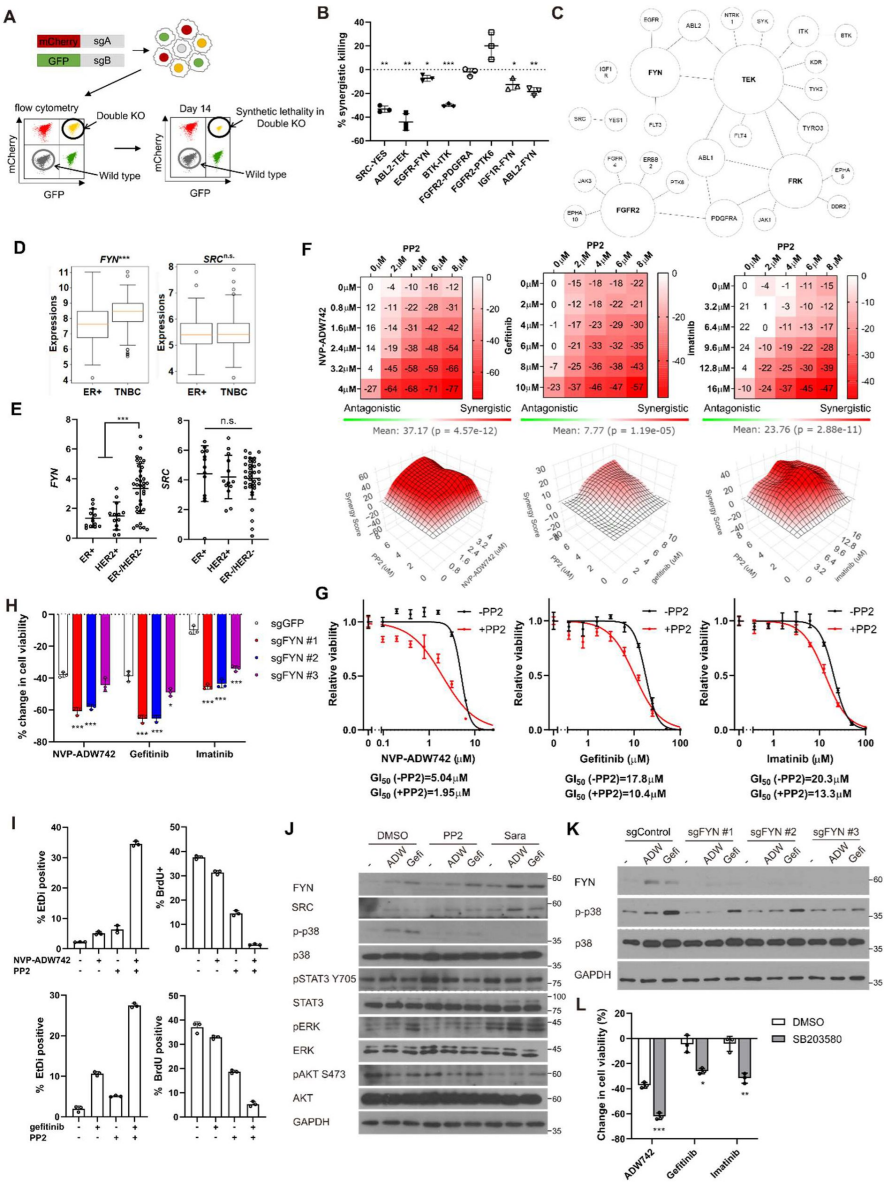

Figure 2.

FYN is critical mediator of TKI resistance.

(A) Schematic diagram of *in vitro* validation of synthetic lethal gene pairs using sgRNAs. (B) Summary of synergistic killing by sgRNAs targeting indicated gene pairs (n=3). (C) Network analysis of the 30 candidate synthetic lethal gene pairs highlighted in **figure 1D**. The size of each node is manually drawn to be proportional to the number of connections the gene has. (D-E) *FYN* and SRC mRNA expressions in (D) microarray data of primary breast cancers of GSE25066 cohort, and (E) in cancer cell line encyclopedia, for indicated subtypes. (F) Summary of MTT assay with MDA-MB-231 cells treated with the TKI combinations at indicated concentrations (n=2). Synergistic killing is calculated using SynergyFinder with Bliss independence model. (G) Dose response curve of the indicated TKI in the presence and absence of 5μM PP2 treated for 72 hours (n=3). (H) MTT assay with MDA-MB-231 Cas9 cells expressing indicated sgRNAs treated with indicated TKIs for 72 hours (n=3). (I) Cell death and cell proliferation in MDA-MB-231 cells treated with NVP-ADW742, gefitinib and PP2 either as single agent or as combination for 72 hours (n=3). (J) western blot analysis of MDA-MB-231 cells treated with indicated drugs for 48 hours (K) western blot analysis of MDA-MB-231 Cas9 cells expressing indicated sgRNA and treated with indicated drugs for 48 hours. (L) MTT assay of MDA-MB-231 cells treated with indicated drugs for 72 hours (SB203580: 10μ M, NVP-ADW742: 4μM, gefitinib: 10μM, imatinib: 10μM) (n=3). PP2, Saracatinib, NVP-ADW742, gefitinib and imatinib were treated at 5μM, 5μM, 4μM, 12μM, 12μM unless otherwise indicated. All data are plotted as mean±s.d. One sample t-test for B, and unpaired two-sided Student's t-test in D,E,H and L. *, p<0.05; **, p<0.01; ***, p<0.001; n.s., p>0.05. All replicates are biological replicates.

to drug resistance may be context dependent. Genetic ablation of *FYN* similarly reduced p38 activation (**Fig. 2K**). Attenuation of p38 activity was also observed in an independent TNBC cell line, Hs578T (Figs. S4I-J). Importantly, treatment of p38 MAPK pathway inhibitor SB203580 markedly sensitized cells to TKI treatment (**Fig. 2L**), while SB203580 as single agent did not significantly change cell viability (Fig. S4K)

FYN mRNA is induced upon TKI treatment in KDM4 dependent manner

Our discovery that inhibition of FYN synergizes with multiple TKIs possessing distinct target profiles suggests that FYN may play a role in general resistance mechanisms against TKI therapy. Consistently, we observed an increase in both protein and mRNA levels of *FYN* following TKI treatment, indicating that upregulation of FYN confers compensatory survival signal in TKI-treated cells (**Fig. 3A-B**). The phosphorylation levels of FYN was increased proportional to FYN protein level, indicating specific kinase activity of FYN did not change (Fig. S5A). Previous study suggested that increased expression of EGR1 transcription factor is responsible for FYN mRNA accumulation in imatinib resistant CML(18). Consistently, EGR1 expression was increased upon TKI treatment in MDA-MB-231 cells. However, EGR1 expression was not increased in TKI treated MDA-MB-231 cells, nor did its knockout significantly downregulated FYN mRNA levels (Fig. S5B). To elucidate the mechanisms underlying the accumulation of *FYN*, we treated MDA-MB-231 cells with inhibitors targeting key epigenetic modifiers and assessed their synergistic effects with NVP-ADW742 in cell killing, as well as their impact on *FYN* mRNA accumulation. Multiple drugs, including pinometostat (DOT1L inhibitor(29)), tazemetostat (EZH2 inhibitor(30)), A366 (G9a inhibitor(31)) and GSK-J4 (KDM6 inhibitor(32)) strongly decreased cell viability upon TKI treatment (Fig. S5C). As the increase in *FYN* mRNA is responsible for TKI resistance, we reasoned that the drug that directly affect FYN mRNA level and hence cell viability should be an inhibitor of epigenetic regulator that enhances transcription. To this end, we focused on pinometostat and GSK-J4 for further validations. Intriguingly, while GSK-J4 decreased FYN mRNA upon NVP-ADW742 treatment, pinometostat failed to decrease it (Fig. S5D).. Consistent with this, treatment with NVP-ADW742 increased the expression of most members of the jumonji domain histone demethylase family (**Fig. 3C**). This observation is consistent with a previous study on taxane-resistant H1299 lung cancer cells(33), suggesting that histone demethylases may play critical roles in activating a drug resistance gene program. However, the ablation of KDM6, the primary targets of GSK-J4, failed to significantly decrease *FYN* expression (Fig. S5E). GSK-J4 is known to inhibit other jumonji domain histone demethylase family proteins including KDM4 and KDM5(34). Therefore, we tested the possibility that other histone demethylase may be involved in regulating *FYN* expression. Among jumonji domain histone demethylases, *KDM4*, and to a lesser extent *KDM3*, were the only gene family members whose ablation inhibited *FYN* upregulation and p38 activation upon TKI treatment (**Figs. 3D** and S5F). Ablation of KDM5, which has been shown to induce drug tolerance in cancer cells(35), did not significantly alter *FYN* expression (Fig. S5G). Like NVP-ADW742 treatment, gefitinib treatment increased *KDM4* demethylase levels (Fig. S5H). We also analyzed two independent TNBC organoids obtained from primary tumors and found concurrent upregulation of *KDM4* with *FYN* mRNAs upon NVP-ADW742 and gefitinib treatment (Fig. S5I). Critically, time course experiment with NVP-ADW742 treated MDA-MB-231 revealed that accumulation of KDM4A protein preceded FYN protein (**Fig. 3E**), suggesting that KDM4A accumulation may be responsible for FYN accumulation. Both KDM3 and KDM4 demethylates methylated H3K9, thereby promoting the opening heterochromatin for transcription(36). Remarkably, expression of *KDM4A*, the most abundantly expressed gene among KDM4 demethylases in TNBC cell lines (Fig. S6A) was enriched in TNBC compared to other breast cancer subtypes (**Fig. 3F**) and was positively correlated with *FYN* expression in CCLE database, suggesting that KDM4 regulates *FYN* mRNA levels (**Fig. 3G**). Genetic ablation of *KDM3* or *KDM4* (Fig. S6B-C) decreased *FYN* and p38 activity. Also, genetic ablation of KDM3 or KDM4 sensitizing MDA-MB-231 cells to TKIs (**Figs. 3H-I**). The Ablation of KDM3 or KDM4 only had modest but statistically insignificant effect on cell viability (Fig. S6D). Likewise, treatment of KDM4 inhibitor

QC6352(37) synergized with TKIs in killing MDA-MB-231 cells (**Fig. 3J**). QC6352 treatment also significantly attenuated *FYN* accumulation upon NVP-ADW742 treatment (**Fig. 3K-L**). This was consistent with the RNA sequencing data results in the previous study with breast cancer stem cells treated with QC6352(38). Specifically, *FYN* was the most significantly downregulated SRC family kinase upon QC6352 treatment (**Fig. 3M**). Analysis of chromatin IP (ChIP) sequencing data from the same study revealed KDM4A enrichment near FYN promoter; and QC6352 treatment increased H3K9me3 enrichment at the same locus (**Fig. 3N**). Indeed, this FYN promoter locus exhibited a reduction in H3K9me3 following NVP-ADW742 treatment, while QC6352 treatment restored H3K9me3 enrichment (**Fig. 3O**). This finding suggests that KDM4 may directly demethylate H3K9me3 at FYN promoter to upregulate *FYN* transcription. In contrast, H3K27me3 marks, which is demethylated by KDM6 family demethylases, were not significantly changed at FYN promoter upon NVP-ADW742 treatment (Fig. S6E). *FYN* accumulation and resistance to TKIs were also confirmed to be attenuated by QC6352 treatment in other independent TNBC cell lines (Figs. S6F-G).

FYN/KDM4 inhibition synergizes with TKI treatment *in vivo*

We proceeded to investigate the potential clinical application of our synthetic lethal gene pairs as combinatorial therapy by assessing the *in vivo* efficacy of pharmacological interventions targeting these gene pairs using MDA-MB-231 xenograft models. Strikingly, co-treatment of saracatinib and NVP-ADW742 synergistically reduced tumor size, whereas treatment with either agent alone was ineffective in slowing tumor growth (**Fig. 4A**). All treatment groups exhibited minimal changes in body weight, indicating that the overall health of the animals was not adversely affected by the combination treatment (Fig. S7A). Saracatinib-gefitinib combination was not tested as saracatinib can inhibit EGFR(28). Similarly, KDM4 inhibitor QC6352 synergized with gefitinib in reducing MDA-MB-231 xenograft tumor growth without causing overt changes in animal health (**Figs. 4B** and S7B). Additionally, the expression levels of *FYN* and *KDM4A* were found to be correlated with poor prognosis in a previously reported breast cancer cohort(24), highlighting the potential of targeting these two genes as therapeutic targets for TNBC (**Fig. 4C**). Collectively, our results demonstrate that upregulation of *KDM4* upon TKI treatment reduces H3K9me3 mark in *FYN* enhancer, thereby increasing *FYN* expression and promoting cell survival under TKI treatment (**Fig. 4D**).

FYN is associated with drug tolerant persister phenotype

The observed epigenetic alterations in regulators conferring resistance to multiple cancer drugs closely resemble non-genetic changes associated with the generation of drug-tolerant persisters(35). Indeed, prolonged incubation of MDA-MB-231 cells treated with TKIs or conventional chemotherapy drugs such as doxorubicin or paclitaxel resulted in increased levels of *FYN* (**Fig. 5A**). Curiously, *KDM4A* expression was only upregulated upon treatment with NVP-ADW742 and gefitinib, suggesting that while *FYN* upregulation is a general feature of drug tolerant cells, the mechanism of *FYN* upregulation may vary depending on the specific drug being used. Analysis of previously published RNA sequencing data from a series of osimertinib tolerant EGFR mutated lung cancer cell lines(39) revealed higher expression levels of *FYN* and *KDM4A* in the drug persisters, but not SRC (**Fig. 5B**). Consistently, we confirmed upregulation of *FYN* at both the protein and mRNA levels in gefitinib and osimertinib resistant PC9 and HCC827 cells (**Figs. 5C-D**). Pharmacological inhibition of FYN or downregulation of *FYN* expression through inhibition of KDM4 sensitized gefitinib resistant PC9 cells to EGFR inhibitor, suggesting that *FYN-KDM4* are responsible for gefitinib resistant phenotype in this cell line (**Figs.5E-G**).

Importantly, upregulation of *FYN* has been consistently observed in multiple independent studies involving drug-tolerant cancer cell lines and patient-derived xenografts treated with various drugs that have distinct target profiles, including TKIs (lapatinib, a HER2 inhibitor, against HER2 positive breast cancer(40)) and chemotherapy drugs (irinotecan, topoisomerase inhibitor against

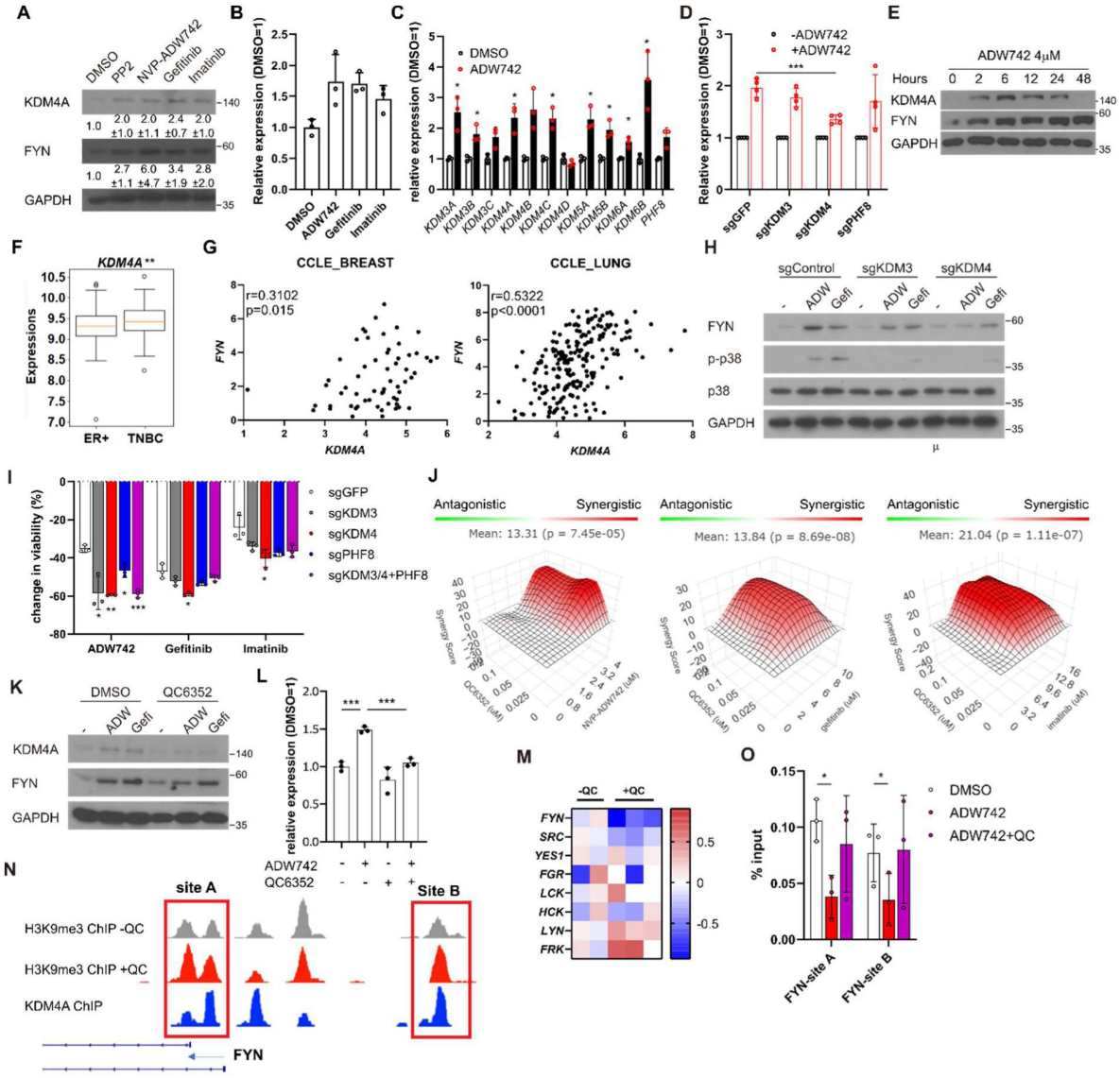

Figure 3.

Activation of *KDM4* upregulates *FYN*, conferring drug resistance.

(A) Western blot analysis of MDA-MB-231 cells treated with indicated drugs. Numbers below blots indicate quantification of average±s.d. expression level normalized to GAPDH in three independent experiments. (B) RT-qPCR analysis of *FYN* expression levels in MDA-MB-231 cells treated with indicated drugs for 48 hours (n=3). (C) RT-qPCR analysis of indicated jumonji family histone demethylase expression levels after 48 hours treatment of NVP-ADW742 (n=3). (D) Changes in *FYN* mRNA levels upon 48 hours of NVP-ADW742 treatment in MDA-MB-231 Cas9 cells expressing indicated sgRNAs (n=4). (E) *KDM4A* mRNA levels in primary tumor tissues of indicated subtypes in GSE25066 cohort. (F) Positive correlation of *FYN* and *KDM4A* mRNA levels in CCLE database. (G) western blot analysis of MDA-MB-231 Cas9 cells expressing indicated sgRNA and treated with indicated drugs for 48 hours. (H) MTT assay of MDA-MB-231 Cas9 cells expressing indicated sgRNAs and treated with indicated drugs for 72 hours (n=3). (I) SynergyFinder analysis of MDA-MB-231 cells treated with indicated drug combinations (n=2). (J-K) western blot analysis (J) and RT-qPCR analysis (K) of MDA-MB-231 cells treated with indicated drugs for 48 hours (n=3). (L) mRNA expression levels of SRC family kinases in breast cancer stem cells treated with QC6352 in RNA sequencing data described in Metzger et. al.(38) (M) H3K9me3 and KDM4A enrichment at genomic locus encoding *FYN* promoter in ChIP sequencing data described in the same study as (L). (N) H3K9me3 Chromatin immunoprecipitation-qPCR analysis of MDA-MB-231 cells treated with indicated drug for 48 hours at specified genomic loci (n=3). QC6352, PP2, NVP-ADW742, gefitinib and imatinib were treated at 10μM, 5μM, 4μM, 12μM, 12μM, respectively, unless otherwise indicated. All data are plotted as mean±s.d. Unpaired two-sided Student's t-test in B,C,D,E,H and K. Paired two-sided Student's t-test in N. *, p<0.05; **, p<0.01; ***, p<0.001; n.s., p>0.05. All replicates are biological replicates.

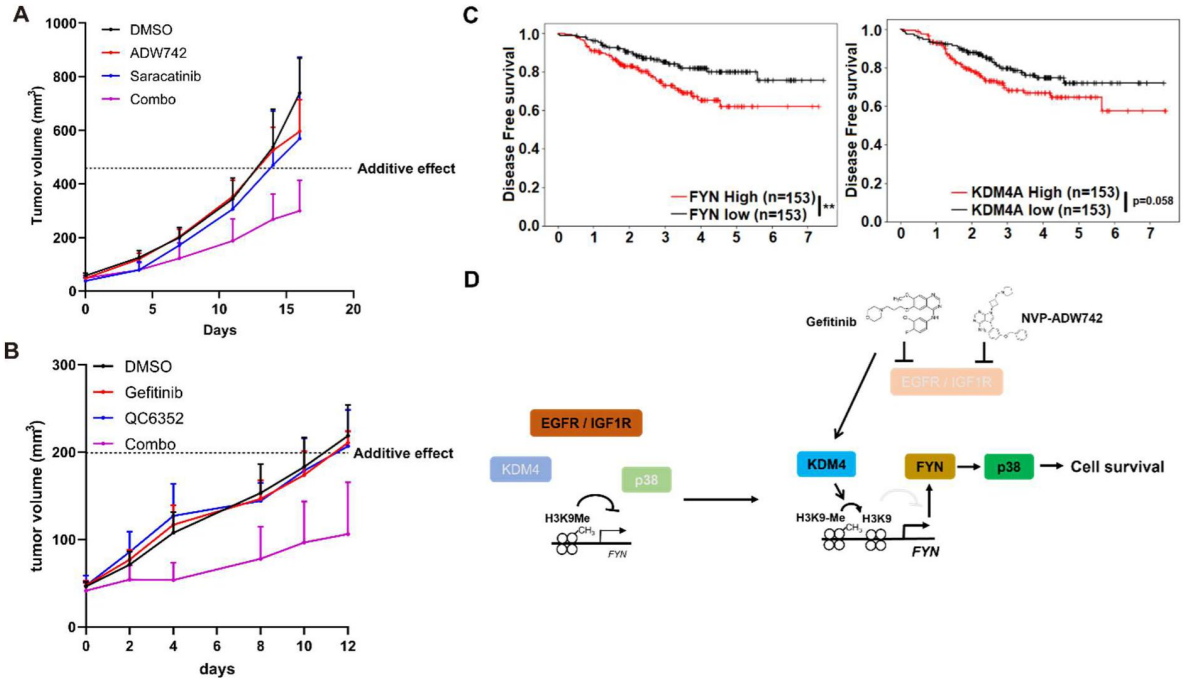

Figure 4.

Combination therapy targeting FYN+IGF1R and KDM4+EGFR synergistically eliminates tumor *in vivo*.

(A-B) Tumor volume for MDA-MB-231 xenografts treated with indicated drugs. Additive effects were calculated by Bliss independence model (n=5). (C) Distant relapse free survival of GSE25066 patient cohort classified by *FYN* (left) and *KDM4A* (right) mRNA expression. (D) Schematics diagram of the mechanism of KDM4-FYN conferring TKI resistance. All data are plotted as mean±s.d. *, p<0.05; **, p<0.01; ***, p<0.001; n.s., p>0.05. All replicates are biological replicates.

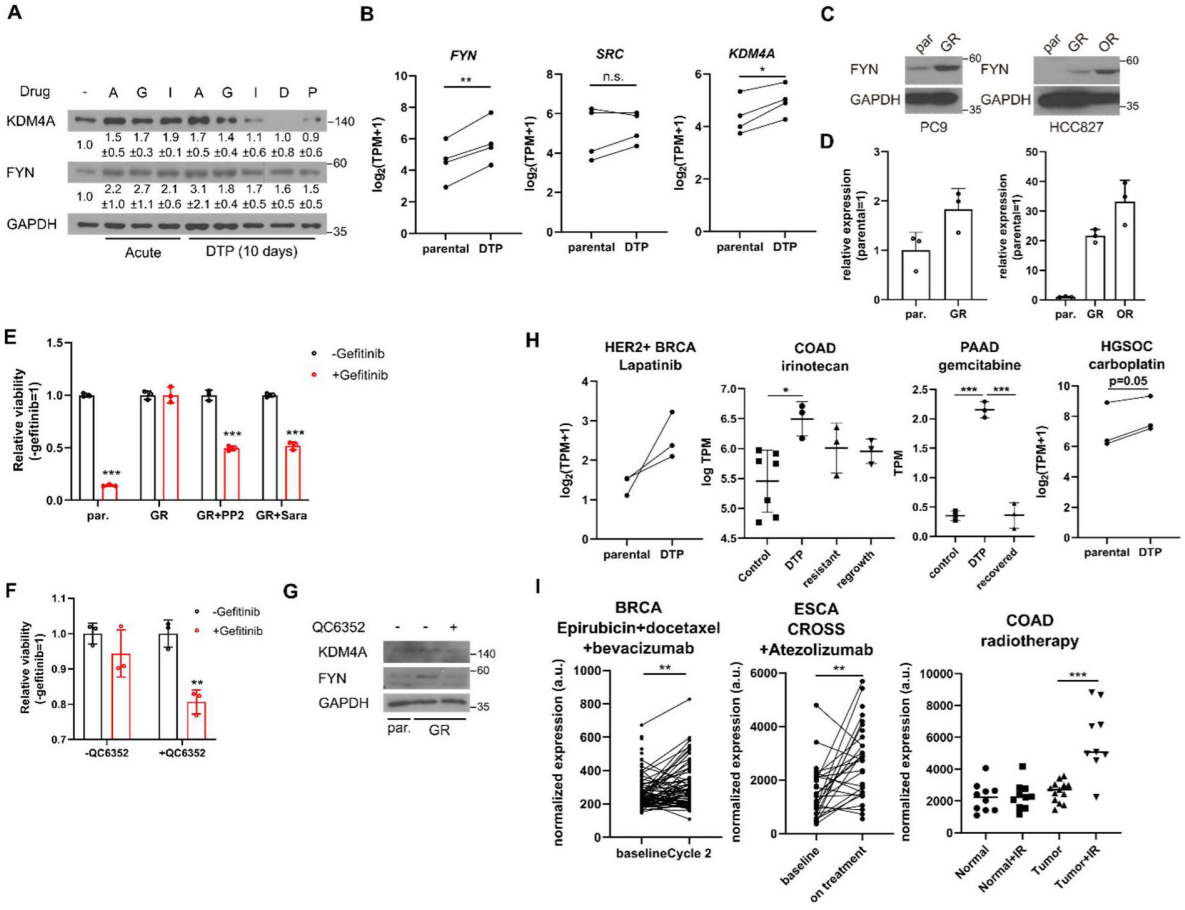

Figure 5.

FYN and *KDM4* are associated with drug tolerance.

(A) MDA-MB-231 cells treated with indicated drugs for short (acute: 2 days) and long (DTP: 10 days) time periods. A: 1μM NVP-ADW742, G: 5μM gefitinib, I: 5μM imatinib, D: 100nM doxorubicin, P: 5nM paclitaxel. Numbers below blots indicate quantification of average±s.d. expression level normalized to GAPDH in three independent experiments. (B) Summary of mRNA expressions of indicated genes in EGFR mutant lung cancer cells (parental) and their derivative osimertinib tolerant persisters (DTP). (C-D) western blots (C) and RT-qPCR (D) analyses of indicated parental and EGFR inhibitor resistant lung cancer cells. (E-F) MTT assay with PC9 parental (par.) and gefitinib resistant (GR) cells treated with indicated drug combinations (gefi: 2μM gefitinib, PP2: 5μM PP2, Sara: 5μM saracatinib, QC6352: 10μM QC6352) for 72 hours. (G) western blot analysis with PC9 cells treated with 10μM QC6352 for 48 hours. (H) *FYN* mRNA expression levels of parental and DTP populations in various cancers treated with indicated drugs.. (I) *FYN* mRNA expression levels of residual disease after indicated treatments. All data are plotted as mean±s.d. Paired two-sided Student's t-test in B, H (HER2+ BRCA set and HGSOC carboplatin set), and I (BRCA set and ESCA set), and unpaired two-sided Student's t-test in H (COAD and PAAD sets) and I (COAD set). The expression data in B,H,I are obtained from NCBI gene expression omnibus. The accession numbers of the expression data analyzed are listed in Supplementary table S2. *, p<0.05; **, p<0.01; ***, p<0.001; n.s., p>0.05. All replicates are biological replicates.

colorectal cancer(41); gemcitabine against pancreatic cancer(42); and carboplatin against high grade serous ovarian carcinoma(43)) (**Fig. 5H**). Moreover, enrichment of *FYN* has also been observed in residual disease following chemotherapy, including neoadjuvant chemostherapy plus bevacizumab treated HER2 negative breast cancer(44), neoadjuvant chemoradiotherapy combined plus atezolizumab treated esophageal cancer(45), and chemoradiotherapy treated colorectal cancer(46), indicating its potential role in mediating drug tolerance during chemotherapy (**Fig. 5I**). While the causal relationship between FYN expression drug tolerance in response to various therapeutic interventions warrants further study, these evidence suggest that FYN expression is associated with drug tolerance. Notably, an analogous increase in *KDM4* was not consistently observed across all tumor models tested in **Figures 5H-I** (Fig. S8A-B). This suggests that, as previously noted in **Figure 5A**, while *FYN* serves as a general mediator of drug tolerance, the specific mechanisms underlying its upregulation may vary depending on the cancer type and the drug being administered. Taken together, these lines of evidence further support our findings in TNBC cell lines and suggest that *FYN* acts as a common mediator of drug tolerance.

Discussion

In this study, we employed combinatorial CRISPR screening to identify combinations of TKIs that exhibit synergistic effects in eliminating triple-negative breast cancer (TNBC). We discovered and validated that concurrent targeting of FYN, along with other tyrosine kinases such as IGF1R, EGFR or ABL2 can synergistically eradicate TNBC and impede cancer growth. Our findings also provide evidence that the transcriptional upregulation of *FYN*, facilitated by the activation of KDM4 histone demethylases, confers resistance and persistence to TKIs. Upregulation of *FYN* is a general feature of drug tolerant cancer cells, suggesting the association of *FYN* expression with drug resistance and tumor recurrence after treatment.

This research provides basis for breakthrough combinatorial therapy achieving effective targeted therapy with minimal risk of developing resistance. Our combinatorial CRISPR screening demonstrates that treatment with TKIs or histone demethylase inhibitors enhances the sensitivity of cells to other TKIs. Consequently, drug combinations exhibit a more potent inhibition of cancer growth than the simple sum of the therapeutic effects of individual drugs. Furthermore, synergistic drug combinations enable a reduction in the dosage of each drug, with minimal compromise in therapeutic efficacy. Such combinations yield a therapeutic response comparable to that achieved with significantly higher doses of each individual agent. We anticipate that combinatorial therapy has the potential to mitigate side effects by minimizing the dosage of each drug, thus widening the therapeutic window. Further studies should elucidate the downstream mechanisms by which FYN upregulation contributes to drug tolerance. SRC family kinases are known to upregulate multiple signaling pathways including ERK, AKT and p38 pathway(47). Although our study showed that, at least in MDA-MB-231 cell line, FYN depends on p38 activity for TKI resistance (figs. 2J-L), it should further be shown while this downstream mechanism is generalizable in other cancers. The downstream mechanism of p38 that contributes to drug resistance would also be of great interest to identify novel therapeutic approaches minimizing drug tolerance. Also, our combinatorial CRISPR screen results warrant further studies with other synthetic lethal gene combinations other than those involving FYN that are not deeply investigated in this study. Particularly, FGFR2, TEK, FRK identified as key nodes in **figure 2C** may be of particular interest as they are also associated with cancer cell survival(48, 49).

It is intriguing to observe that *FYN* is specifically upregulated in various models of drug resistance and tolerance. SRC family kinases, which includes FYN, have been linked to drug resistance(47, 50). In line with this, phosphoproteomic analysis of neoadjuvant chemotherapy resistant TNBC patient derived xenografts showed upregulated SRC family kinase networks including kinases and their substrates and adaptors(51). Our findings reveal that *FYN* is specifically upregulated at the mRNA level possibly through epigenetic regulations, providing further depth to our understanding

of drug resistance in cancer therapy. The epigenetic reprogramming of the drug tolerant cells may be distinct depending on the tumor type or the therapeutic interventions, as KDM4A, which we show is increased upon TKI treatment in TNBC cell line in **figure 3**, is not significantly regulated in other cancers analyzed in **figure 5**. Therefore, the context dependent mechanisms underlying FYN upregulation, and its essentiality in constituting drug tolerance remains as subjects for further study.

Furthermore, our work highlights the significance of histone demethylases in TKI resistance. Numerous histone demethylases have been implicated in drug resistance and tolerance across different cancer drug types. For instance, the KDM5 family of H3K4 demethylases has been associated with the drug-tolerant persister phenotype against multiple TKIs(35). In our study, we identify *KDM4* as a critical factor in the generation of drug-tolerant persisters in breast cancer. *KDM4* is known to be upregulated in various cancers, including breast cancer, and promotes key malignant traits. Previous studies have demonstrated the essential role of KDM4 in induced pluripotency through its interaction with pluripotency factors(52). These findings suggest that an KDM4 inhibitor could be a promising therapeutic target with specific activity against cancer stem cells. Consistently, specific inhibitors targeting KDM4 have recently been developed and shown to inhibit the generation of breast cancer stem cells(38). The mechanisms underlying KDM4 upregulation upon drug treatment is currently unclear and remains as subjects for further study. Nevertheless, given our discoveries regarding the involvement of *KDM4* in drug resistance in breast and lung cancer, the development of novel drugs targeting KDM4 holds significant therapeutic potential.

Materials and methods

Cell Culture

HEK293T, MDA-MB-231, Hs578T cells were obtained from American Type Cell Culture (ATCC) HCC1143, HCC1395, HCC1937 were obtained from Korean cell line bank. HEK293T and MDA-MB-231 were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. Hs578T and HCC1143, HCC1395, HCC1937 were grown in RPMI1640 supplemented with 10% FBS. Parental and gefitinib-, and Osimertinib-resistant PC9 and HCC827 cells were kind gifts from Jae Cheol Lee (Asan Medical Center, Seoul, Korea) and were grown in RPMI1640 supplemented with 10% FBS. Gefitinib and osimertinib resistant cells are maintained in the presence of 1μM gefitnib and 0.5μM osimertinib, respectively. Gefitinib resistant derivatives of PC9 and HCC827 cells were generated as described previously by treating cells with escalating dose of gefitinib (53). All cell culture medium and supplements are purchased from Welgene Inc.

Combinatorial library construction

Combinatorial library was constructed as previously described(15). The sgRNAs used in the screens were cloned in pAWp28 storage vector in two versions: one version containing human U6 driven sgRNA with wild type scaffold, and another containing mouse U6 driven sgRNA with cr2 variant scaffold. The sgRNA expression cassette consisting of U6 promoter and sgRNA were subject to one-pot, iterative cloning into lentiviral pTK799 vector using BglII-MfeI restriction sites flanking the sgRNA expression cassette and BamHI-EcoRI sites in pTK799. pTK799 vector is derived from pAWp12(15) by replacing CMV-GFP selectable marker to EFS-Puro.

Combinatorial CRISPR screening procedure

Lentivirus was generated in HEK293T cells by transfecting lentiviral transfer vector, and helper vectors (psPAX2, and pVSV-G) using Fugene HD (Promega). Lentiviral supernatant was collected 48 hours after transfection, and was frozen and stored in −80C. The appropriate titer for lentiviral transduction was determined by transducing MDA-MB231 cells with two-fold serial dilution of

lentiviral supernatant, selecting with puromycin 2 days after transduction for 2 days, and determining cell viability with AQuaeous one cell viability, MTS assay (Promega). After determining the titer of lentiviral supernatant, 100 million MDA-MB231 cells carrying constitutively expressed Cas9 were transduced with CombiGEM library at MOI of 0.3. The expected initial coverage is 100 million x 0.3/ (54,289 different sgRNA combinations)= 553. Three days after transduction, the cells were either harvested as day 0 sample or selected with 2μg/mL puromycin (Invitrogen). Cells were treated with benzonase before harvesting to minimize carryover of plasmid DNA in lentiviral supernatant. Cells were grown in the presence of 2μg/mL puromycin for 20 days before harvesting.

The genomic DNA of harvested cells were isolated using Blood & Cell Culture Maxi kit (Qiagen). The PCR amplicon spanning the two sgRNAs were generated with PCR using Q5 High Fidelity DNA polymerase (New England Biolabs) and the following primers:

F: CAAGCAGAAGACGGCATACGAGAT CCTAGTAACTATAGAGGCTTAATGTGCG

R: AATGATACGGCGACCACCGAGATCTACAC NNNNNN ACACGAATTCTGCCGTGGATCCAA

The six nucleotides described as “NNNNNN” in reverse primer above represents unique index to identify biological replicates in multiplexed NGS run.

The PCR protocol involves 60 seconds of initial DNA denaturation at 98C, and 20 cycles of 10 seconds denaturation at 98C, 10 seconds annealing at 67C, and 120 seconds elongation at 72C. All genomic DNA isolated were used in PCR reaction at concentration of 40μg/mL. All PCR products were combined and precipitated with isopropanol at room temperature. The precipitated DNA was resuspended in 400μL EB buffer (Qiagen) and gel purified. The purified PCR products were sent for NGS by NextSeq500 paired end sequencing with the following sequencing primers:

Forward read: GGACTAGCCTTATTTGAACTTGCTATGCAGCTTTCTGCTTAGCTCTCAAAC

Forward index read:
CGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

Reverse read: GCA CCG AGT CGG TGC TTT TTT GGA TCC ACG GCA GAA TTC GTGT

The raw GI scores calculated as deviation from the quadratic fit of the expected-observed Z score plot. The GI scores were normalized by dividing the raw GI scores with the standard deviation of the GI scores obtained from the 200 nearest neighbors in terms of expected Z scores (Fig. S1I) (11).

Data analysis

The sgRNA sequences were identified and their occurrences were counted with C++ script deposited in Github (*https://github.com/tackhoonkim/combinatorial-CRISPR-screens-2023*).

Validation of screens using sgRNAs

Individual sgRNA was cloned to either pTK1329, and pTK1336 that are both derived from pAWp12 with EFS-GFP and EFS-mCherry, respectively, as selectable markers. Validation of synthetic lethality between gene A and B were analyzed by transducing MDA-MB-231 Cas9 cells with four combinations of lentiviral supernatant pairs (MOI ~ 0.5 each) containing (1) GFP-sgA and mCherry-sgB; (2) GFP-sgA and mCherry-sgCon; (3) GFP-sgCon and mCherry-sgB; (4) GFP-sgCon and mCherry-sgCon. The fraction of GFP/mCherry double positive cells were analyzed using BD Accuri

C6 and its accompanying software. The expected fold change in sgA+sgB were calculated as $FC_{sgA+sgCon}$ x $FC_{sgCon+sgB,}$ where FC is normalized fold change in fraction of GFP/mCherry double positive cells relative to those transduced with GFP-sgCon and mCherry-sgCon.

MTT Cell viability assay

Cells were seeded at 1000-2000 cells/well in 96 well plate. Tyrosine kinase inhibitors at indicated combination of dose were treated 12 hours after seeding, and cells were grown for 3 days. The relative viability was measured by EzCytox cell viability assay (Dojindo). The absorbance at 450nm wavelength was measured using EnVision multimode plate reader (PerkinElmer).

Cell death and cell proliferation assay

Cells were incubated with tyrosine kinase inhibitors for 48 hours. Cell proliferation was quantified with BrdU assay using FITC conjugated BrdU antibody (Biolegend, 364103) and propidum iodide/RNase A solution (Cell Signaling), analyzed with BD Accuri C6 and accompanied software. Cell death was quantified with Live-Dead cell staining kit (Molecular Probes) by flow cytometry analysis using BD Accuri C6 and accompanied software.

Western blot analysis

Cells were treated with drugs for 48 hours unless otherwise indicated. Western blots were performed as previously reported. Briefly, Cells were lysed in RIPA buffer (50mM Tris-HCl pH 7.5, 150mM sodium chloride, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% NP-40, 1mM EDTA) supplemented with protease inhibitor (aprotinin, leupeptin, pepstatin A, and phenylmethylsulfonyl fluoride [PMSF]) and phosphatase inhibitor (sodium fluoride and sodium orthovanadate) cocktail. Antibodies used for western blot analysis were: anti-SRC (Santa Cruz biotechnology, mouse sc-), anti-FYN (Cell Signaling Technology, rabbit #4023), anti-phospho-FYN Y530 (Invitrogen, PA5-36644), anti-GAPDH (Cell Signaling Technology, rabbit #5174), anti-KDM4A (Bethyl Laboratories, rabbit A300-861A), anti-phospho-ERK T202/Y204 (Cell Signaling Technology, rabbit #9101), anti-ERK (Cell Signaling Technology, rabbit #9102), anti-phospho-STAT3 Y705 (Cell Signaling Technology, rabbit #9145), anti-STAT3 (Cell Signaling Technology,), anti-phospho-AKT (Cell Signaling Technology, mouse #4051), anti-AKT (Cell Signaling Technology,), anti-phospho-p38 (Cell Signaling Technology,), anti-p38 (Cell Signaling Technology,).

RT-qPCR analysis

RNA is extracted from cultured cells with Trizol (Invitrogen) according to the manufacturer's instructions. The precipitated RNA pellet was resuspended in RNase free water (Enzynomics), and was subject to reverse transcription with M-MLV reverse transcriptase (Enzynomics) at 37C for 2 hours, followed by heat inactivation at 95C for 5 minutes. The resulting cDNA was used for quantitative real time PCR using TOPreal SYBR Green qPCR premix (low ROX, Enzynomics) reagent and CFX96 Real-Time PCR detection system (Bio-Rad).

Xenograft assay

All animal experiments were approved by IACUC of Korea Institute of Science and Technology (KIST). Six-week-old female nude mice were injected with 5 x 10^6 MDA-MB-231 cells suspended in 1:1 (w/w) mixture of PBS and growth factor reduced Matrigel (Corning) in fourth inguinal mammary fat pad. Starting two weeks after tumor cell injection, saracatinib (50mg/kg mouse body weight, MedChemExpress), NVP-ADW742 (20mg/kg, Sigma), gefitinib (20mg/kg, MedChemExpress), QC6352 (10mg/kg, MedChemExpress) in 45% saline+40% polyethyleneglycol 300 (sigma)+5% Tween-80 (sigma)+5% DMSO (sigma) were injected intraperitoneally every 24 hours for two weeks. Tumor volume was measured by digital caliper and calculated as $(width)^2$ x length x 0.5.

Public database analyses

Gene Expression Omnibus (GEO) data with breast cancer cohort (GSE25066(24)) were analyzed using web based platform Cancer Target Gene Screening (*https://ctgs.biohackers.net*)(54). Cancer Cell Line Encyclopedia (CCLE) data were analyzed using depmap R package version 1.14. The list of GEO data used for analysis are listed in table S2.

Primary TNBC organoid culture and drug treatment

Specimens was obtained from enrolled patients with TNBC breast cancer with IRB approval. Tumor tissue was collected and transferred using cold RPMI1640 media at the National Cancer Center (Goyang, Republic of Korea). Then the tissues were dissected with a blade on Petri dish and enzymatically digested with dissociated kit (Multi Tissue Dissociation Kit 1, 130-110-201) by gentle MACS Dissociators (Miltenyi Biotec, Germany). After cell counting, 1×10^5 cells were embedded in 40 µl of Matrigel (Corning) and seeded into each well of a 24-well cell culture plate. After the matrigel was solidified, 500 µL organoid medium supplemented with advanced DMEM/F12 medium (Invitrogen, USA), B27 (Invitrogen, USA, 17504), 1.25 mM N-acetylcysteine (Sigma, USA, A9165), 5 ng/mL EGF (PeproTech, USA, AF-100-15), 20 ng/mL FGF-10 (Peprotech, 100-26), 5ng/ml FGF7 (Peprotech, AF-100-19), 50ng/ml R-spondin 1 (Qkine, Qk006), 5 mM nicotinamide (Sigma, N0636), 500 nM A83-01 (Tocris, 2939), 1X GlutaMAX (Gibco, 35050-061), 100ug/ml primocin (Invivogen, ant-pm), 10mM HEPES (Gibco, 15630-080), 1X Noggin (U-Protein Express BV, N002), 1X ITS (Gibco, 12585014), 100nM β-estradiol (Sigma, E2758), 1ug/ml Hydrocortisone (Sigma, H0888), 5nM Heregulin (PeproTech, AF-100-03), 500nM SB202190 (R&D system, 1264), 10uM Y27632 (TOCRIS, 1254) as described by Clevers and colleagues (55), was added to each well and the cells grown under standard culture conditions (37 ℃, 5% CO2).

Availability of data and materials

The NGS data for CRISPR screening results are available under NCBI SRA accession code PRJNA976939.

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Additional information

Author contributions

T.K., B.-S.P., H.J. and J.K. performed experiments. T.K. and T.K.L. supervised the research. S.H. S.-Y.J. and S.Y.K. performed experiments with TNBC patient derived organoids. D.K., S.K.L. generated and provided osimertinib resistant HCC827 cell line.

Ethics Statement

All experiments with human tumor organoids were conducted in accordance with the requirements of the National Cancer Center Institutional Review Board (IRB).

Additional files

Supplementary dataset S2

Supplementary dataset S2

Supplementary figures S1-8, tables S1-3

References

1. Wu P, Nielsen TE, Clausen MH (2016) **Small-molecule kinase inhibitors: an analysis of FDA-approved drugs** *Drug Discovery Today* **21**:5–10

2. Hanahan D, Weinberg Robert A (2011) **Hallmarks of Cancer: The Next Generation** *Cell* **144**:646–74

3. Li Y, Zhan Z, Yin X, Fu S, Deng X (2021) **Targeted Therapeutic Strategies for Triple-Negative Breast Cancer** *Frontiers in Oncology* **11**

4. Litzenburger BC, Creighton CJ, Tsimelzon A, Chan BT, Hilsenbeck SG, Wang T, et al. (2011) **High IGF-IR Activity in Triple-Negative Breast Cancer Cell Lines and Tumorgrafts Correlates with Sensitivity to Anti–IGF-IR Therapy** *Clinical Cancer Research* **17**:2314–27

5. Morgillo F, Della Corte CM, Fasano M, Ciardiello F (2016) **Mechanisms of resistance to EGFR-targeted drugs: lung cancer** *ESMO Open* **1**

6. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. (2010) **Gefitinib or Chemotherapy for Non–Small-Cell Lung Cancer with Mutated EGFR** *New England Journal of Medicine* **362**:2380–8

7. Leonetti A, Sharma S, Minari R, Perego P, Giovannetti E, Tiseo M (2019) **Resistance mechanisms to osimertinib in EGFR-mutated non-small cell lung cancer** *British Journal of Cancer* **121**:725–37

8. Jin H, Wang L, Bernards R (2023) **Rational combinations of targeted cancer therapies: background, advances and challenges** *Nature Reviews Drug Discovery* **22**:213–34

9. Wong ASL, Choi GCG, Cui CH, Pregernig G, Milani P, Adam M, et al. (2016) **Multiplexed barcoded CRISPR-Cas9 screening enabled by CombiGEM** *Proceedings of the National Academy of Sciences* **113**:2544–9

10. Horlbeck MA, Xu A, Wang M, Bennett NK, Park CY, Bogdanoff D, et al. (2018) **Mapping the Genetic Landscape of Human Cells** *Cell* **174**:953–67

11. Han K, Jeng EE, Hess GT, Morgens DW, Li A, Bassik MC (2017) **Synergistic drug combinations for cancer identified in a CRISPR screen for pairwise genetic interactions** *Nature Biotechnology* **35**:463–74

12. Shen JP, Zhao D, Sasik R, Luebeck J, Birmingham A, Bojorquez-Gomez A, et al. (2017) **Combinatorial CRISPR–Cas9 screens for de novo mapping of genetic interactions** *Nature Methods* **14**:573–6

13. Corsello SM, Bittker JA, Liu Z, Gould J, McCarren P, Hirschman JE, et al. (2017) **The Drug Repurposing Hub: a next-generation drug library and information resource** *Nature medicine* **23**:405–8

14. Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, et al. (2016) **Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9** *Nature Biotechnology* **34**:184–91

15. Wong ASL, Choi GCG, Cheng AA, Purcell O, Lu TK (2015) **Massively parallel high-order combinatorial genetics in human cells** *Nat Biotech* **33**:952–61

16. Luo B, Cheung HW, Subramanian A, Sharifnia T, Okamoto M, Yang X, et al. (2008) **Highly parallel identification of essential genes in cancer cells** *Proceedings of the National Academy of Sciences* **105**

17. Stein PL, Vogel H, Soriano P (1994) **Combined deficiencies of Src, Fyn, and Yes tyrosine kinases in mutant mice** *Genes & Development* **8**:1999–2007

18. Irwin ME, Johnson BP, Manshouri R, Amin HM, Chandra J (2015) **A NOX2/Egr-1/Fyn pathway delineates new targets for TKI-resistant malignancies** *Oncotarget* **6**

19. Fenouille N, Puissant A, Dufies M, Robert G, Jacquel A, Ohanna M, et al. (2010) **Persistent Activation of the Fyn/ERK Kinase Signaling Axis Mediates Imatinib Resistance in Chronic Myelogenous Leukemia Cells through Upregulation of Intracellular SPARC** *Cancer Research* **70**:9659–70

20. Airiau K, Turcq B, Mahon F-X, Belloc F (2017) **A new mechanism of resistance to ABL1 tyrosine kinase inhibitors in a BCR-ABL1-positive cell line** *Leukemia Research* **61**:44–52

21. Grosso S, Puissant A, Dufies M, Colosetti P, Jacquel A, Lebrigand K, et al. (2009) **Gene expression profiling of imatinib and PD166326-resistant CML cell lines identifies Fyn as a gene associated with resistance to BCR-ABL inhibitors** *Molecular Cancer Therapeutics* **8**:1924–33

22. Lee G-H, Yoo K-C, An Y, Lee H-J, Lee M, Uddin N, et al. (2018) **FYN promotes mesenchymal phenotypes of basal type breast cancer cells through STAT5/NOTCH2 signaling node** *Oncogene* **37**:1857–68

23. Xie Y-G, Yu Y, Hou L-K, Wang X, Zhang B, Cao X-C (2016) **FYN promotes breast cancer progression through epithelial-mesenchymal transition** *Oncol Rep* **36**:1000–6

24. Hatzis C, Pusztai L, Valero V, Booser DJ, Esserman L, Lluch A, et al. (2011) **A Genomic Predictor of Response and Survival Following Taxane-Anthracycline Chemotherapy for Invasive Breast Cancer** *JAMA* **305**:1873–81

25. Ghandi M, Huang FW, Jané-Valbuena J, Kryukov GV, Lo CC, McDonald ER, et al. (2019) **Next-generation characterization of the Cancer Cell Line Encyclopedia** *Nature* **569**

26. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, et al. (1996) **Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation** *J Biol Chem* **271**:695–701

27. Zheng S, Wang W, Aldahdooh J, Malyutina A, Shadbahr T, Tanoli Z, et al. (2022) **SynergyFinder Plus: Toward Better Interpretation and Annotation of Drug Combination Screening Datasets** *Genomics, Proteomics & Bioinformatics* **20**:587–96

28. Green TP, Fennell M, Whittaker R, Curwen J, Jacobs V, Allen J, et al. (2009) **Preclinical anticancer activity of the potent, oral Src inhibitor AZD0530** *Molecular Oncology* **3**:248–61

29. Daigle SR, Olhava EJ, Therkelsen CA, Basavapathruni A, Jin L, Boriack-Sjodin PA, et al. (2013) **Potent inhibition of DOT1L as treatment of MLL-fusion leukemia** *Blood* **122**:1017–25

30. Knutson SK, Warholic NM, Wigle TJ, Klaus CR, Allain CJ, Raimondi A, et al. (2013) **Durable tumor regression in genetically altered malignant rhabdoid tumors by inhibition of methyltransferase EZH2** *Proceedings of the National Academy of Sciences* **110**:7922–7

31. Sweis RF, Pliushchev M, Brown PJ, Guo J, Li F, Maag D, et al. (2014) **Discovery and Development of Potent and Selective Inhibitors of Histone Methyltransferase G9a** *ACS Medicinal Chemistry Letters* **5**:205–9

32. Kruidenier L, Chung C-w, Cheng Z, Liddle J, Che K, Joberty G, et al. (2012) **A selective jumonji H3K27 demethylase inhibitor modulates the proinflammatory macrophage response** *Nature* **488**

33. Dalvi MP, Wang L, Zhong R, Kollipara RK, Park H, Bayo J, et al. (2017) **Taxane-Platin-Resistant Lung Cancers Co-develop Hypersensitivity to JumonjiC Demethylase Inhibitors** *Cell Reports* **19**:1669–84

34. Heinemann B, Nielsen JM, Hudlebusch HR, Lees MJ, Larsen DV, Boesen T, et al. (2014) **Inhibition of demethylases by GSK-J1/J4** *Nature* **514**:E1–E2

35. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, et al. (2010) **A Chromatin-Mediated Reversible Drug-Tolerant State in Cancer Cell Subpopulations** *Cell* **141**:69–80

36. Hyun K, Jeon J, Park K, Kim J (2017) **Writing, erasing and reading histone lysine methylations** *Experimental & Molecular Medicine* **49**

37. Chen YK, Bonaldi T, Cuomo A, Del Rosario JR, Hosfield DJ, Kanouni T, et al. (2017) **Design of KDM4 Inhibitors with Antiproliferative Effects in Cancer Models** *ACS Medicinal Chemistry Letters* **8**:869–74

38. Metzger E, Stepputtis SS, Strietz J, Preca B-T, Urban S, Willmann D, et al. (2017) **KDM4 Inhibition Targets Breast Cancer Stem–like Cells** *Cancer Research* **77**:5900–12

39. Gogleva A, Polychronopoulos D, Pfeifer M, Poroshin V, Ughetto M, Martin MJ, et al. (2022) **Knowledge graph-based recommendation framework identifies drivers of resistance in EGFR mutant non-small cell lung cancer** *Nature Communications* **13**

40. Chang CA, Jen J, Jiang S, Sayad A, Mer AS, Brown KR, et al. (2022) **Ontogeny and Vulnerabilities of Drug-Tolerant Persisters in HER2+ Breast Cancer** *Cancer Discovery* **12**:1022–45

41. Rehman SK, Haynes J, Collignon E, Brown KR, Wang Y, Nixon AML, et al. (2021) **Colorectal Cancer Cells Enter a Diapause-like DTP State to Survive Chemotherapy** *Cell* **184**:226–42

42. Liu Y, Azizian NG, Sullivan DK, Li Y (2022) **mTOR inhibition attenuates chemosensitivity through the induction of chemotherapy resistant persisters** *Nature Communications* **13**

43. du Manoir S, Delpech H, Orsetti B, Jacot W, Pirot N, Noel J, et al. (2022) **In high-grade ovarian carcinoma, platinum-sensitive tumor recurrence and acquired-resistance derive from quiescent residual cancer cells that overexpress CRYAB, CEACAM6, and SOX2** *The Journal of Pathology* **257**:367–78

44. Kimbung S, Markholm I, Bjöhle J, Lekberg T, von Wachenfeldt A, Azavedo E, et al. (2018) **Assessment of early response biomarkers in relation to long-term survival in patients with HER2-negative breast cancer receiving neoadjuvant chemotherapy plus bevacizumab: Results from the Phase II PROMIX trial** *International Journal of Cancer* **142**:618–28

45. van den Ende T, de Clercq NC, van Berge Henegouwen MI, Gisbertz SS, Geijsen ED, Verhoeven RHA, et al. (2021) **Neoadjuvant Chemoradiotherapy Combined with Atezolizumab for Resectable Esophageal Adenocarcinoma: A Single-arm Phase II Feasibility Trial (PERFECT)** *Clinical Cancer Research* **27**:3351–9

46. Snipstad K, Fenton CG, Kjæve J, Cui G, Anderssen E, Paulssen RH (2010) **New specific molecular targets for radio-chemotherapy of rectal cancer** *Molecular Oncology* **4**:52–64

47. Zhang S, Yu D (2012) **Targeting Src family kinases in anti-cancer therapies: turning promise into triumph** *Trends in Pharmacological Sciences* **33**:122–8

48. Formisano L, Lu Y, Servetto A, Hanker AB, Jansen VM, Bauer JA, et al. (2019) **Aberrant FGFR signaling mediates resistance to CDK4/6 inhibitors in ER+ breast cancer** *Nature Communications* **10**

49. Zhang L, Yang Y, Chai L, Bu H, Yang Y, Huang H, et al. (2020) **FRK plays an oncogenic role in non-small cell lung cancer by enhancing the stemness phenotype via induction of metabolic reprogramming** *International Journal of Cancer* **146**:208–22

50. Girotti MR, Pedersen M, Sanchez-Laorden B, Viros A, Turajlic S, Niculescu-Duvaz D, et al. (2013) **Inhibiting EGF Receptor or SRC Family Kinase Signaling Overcomes BRAF Inhibitor Resistance in Melanoma** *Cancer Discovery* **3**:158–67

51. Kohale IN, Yu J, Zhuang Y, Fan X, Reddy RJ, Sinnwell J, et al. (2022) **Identification of Src Family Kinases as Potential Therapeutic Targets for Chemotherapy-Resistant Triple Negative Breast Cancer** *Cancers [Internet]* **14**

52. Loh Y-H, Zhang W, Chen X, George J, Ng H-H (2007) **Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells** *Genes & Development* **21**:2545–57

53. Zhang Z, Lee JC, Lin L, Olivas V, Au V, LaFramboise T, et al. (2012) **Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer** *Nature Genetics* **44**:852–60

54. Kim H-Y, Choi H-J, Lee J-Y, Kong G (2020) **Cancer Target Gene Screening: a web application for breast cancer target gene screening using multi-omics data analysis** *Briefings in Bioinformatics* **21**:663–75

55. Sachs N, de Ligt J, Kopper O, Gogola E, Bounova G, Weeber F, et al. (2018) **A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity** *Cell* **172**:373–86

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Reviewer #1 (Public review):

Summary:

The authors employed a combinatorial CRISPR-Cas9 knockout screen to uncover synthetically lethal kinase genes that could play a role in drug resistance to kinase inhibitors in triple-negative breast cancer. The study successfully reveals FYN as a mediator of resistance to depletion and inhibition of various tyrosine kinases, notably EGFR, IGF-1R, and ABL, in triple-negative breast cancer cells and xenografts. Mechanistically, they demonstrate that KDM4 contributes to the upregulation of FYN and thereby is an important mediator of the drug resistance. All together, these findings suggest FYN and KDM4A as potential targets for combination therapy with kinase inhibitors in triple-negative breast cancer. Moreover, the study may also have important implications for other cancer types and other inhibitors, as the authors suggest that FYN could be a general feature of drug-tolerant persister cells.

Strengths:

(1) The authors used a large combination matrix of druggable tyrosine kinase gene knockouts, enabling studying of co-dependence of kinase genes. This approach mitigates off-target effects typically associated with kinase inhibitors, enhancing the precision of the findings.

(2) The authors demonstrate the importance of FYN in drug resistance in multiple ways. They demonstrate synergistic interactions using both knockouts and inhibitors, while also revealing its transcriptional upregulation upon treatment, strengthening the conclusion that FYN plays a role in the resistance.

(3) The study extends its impact by demonstrating the potent in vivo efficacy of certain combination treatments, underscoring the clinical relevance of the identified strategies.

Weaknesses:

(1) The combination of FYN knockout with other gene knockouts exhibits only very modest synergy. The high standard deviation observed for FYN knockout in Figure S2A weakens the robustness of these findings. As combination treatments involving inhibitors did demonstrate stronger synergistic effects, the data still support the role of FYN in regulating sensitivity to the described drugs.

(2) While the study identifies KDM4A as a key contributor to FYN upregulation, it does not fully explore the upstream mechanisms regulating KDM4A or the downstream pathways through which FYN upregulation confers drug resistance. These unaddressed questions limit the mechanistic understanding that can be obtained from this study.

(3) FYN has been implicated in drug resistance in previous studies, and other mechanisms for its upregulation and downstream effects have already been described. While this study adds

value to the existing literature in the context of breast cancer, it does not present entirely novel findings regarding FYN's role in drug resistance.

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Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *The authors employed a combinatorial CRISPR-Cas9 knockout screen to uncover synthetically lethal kinase genes that could play a role in drug resistance to kinase inhibitors in triple-negative breast cancer. The study successfully reveals FYN as a mediator of resistance to depletion and inhibition of various tyrosine kinases, notably EGFR, IGF-1R, and ABL, in triple-negative breast cancer cells and xenografts. Mechanistically, they demonstrate that KDM4 contributes to the upregulation of FYN and thereby is an important mediator of drug resistance. All together, these findings suggest FYN and KDM4A as potential targets for combination therapy with kinase inhibitors in triple-negative breast cancer. Moreover, the study may also have important implications for other cancer types and other inhibitors, as the authors suggest that FYN could be a general feature of drug-tolerant persister cells.*
>
> *Strengths:*
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> *(2) The authors demonstrate the importance of FYN in drug resistance in multiple ways. They demonstrate synergistic interactions using both knockouts and inhibitors, while also revealing its transcriptional upregulation upon treatment, strengthening the conclusion that FYN plays a role in the resistance.*
>
> *(3) The study extends its impact by demonstrating the potent in vivo efficacy of certain combination treatments, underscoring the clinical relevance of the identified strategies.*
>
> *Weaknesses:*
>
> *(1) The methods and figure legends are incomplete, posing a barrier to the reproducibility of the study and hindering a comprehensive understanding and accurate interpretation of the results.*

We thank the reviewer for pointing this out. We tried adding as much detail in methods and figures legends as possible to maximize reproducibility and accuracy in interpreting our results as will be described for our responses for the recommendations for authors.

> *(2) The authors make use of a large quantity of public data (Fig. 2D/E, Fig. 3F/L/M, Fig 4C, Fig 5B/H/I), whereas it would have strengthened the paper to perform these experiments themselves. While some of this data would be hard to generate (e.g. patient data) other data could have been generated by the authors. The disadvantage of the use of public*

> *data is that it merely comprises associations, but does not have causal/functional results (e.g. FYN inhibition in the different cancer models with various drugs). Moreover, by cherry-picking the data from public sources, the context of these sources is not clear to the reader, and thus harder to interpret correctly. For example, it is not directly clear whether the upregulation of FYN in these models is a very selective event or whether it is part of a very large epigenetic re-programming, where other genes may be more critical. While some of the used data are from well-known curated databases, others are from individual papers that the reader should assess critically in order to interpret the data. Sometimes the public data was redundant, as the authors did do the experiments themselves (e.g. lung cancer drug-tolerant persisters), in this case, the public data could also be left out.*
>
> *More importantly, the original sources are not properly cited. While the GEO accession numbers are shown in a supplementary table, the articles corresponding to this data should be cited in the main text, and preferably also in the figure legend, to clarify that this data is from public sources, which is now not always the case (e.g. line 224-226). If these original papers do already mention the upregulation of FYN, and the findings from the authors are thus not original, these findings should be discussed in the Discussion section instead of shown in the Results.*

We welcome the reviewer's concern. As reviewer pointed out, our analysis with FYN expression levels in multiple studies with drug tolerant cells may merely reflect association and not causal relationships. We had at least shown that FYN inhibition may reduce drug tolerance in TNBC and EGFR inhibitor treated lung cancer cells (figures 2H, 5E). The causal role of FYN in emergence of drug tolerance in other cancers treated with different drugs (such as irinotecan treated colon adenocarcinoma and gemcitabine treated pancreatic adenocarcinoma) may be beyond scope of this study. We made a brief discussion addressing this concern in lines 273-275.

We also added proper citations of the public data used in this study in main text and figure legends in lines 267-269. The GEO accession numbers are listed in supplementary table S2. Importantly, none of the referenced studies identified FYN as key factor in generating drug tolerant cells.

> *(3) The claim in the abstract (and discussion) that the study "highlights FYN as broadly applicable mediator of therapy resistance and persistence", is not sufficiently supported by the results. The current study only shows functional evidence for this for an EGFR, IGF1R, and Abl inhibitor in TNBC cells. Further, it demonstrates (to a limited extent) the role of FYN in gefitinib and osimertinib resistance (also EGFR inhibitors) in lung cancer cells. Thus, the causal evidence provided is only limited to a select subset of tyrosine kinase inhibitors in two cancer types. While the authors show associations between FYN and drug resistance in other cancer types and after other treatments, these associations are not solid evidence for a causal connection as mentioned in this statement. Epigenetic reprogramming causing drug resistance can be accompanied by altered gene expression of many genes, and the upregulation of FYN may be a consequence, but not a cause of the drug resistance. Therefore, the authors should be more cautious in making such statements about the broad applicability of FYN as a mediator of therapy resistance.*

We fully agree with the reviewer's concern that FYN upregulation is simply an association, and may not be the cause of drug tolerance and resistance. Therefore, to accurately convey our findings, we edited our manuscript in lines 34-36 in abstract to "*FYN* expression is associated with therapy resistance and persistence by demonstrating its upregulation in various experimental models of drug-tolerant persisters and residual disease following targeted therapy, chemotherapy, and radiotherapy" and lines 288-290 in discussion to "

Upregulation of *FYN* is a general feature of drug tolerant cancer cells, suggesting the association of *FYN* expression with drug resistance and tumor recurrence after treatment." We hope this satisfies the reviewer.

> *(4) The rationale for picking and validating FYN as the main candidate gene over other genes such as FGFR2, FRK2, and TEK is not clear.*
>
> *a. While gene pairs containing FGFR2 knockouts seemed to be equally effective as FYN gene pairs in the primary screening, these could not be validated in the validation experiment. It is unclear whether multiple individual or a pool of gRNAs were used for this validation, or whether only 1 gRNA sequence was picked per gene for this validation. If only 1 gRNA per gene was used, this likely would have resulted in variable knockout efficiencies. Moreover, the T7 endonuclease assay may not have been the best method to check knockout efficiency, as it only implies endonuclease activity around a gene (but not to the extent of indels that can cause frameshifts, such as by TIDE analysis, or extent of reduction in protein levels by western blot).*
>
> *b. Moreover, FRK2 and TEK, also demonstrated many synergistic gene pairs in the primary screen. However, many of these gene pairs were not included in the validation screening. The selection criteria of candidate gene pairs for validation screening is not clear. Still, TEK-ABL2 was also validated as a strong hit in the validation screen. The authors should better explain the choice of FYN over other hits, and/or mention that TEK and FRK2 may also be important targets for combination treatment that can be further elucidated.*

We thank the reviewer for improving our manuscript. We had concerns with the generalizability of FGFR2, FRK and TEK in TNBC as their expressions are very low in MDA-MB-231, nor were they enriched in TNBC compared to cancer cell lines of other subtypes. We added a brief comment on this concern in results section and discussion section (lines 150-154, figure S3). Although we acknowledge that the validations done in figure 2B is a result of only one guide RNA, with validations with pharmacological inhibition of FYN (figure 2F-I), we hope the reader and reviewer can be convinced with our key findings in synthetic lethality between FYN and other tyrosine kinases.

> *(5) On several occasions, the right controls (individual treatments, performed in parallel) are not included in the figures. The authors should include the responses to each of the single treatments, and/or better explain the normalization that might explain why the controls are not shown.*
>
> *a. Figure 2G: The effect of PP2 treatment, without combined treatment, is not shown.*
>
> *b. Figure 2H/3G: The effect of the knockouts on growth alone, compared to sgGFP, is not demonstrated. It is unclear whether the viability of knockouts is normalized to sgGFP, or to each untreated knockout.*
>
> *c. Figure 2L: The effect of SB203580 as a single treatment is not shown.*

We thank the reviewer for pointing this out. The data shown for all figures listed in these concerns were normalized by the changes in viability by pharmacological or genetic perturbations that synergized with TKIs (NVP-ADW742, gefitinib…etc.) used in the figures in the original manuscript. As reviewer had suggested, we newly added the effect of SB203580 and PP2 treatment on cell viability in supplementary figures S4A, S4K. SB203580 had no significant effect on cell viability, while PP2 treatment caused significant decrease in cell viability, which is expected as PP2 can inhibit activity of multiple Src family kinases. Regardless of the effect of SB203580 and PP2 on cell viability as single agent, it is evident that treatment of TKIs synergistically decreased cell viability in cancer cell lines. The change in

viability by FYN or histone lysine demethylase knockout was also provided in newly added figure S4D and S6C. Notably, genetic ablation of FYN or histone lysine demethylases had modest, if any, influences on cell viability.

> *(6) The study examines the effects at a single, relatively late time point after treatment with inhibitors, without confirming the sequential impact on KDM4A and FYN. The proposed sequence of transcriptional upregulation of KDM4A followed by epigenetic modifications leading to FYN upregulation would be more compellingly supported by demonstrating a consecutive, rather than simultaneous, occurrence of these events. Furthermore, the protein level assessment at 48 hours (for RNA levels not clearly described), raises concerns about potential confounding factors. At this late time point, reduced cell viability due to the combination treatment could contribute to observed effects such as altered FYN expression and P38 MAPK phosphorylation, making it challenging to attribute these changes solely to the specific and selective reduction of FYN expression by KDM4A.*

We thank the reviewer for pointing this out. We performed time course experiment for NVP-ADW742 treatment on MDA-MB-231 cells in our newly added figure 3E. Surprisingly, treatment of NVP-ADW742 increased KDM4A protein level within two hours. FYN protein accumulation followed KDM4A accumulation after 24 hours. This observation, with our chromatin immunoprecipitation data in figure 3O, provide evidence that FYN accumulation is a consequence of KDM4A accumulation and H3K9me3 demethylation upon TKI treatment. We newly discussed this data in results and discussion section in lines 214-216.

> *(7) The cut-off for considering interactions "synergistic" is quite low. The manual of the used "SynergyFinder" tool itself recommends values above >10 as synergistic and between -10 and 10 as additive (https://synergyfinder.fimm.fi/synergy/synfin_docs/). Here, values between 5-10 are also considered synergistic. Caution should be taken when discussing those results. Showing the actual dose response (including responses to each single treatment) may be required to enable the reader to critically assess the synergy, along with its standard deviation.*

We thank the reviewer for careful comments. We reanalyzed our data with SynergyFinder plus tool (Zheng, Genomics, Proteomics, and Bioinformatics 2022), which implements mathematical models distinct from SynergyFinder 3, for more faithful implementation of Bliss, Loewe independence models, and more critically, calculates statistical significance of the synergy. We provide updates synergy plots with statistics in figures 2F, 3J, and S4B. All drug combinations show statistically significant synergy ($p<0.01$). We also add raw data used to calculate synergy in figures 2F, 3J and S4B in supplementary dataset S2.

> *(8) As the effect size on Western blots is quite limited and sometimes accompanied by differences in loading control, these data should be further supported by quantifications of signal intensities of at least 3 biological replicates (e.g. especially Figure 3A/5A). The figure legends should also state how many independent experiments the blots are representative of.*

We added quantifications for figure 3A and 5A for better depiction of our results. Figure legends were edited to indicate this is a representative of three independent experiments.

> *(9) While the article provides mechanistic insights into the likely upregulation of FYN by KDM4A, this constitutes only a fragment of the broader mechanism underlying drug resistance associated with FYN. The study falls short in investigating the causes of KDM4A upregulation and fails to explore the downstream effects (except for p38 MAPK phosphorylation, which may not be complete) of FYN upregulation that could potentially*

> *drive sustained cell proliferation and survival. These omissions limit the comprehensive understanding of the complete molecular pathway, and the discussion section does not address potential implications or pathways beyond the identified KDM4A-FYN axis. A more thorough exploration of these aspects would enhance the study's contribution to the field.*

We welcome the reviewer's careful concern. We agree our delineation of mechanisms underlying TKI resistance in TNBC involving KDM4 and FYN is far from complete. The increases in expression of histone demethylases were observed in cancers treated with different drugs. The mechanisms governing the increase in histone demethylase expression is not known and is beyond the scope of this paper. We newly added this in discussion section in lines 299-304.

> *(10) FYN has been implied in drug resistance previously, and other mechanisms of its upregulation, as well as downstream consequences, have been described previously. These were not evaluated in this paper, and are also not discussed in the discussion section. Moreover, the authors did not investigate whether any of the many other mechanisms of drug resistance to EGFR, IGF1R, and Abl inhibitors that have been described, could be related to FYN as well. A more comprehensive examination of existing literature and consideration of alternative or parallel mechanisms in the discussion would enhance the paper's contribution to understanding FYN's involvement in drug resistance.*

FYN has been implicated in TKI resistance in CML cell lines (Irwin, Oncotarget, 2015). In this study, FYN is similarly transcriptionally upregulated in imatinib resistant CML, and this upregulation is dependent on EGR1 transcription factor. To address this concern, we generated EGR1 KO MDA-MB-231 cells and tested whether these cells retain the ability to accumulate FYN. Consistent with the previous study, imatinib treatment increased EGR1 protein level. However, EGR1 knockout did not influence FYN accumulation in MDA-MB-231 cells. EGR1 mediated accumulation of FYN may be context specific phenomenon to CML (Figure S5B). We newly discussed this result in result sections in lines 187-190. We also acknowledge that SRC family kinases are generally involved in drug resistance in many cancers. We discuss the recent findings regarding SRC family kinases in drug resistance in result section in lines 145-147 and discussion sections in lines 315-317.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *Kim et al. conducted a study in which they selected 76 tyrosine kinases and performed CRISPR/Cas9 combinatorial screening to target 3003 genes in Triple-negative breast cancer (TNBC) cells. Their investigation revealed a significant correlation between the FYN gene and the proliferation and death of breast cancer cells. The authors demonstrated that depleting FYN and using FYN inhibitors, in combination with TKIs, synergistically suppressed the growth of breast cancer tumor cells. They observed that TKIs upregulate the levels of FYN and the histone demethylase family, particularly KDM4, promoting FYN expression. The authors further showed that KDM4 weakens the H3K9me3 mark in the FYN enhancer region, and the inhibitor QC6352 effectively inhibits this process, leading to a synergistic induction of apoptosis in breast cancer cells along with TKIs. Additionally, the authors discovered that FYN is upregulated in various drug-resistant cancer cells, and inhibitors targeting FYN, such as PP2, sensitize drug-resistant cells to EGFR inhibitors.*
>
> *Strengths:*

> *This study provides new insights into the roles and mechanisms of FYN and KDM4 in tumor cell resistance.*
>
> *Weaknesses:*
>
> *It is important to note that previous studies have also implicated FYN as a potential key factor in drug resistance of tumor cells, including breast cancer cells. While the current study is comprehensive and provides a rich dataset, certain experiments could be refined, and the logical structure could be more rigorous. For instance, the rationale behind selecting FYN, KDM4, and KDM4A as the focus of the study could be more thoroughly justified.*
>
> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) The methods and figure legends are incomplete, posing a barrier to the reproducibility of the study and hindering a comprehensive understanding and accurate interpretation of the results. A critical revision of these aspects is needed, for example:*
>
> *a. Catalogue numbers of certain products critical to reproduce the study (e.g. antibodies) and/or at what company they have been purchased (e.g. used compounds)*
>
> *b. On several occasions the used concentrations of drugs or exposure time are not mentioned (e.g. Figure 2H, G (PP2), I, J, K, L, etc.)*
>
> *c. Figure legend of figure panels E-I in Figure 5 seems to be completely incorrect and not consistent with the figure axis etc.*
>
> *d. RT-qPCR methodology is not described in Methods.*
>
> *e. Western blot methods are very limited: these should be described in more detail or cite an article that does.*
>
> *f. Organoid culture: Information about the source of tumour cells (e.g. pre-treatment biopsy, material after surgery), isolation of tumor cells (e.g. methodology, characterization of material) and culture conditions (e.g. culture time before the experiment) is lacking.*
>
> *g. Information about how gefitinib/osimertinib-resistant PC9 and HCC827 cells are generated (as well as culture conditions and where they are from) is missing.*

We thank the reviewer for pointing these out. We have done our best to add experimental details for reproducibility in methods section and figure legends in lines 343-348, 408-426, 431-432, 439-453, 648-650, 671-672 and 691-693.

> *(2) Figure 1B/C/D: it would be more meaningful if the most important hits (at least in one of these panels) were highlighted (e.g. line with gene-pair named), or visualized separately, so that the reader does not have to read the supplementary table to know what the most important hits were.*

We thank the reviewer for careful concern. We newly added labels for key synergistic gene pairs in figures 1D as reviewer suggested.

> *(3) qPCR data shown in Figure S4 is from 1 independent experiment. As these experiments (especially qPCR) can be rather variable and the effect size is not very large, I*

> *would highly recommend repeating these experiments, or excluding them, as conclusions from them are not solid.*

We found performing qPCR with many drugs that did not cause substantial synergistic cell death with NVP-ADW742 in figure S5C (figure S4A in previous version of manuscript) will not provide much additional insights. Also, as we were more interested in finding direct regulators of FYN expression, we focused on drugs that inhibit epigenetic regulator that activate transcription. Therefore, we focused on performing FYN qPCR with drug combinations involving GSK-J4 (KDM6 inhibitor) and pinometostat(DOT1L inhibitor). As shown in our newly added figure in S5D, while GSK-J4 inhibited FYN expression, pinometostat failed to do so. Also, we also confirm that knockout of KDM5 or KDM6 reproducibly failed to decrease FYN expression upon TKI treatment (figure S5E and S5G). The new results are discussed in lines 193-198. We hope these additions satisfy the reviewer.

> *(4) For validation of synergistic knockouts, it would be helpful for the interpretation to also show the viability/growth of each knockout (or treatment), instead of mostly normalized scores. For example, the reader now has no insight into whether FYN knockout itself already affects cell viability, or not. If it (or EGFR/IGF1R/ABL knockout) would already substantially affect cell viability, a further reduction in cell viability may not be as relevant as when it would not affect cell viability at all.*

We thank the reviewer for pointing this out. We replaced our figure in figure 2A to indicate raw changes in cell viability in each single and double knockout cells in figure S2A. We hope this satisfies the reviewer.

> *(5) The curve fitting as in Figure 2G is somewhat misleading. While the curve seems to be forced to go from 1-0, the +PP2 dose-response curve does actually not seem to start at 1, but rather at 0.8, likely resulting from the effect of PP2 as a single treatment, thus, effects may be interpreted as more synergistic than that they truly are.*

The results shown in figure 2G is actually normalized to cells treated or not with PP2 to better reflect the effect of NVP-ADW742, gefitinib and imatinib in the presence of PP2. So viability value starting at 0.8 is not because of the effect of PP2 treatment as single agent (because it is normalized to PP2 treated cells), but is actually because very small dose of particularly NVP-ADW742 resulted in modest decrease in viability. To more accurately depict our findings, we added the data point in figure 2G with TKI dose of 0uM at viability 1. We also added details for normalization of viability in figure legends.

> *(6) The readability of the paper could be enhanced by higher-quality images (now the text is quite pixelated).*

We had technical difficulties in converting file types. We have replaced figures for better resolution for all main and supplementary figures.

> *(7) The discussion now contains one paragraph about the selectivity of kinase inhibitors, and that repurposing of inhibitors with more relaxed specificity or multi-kinase inhibitors can be beneficial. This does not seem to fall within the scope of the study, as there was no comparison between selective and non-selective inhibitors. It was also not clearly mentioned that the non-selective inhibitors worked better than the gene knockouts, or that for example, KDM3 and KDM4 knockout together worked better than only KDM4 knockout. It is recommended to either remove this paragraph, or rephrase it so that it better fits the actual results*

We agree with the reviewer. We chose to remove this paragraph in lines 308-313.

> *(8) The entire paper does not discuss any known functions of FYN. Its function could be very briefly introduced in the results section when highlighting it as an important hit. More importantly, its known role in cancer and especially drug resistance should be discussed in the discussion (see also Public review).*

We thank the reviewer for pointing this out. We added brief description of the role of FYN in cancer malignancy and drug resistance in lines 145-147. Particularly, FYN accumulation by EGR1 transcription factor had been described in the context of imatinib resistant chronic myeloid leukemia (Irwin, Oncotarget, 2015). To address this, we tested whether EGR1 knockout decreases FYN level in MDA-MB-231 (Figure S5A). Notably EGR1 knockout failed to decrease FYN protein level. This result was discussed in lines 187-190.

> *(9) Textual changes including:*
>
> *a. Line 29 (and others) "Massively parallel combinatorial CRISPR screens": I would rather choose a more descriptive term, such as "combinatorial tyrosine kinase knockout CRISPR screen", which already clarifies the screen used knockouts of (druggable) tyrosine kinases only. Using both "Parallel" and "combinatorial" is somewhat redundant, and "massively" is subjective, in my opinion.*

Manuscript edited as suggested (lines 29, 63, 86, 283). The term “massively parallel” have been removed as they don’t significantly change our scientific findings.

> *b. Line 67 (and others): "to identify ... for elimination of TNBC": while this may be its potential implication, this study has identified genes in (mostly) TNBC cell lines and cell line xenografts. Please rephrase to something more within the scope of this research.*

Manuscript edited as suggested (lines 68-69) as “we utilize CombiGEM-CRISPR technology to identify tyrosine kinase inhibitor combinations with synergistic effect in TNBC cell line and xenograft models for potential combinatorial therapy against TNBC.” We hope it satisfies the reviewer.

> *c. Line 31 (and others): Please check the capitals of words describing inhibitors, and make them consistent (e.g. Imatinib written with capital I, other inhibitors without capitals).*

We thank the reviewer for catching this error. We changed all “imatinib” and “osimertinib” to lowercase.

> *d. Line 71: "... combining PP2, saracatinib (FYN inhibitor), .." ..." Here it is not clear PP2 is a FYN inhibitor, and, as saracatinib is a well-known Src-inhibitor, it is not correct to just say "FYN inhibitor". Better to rephrase to something such as: "combining PP2 (Lck/Fyn inhibitor), saracatinib (Src/FYN inhibitor).*

As reviewer noted, most Src family kinase inhibitors are not selective against specific member among other Src family members. Therefore, we changed line 73 to “PP2, saracatinib (Src family kinase / FYN inhibitor).”

> *e. Line 81: "The resulting library enabled massively parallel screens of pairwise knockouts, .." To clarify this is for the selected kinases only: "The resulting library enabled screens of pairwise knockouts of the 76 tyrosine kinase genes, .."*

Manuscript edited as suggested by the reviewer in line 86.

f. Line 88 (and others): "after infection" consider rephrasing to "after transduction" as this is more commonly used when using lentiviral vectors only.

We thank the reviewer for this. Every “infection” that designates lentiviral transduction were changed to “transduction”.

> *g. Line 97-99: While being described as "good" correlation, a correlation of the same sgRNA pair, yet in a different order, of r=0.5 does not seem to be very good, neither does a correlation of r=0.74 for biological replicates. Please consider describing in a less subjective way.*

We removed the subjective terms and changed the manuscript as follows: “sgRNA pair (e.g., sgRNA-A + sgRNA-B and sgRNA-B + sgRNA-A) were positively correlated (r = 0.50) and were combined when calculating Z (Fig. S1D). The Z scores for three biological replicates were also correlated with r = 0.74 between replicates #2 and #3 (Fig. S1E).” in lines 97-101.

> *h. Lines 92-96 and lines 102-115: The results section here contains quite a lot of technical information. While some information may be directly needed to understand the described results (such as a very short and simple explanation of how to interpret gene interaction score), other information may be more appropriate for the Methods section, to enhance the readability of the paper. Consider simplifying here and giving a more detailed overview in the Methods section. Also, the text is not entirely clear. You seem to give two separate explanations of how the GI scores were calculated (Starting in lines 106 and 111): please rephrase and clearly indicate the connections between those two explanations (in the Methods section).*

We thank the reviewer for valuable suggestion. We moved significant portions of the technical descriptions in methods section. We also clarified the text regarding the procedures for calculating GI scores in lines 385-387.

> *i. Line 142: "These findings suggest that gene A could represent an attractive drug target.." "Gene A" should be "FYN"?*

We thank the reviewer for catching this. Indeed, it is “FYN” and we changed it in line 154.

> *j. Line 149: Introduce Saracatinib, and make the reader aware that it actually mostly targets Src, and FYN with lower affinity.*

We newly added text in lines 73 and 164 to indicate that saracatinib is an inhibitor against Src family kinases.

> *k. Line 469: "by the two sgRNA."□ "by the two sgRNAs".*

Corrected

> *l. Throughout text/figures/figure legends, please check for consistency in the naming of cell lines, compounds, referring to figures etc. (E.g. MDA-MB-231/MDA MB 231/MDAMB-231 ; Fig. 1/Figure 1).*

Corrected. Thank you for catching this error.

> *m. In Methods, frequently ug or uL are used instead of µg or µL*

Corrected.

> *n. Legend Figure 5: Clarify what A, G, I, D, and P mean.*

Corrected in line 685-686 to: “A: NVP-ADW742, G: gefitinib, I: imatinib, D: doxorubicin, P: Paclitaxel.”

> *o. Line 303: What is meant by: "The six variable nucleotides were added in reverse primer for multiplexing". Could you clarify this in the text?*

We apologize for confusion the six nucleotides is index sequence for multiplexed run in NGS. The text in lines 373-374 is edited to: “The six nucleotides described as “NNNNNN” in reverse primer above represents unique index to identify biological replicates in multiplexed NGS run.”

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *To enhance the robustness of the conclusions drawn from this study, certain concerns merit attention.*
>
> *Concerns:*
>
> *(1) Line 130 indicates that eight synergistic target gene combinations were validated. It would be helpful to clarify the criteria used to select these gene pairs and provide the rationale for studying these specific combinations of genes.*

In fact, we had selected the gene pairs that we had the sgRNAs against available when we performed the experiments, so we did not have very good reason to explain our selections. Instead we added a brief discussion in lines 304-306 that further validations are required for the gene pairs not experimentally tested.

> *(2) According to Figure 2C, FYN was identified as crucial among the 30 gene pairs, and its upregulation in TNBC prompted further investigation. It would be informative to discuss the expression levels of TEK, FRK, and FGFR2 in TNBC and explain why these nodes were not studied. Is there existing evidence demonstrating the superiority of FYN over these other genes?*

The similar concern was raised by reviewer #1. The expression levels of TEK, FRK and FGFR2 were relatively low in MDA-MB-231 and TNBCs in general, and we were concerned about the generalizability of these targets for treating TNBC. While the validation of these genes for possible synthetic lethality may lead to valuable insight, this may be beyond scope of this paper. This concern is newly discussed in result and discussion sections in lines 150-154.

> *(3) The screening process employed only one cell line, and validation was conducted with only one cell line (Figure 2A). Consider supplementing the findings with more convincing evidence from other breast cancer cell lines to strengthen the conclusions.*

Although the CRISPR screens and primary validations were done with only one cell line, further validations with drug combinations were done in independent cancer cell lines such as Hs578T (figures S4E-J). Also, the possible association of FYN expression in drug tolerant cells were also demonstrated in lung cancer cells. We hope this satisfies the reviewer.

> *(4) The network analysis in Figure 2C lacks a description of the methodology used. It would be beneficial to provide a brief explanation of the methods employed for this analysis.*

The network analysis was done manually with the size of each node proportional to the number of gene pairs. We newly added text in figure legend in line 638 to clarify this.

> *(5) The significance of gene A mentioned in line 142 is unclear. Please provide a clear explanation or context for the importance of this gene.*

This is a mistake that were also pointed out by reviewer #1. The “gene A” should have been “FYN”. We corrected this in line 154.

> *6. In Figure 2J and Figure 2K, it would be more informative to measure the phosphorylation levels of FYN and SRC rather than just their baseline levels. Consider revising the figures accordingly.*

We thank the reviewer for a careful comment. We newly provide supplementary figure S5A to show that phosphorylation level of FYN is increased, but this increase was proportional to the increase in FYN protein level, so the ratio of pFYN/FYN did not change significantly. We discussed this result in lines 187-190.

> *(7) Figure S4B lacks biological replicates, which could impact the reliability of the experimental results. Consider adding biological replicates to enhance the robustness of the findings.*

This was also pointed out by reviewer #1. Instead of performing qPCR for all drugs, we focused on validating the decrease in FYN mRNA level for drug combinations that synergistically kill cancer cells. We were also aiming to identify direct mediator of FYN mRNA upregulation, so we focused on drug combination that involves inhibitor of epigenetic regulator that promotes transcription. To this end, we tested the impact of GSK-J4(KDM6 inhibitor) and pinometostat (DOT1L inhibitor) in combination with TKI in regulating FYN expression level. Notably, while GSK-J4 attenuated FYN mRNA accumulation by NVP-ADW742 treatment, pinometostat failed to do so (figure S5C). We newly described these results in lines 192-197 in results section.

> *(8) Line 186 indicates that KDM3 knockout was not tested in Figure S5A. It would be helpful to provide an explanation for this omission or consider including the data if available.*

We thank the reviewer for pointing this out. The T7 endonuclease assay results for KDM3, KDM4 and PHF8 are added in figure S6B. All guide RNAs used in the study efficiently generated indel mutations.

> *(9) In line 206, KDM4A is introduced, but Figures 3J and 3M had already pointed to KDM4A. The authors did not analyze the ChIP results for other members of the KDM4 family at this point. Please address this inconsistency and provide a rationale for focusing on KDM4A. Additionally, in Figure 3M, consider adding peak labeling to the enriched portion for clarity.*

We welcome the reviewer’s careful concern. KDM4 family enzymes perform catalytically identical reactions, and are thought to be redundant. Therefore, we judged that the most abundantly expression genes among KDM4 family should be the primary target to focus on. To this end, we analyzed the expression levels of KDM4 family genes in supplementary figure S6A. Indeed KDM4A expression was the highest among other KDM4 family genes. We discussed this in results section in lines 218-220.

> *(10) The author only indicated the relationship between the H3K9me3 level in the enhancer region and FYN expression. It would be valuable to verify the activity of the enhancers and investigate additional markers such as H3K27ac and H3K4me1. Consider discussing these aspects to provide a more comprehensive understanding.*

Since we and others had shown that histone dementhylases are increased upon drug treatment, we focused on histone methylation marks which are associated with gene repression and whose removal by demethylases are associated with drug resistance. To this end, KDM6 demethylases removing H3K27me3 may serve as attractive alternative. In our newly added supplementary figure S6E, ADW742 treatment did not decrease H3K27me3 level in FYN promoter, indicating that H3K9me3 may be the dominant epigenetic change that modulates FYN expression upon drug treatment. This was briefly discussed in lines 233-235.

> *(11) In Figure 4A, the addition of the drug alone does not inhibit tumor growth. Please provide an explanation for this result and consider discussing potential reasons for the observed lack of inhibition.*

The drug dose was adjusted carefully to minimize tumor shrinkage by single drug so that synergistic tumor shrinkage can be clearer.

> *(12) Line 208 indicates missing parentheses in the text describing Figure 4C. Please correct the text accordingly to ensure clarity.*

Corrected. Thank you for catching this error.

> *(13) The figure legends for Figures 5E, F, G, and H contain errors. Please correct the figure legends to accurately describe the respective figures.*

We thank the reviewer for catching this error. We have changed the figure legends in lines 691-697 to accurately describe the figures.

> *(14) It may be beneficial for the authors to divide the results section into several subsections and add headings to improve the overall understanding of the findings.*

This is an excellent suggestion. We divided our results section into subsections and added headings in lines 80, 141, 181, 237 and 251 to help readers understand our findings.

> *(15) The authors should include the sgRNA sequences used for gene targeting, along with details of the target genes and negative/positive controls, in the Supplementary Materials to enhance reproducibility and transparency.*

This is a critical point for improving reproducibility of our work. The sgRNA sequences used in the study are newly added in supplementary table S3.

> *(16) The resolution of the figures in the Supplementary Materials is too low, which may impede the authors' ability to interpret the data. Consider providing higher-resolution figures for better readability.*

We had similar concern posed by reviewer #1, we provided higher resolution image for all main and supplementary figures.

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