**Treatment of Triple-Negative Breast Cancer with TORC1/2 Inhibitors Sustains a Drug-Resistant and Notch-Dependent Cancer Stem Cell Population**

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**Abstract**

Approximately 30% of triple-negative breast cancers (TNBC) harbor molecular alterations in PI3K/mTOR signaling, but therapeutic inhibition of this pathway has not been effective. We hypothesized that intrinsic resistance to TORC1/2 inhibition is driven by cancer stem cell (CSC)-like populations that could be targeted to enhance the antitumor action of these drugs. Therefore, we investigated the molecular mechanisms by which PI3K/mTOR inhibitors affect the stem-like properties of TNBC cells. Treatment of established TNBC cell lines with a PI3K/mTOR inhibitor or a TORC1/2 inhibitor increased the expression of CSC markers and mammosphere formation. A CSC-specific PCR array revealed that inhibition of TORC1/2 cophosphorylates S6K and 4EBP1, signal transducers involved in RNA translation and protein synthesis, while TORC2 phosphorylates and activates Akt, a major effector of PI3K signaling (8). Inhibitors of PI3K/mTOR, TORC1/2 and TORC1, are currently being developed in breast cancer patients (9). Preclinical studies using patient-derived and cell line–generated TNBC xenografts suggest an antitumor effect of PI3K/mTOR (4) and mTOR inhibitors (10). However, clinical efficacy of these drugs in patients with TNBC has been limited. Recent publications have implicated various mechanisms of resistance to PI3K/mTOR inhibitors, such as BEZ235. These mechanisms included activation of JAK2/STAT5, STAT3, and eif4E in various tumor models (11, 12). The PI3K/mTOR inhibitor BEZ235 binds to the kinase domain of mTOR, thus potently inhibiting both TORC1 and TORC2 complexes in addition to PI3K (13, 14). Cancer stem cells (CSC) are a subpopulation of drug-resistant cells with self-renewing and tumor-initiating capabilities (15, 16). Based on these concepts, we first identified that resistance to BEZ235 was driven more by TORC1/2 inhibition than PI3K inhibition and, second, we asked whether this resistance was due to the survival of a CSC-like population. We hypothesized that TORC1/2 inhibition promotes the survival of CSCs and, therefore, targeting molecular pathways utilized by these CSCs should enhance the antitumor effect of these inhibitors against TNBC cells. We show herein that TORC1/2 inhibition results in activation of Notch 1, which, in turn, increases CSCs. Further, we show that Notch1 activation is dependent on FGFR1 and mitochondrial activity. These results point to an intrinsic limitation of TORC1/2 inhibitors in TNBC but also suggest that combinations of TORC1/2 inhibitors with antagonists of the FGFR–mitochondrial metabolism–Notch1 axis are worthy of clinical investigation in appropriately selected tumors.

**Introduction**

Triple-negative breast cancer (TNBC) accounts for approximately 15% of all breast cancers and is considered the most virulent clinical subtype of this neoplasm. Most of these tumors exhibit a basal-like gene expression signature (1). Patients with metastatic TNBC respond transiently to chemotherapy but almost invariably progress and exhibit a poor prognosis (2). Currently there are no approved targeted therapies in TNBC, underscoring the need to identify pathogenic pathways in this breast cancer subtype. Genomic and proteomic studies have identified PI3K/Akt/mTOR pathway alterations in the basal-like subtype of breast cancer, of which, approximately 80% are TNBC (3–6). However, the molecular blockade of this pathway with single-agent inhibitors has not been effective.

Mammalian target of rapamycin (mTOR) signals via two different complexes, TORC1 and TORC2 (7). TORC1 phosphorylates S6K and 4EBP1, signal transducers involved in RNA translation and protein synthesis, while TORC2 phosphorylates and activates Akt, a major effector of PI3K signaling (8). Inhibitors of PI3K/mTOR, TORC1/2 and TORC1, are currently being developed in breast cancer patients (9). Preclinical studies using patient-derived and cell line–generated TNBC xenografts suggest an antitumor effect of PI3K/mTOR (4) and mTOR inhibitors (10). However, clinical efficacy of these drugs in patients with TNBC has been limited.

Recent publications have implicated various mechanisms of resistance to PI3K/mTOR inhibitors, such as BEZ235. These mechanisms included activation of JAK2/STAT5, STAT3, and eif4E in various tumor models (11, 12). The PI3K/mTOR inhibitor BEZ235 binds to the kinase domain of mTOR, thus potently inhibiting both TORC1 and TORC2 complexes in addition to PI3K (13, 14). Cancer stem cells (CSC) are a subpopulation of drug-resistant cells with self-renewing and tumor-initiating capacities (15, 16). Based on these concepts, we first identified that resistance to BEZ235 was driven more by TORC1/2 inhibition than PI3K inhibition and, second, we asked whether this resistance was due to the survival of a CSC-like population. We hypothesized that TORC1/2 inhibition promotes the survival of CSCs and, therefore, targeting molecular pathways utilized by these CSCs should enhance the antitumor effect of these inhibitors against TNBC cells. We show herein that TORC1/2 inhibition results in activation of Notch 1, which, in turn, increases CSCs. Further, we show that Notch1 activation is dependent on FGFR1 and mitochondrial activity. These results point to an intrinsic limitation of TORC1/2 inhibitors in TNBC but also suggest that combinations of TORC1/2 inhibitors with antagonists of the FGFR–mitochondrial metabolism–Notch1 axis are worthy of clinical investigation in appropriately selected tumors.
Materials and Methods

Cell lines and reagents

All cell lines were obtained from the ATCC and cultured according to the instructions provided by ATCC for no longer than 6 months. Cell lines were tested and authenticated by short tandem repeat (STR) profiling by the ATCC. The human Notch1 intracellular domain (hNICD) construct was a gift from Linzhao Cheng, Johns Hopkins University School of Medicine, Baltimore, MD (Addgene plasmid #17626; ref. 17). RBP-Jk firefly luciferase lentiviral particles were obtained from Sigma-Aldrich. The 4X-CSL luciferase plasmid was a kind gift from Raphael Kopan, Washington University School of Medicine, St. Louis, MO (Addgene plasmid #41726; ref. 18). BEZ235, MLN128, RAD001 (everolimus), and GSI-IX were obtained from SelleckChem. Lucitanib was provided by Clovis Oncology. Paclitaxel and oligomycin A were obtained from Sigma-Aldrich. The Hes1 firefly luciferase plasmid was a kind gift from Scott Hiebert (Vanderbilt University, Nashville, TN).

Viability assays

Cells were seeded in 96-well black plates and treated with inhibitors or siRNAs. At variable time points, 10 μL of Alamar Blue reagent was added to each well. Plates were incubated at 37°C for 4 hours in the dark. After 4 hours, the plates were read in a GloMax Multi Detection plate reader.

Flow cytometry of stem cell markers

The ALDEFLUOR assay (Stemcell Technologies) was performed according to the manufacturer’s guidelines to identify cells with high ALDH activity. Cells were passed through a 35-μm filter, suspended in Aldefluor assay buffer + BODIPY-aminocetaldehyde (BAA) and incubated for 45 minutes at 37°C in the presence or absence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB). CD44-APC (BD Biosciences), PROCR-PE (BD Biosciences), ESA-FITC (BD Biosciences), CD24-PE (BD Biosciences), and CD133-APC (Biologend) antibodies were incubated with single cells in PBS/1% FCS for 30 minutes at 4°C. Cells were stained with propidium iodide (PI) or 7-AAD to exclude nonviable cells. For experiments using cells transfected with GFP-tagged hNICD, CD44-APC was used to detect the CScs. GFP analysis by FACS was used to verify transfection of the hNICD construct. The CSC markers representing tumor-initiating populations in the cell lines used and their respective references are given in Table 1.

Mammosphere assay

Single-cell suspensions were seeded in 6-well ultra-low attachment plates (Corning) in serum-free DMEM/F12 containing 20 ng/mL EGF (R&D Systems) and 1 × B27 (Life Technologies). Fresh mammosphere media were added every 3 days. Mammosphere number and volume were determined using the GelCount mammalian cell colony counter (Oxford Optronix). For microscopic images of mammospheres the original magnification of the images is × 40 (4 × 10×). For generation of secondary mammospheres, primary mammospheres were enzymatically (0.05% Trypsin) and mechanically digested by pipette titration, imaged to assess dissociated single cells, counted and re-seeded in mammosphere media.

Immunoblot analysis

Cell monolayers were washed and then lysed with RIPA lysis buffer (150 mmol/L Tris, pH 7.4, 100 mmol/L NaF, 120 mmol/L NaCl, 100 μmol/L sodium vanadate, and 1 × protease inhibitor cocktail; Roche). Lysates (40 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes; these were first incubated with primary antibodies at 4°C overnight or at room temperature for 2 hours followed by incubation with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Thermo Scientific). All antibodies were from Cell Signaling Technology with the exception of FGFR1, which was obtained from Abcam.

RNA interference

Cells were seeded in 6-well plates or 10-cm dishes. The following day, cells were transfected with siRNA oligonucleotides mixed with Lipofectamine RNAiMax (Life Technologies). Notch1, TAFM, JAG1, Rictor, and Raptor siRNA oligonucleotides were obtained from Dharmacon. FRS2 and FGFRI siRNA was obtained from Ambion.

Dye exclusion assay

Cells were grown in monolayer up to 70% to 80% confluence, treated with 10 μmol/L Cell Tracker Green dye (Life Technologies), and incubated in serum-free media for 30 minutes at 37°C, after which media were replaced with regular growth media for additional 45 minutes. Cells were then trypsinized and seeded at 20% confluence. Cells were analyzed by FACS on days 1 and 10 for FITC expression.

Quantitative real-time PCR

RNA was harvested using the RNAeasy Kit (Qiagen) and used to synthesize cDNA (iSCRIPT cDNA synthesis kit, Bio-Rad) according to the manufacturer’s instructions. PCR reactions were performed using SYBR Green Master Mix (Bio-Rad) and the Bio-Rad IQ5 cycler. Relative mRNA levels were standardized to GAPDH mRNA levels. The Stem Cell, Cancer Stem Cell, and Mitochondrial Metabolism PCR Arrays (SA Biosciences), containing primers for 84 pathway-specific genes each, were performed according to the manufacturer’s instructions. Gene expression was verified in cells independently using primers from SA Biosciences.

Nanostring analysis

RNA was extracted from formalin-fixed paraffin-embedded blocks of tumor biopsies obtained from patients before and after neoadjuvant chemotherapy. Nanostring Analysis was performed as previously described (19).

Luciferase reporter assays

Cells were transiently transfected for 5 hours with the RBP-Jk, 4X-CSL or Hes1 firefly luciferase reporter (SA Biosciences), each
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A

Percentage cell viability vs. CT-5

SUM159  BT549

CTL BEZ MLN

** **

B

SUM159  BT549  MDA231

P-S6

P-4EBP1

Actin

C

SUM159

Side scatter

BAAA-FITC

CTL BEZ235 MLN128 RAD001

% ALDH+ cells

CTL BEZ235 MLN128 RAD001

% CD4+ APC

PROCR-PE

CTL BEZ235 MLN128 RAD001

% CD4+ CD86

ESA-FITC

CTL BEZ235 MLN128

% PROCR+ESA

D

SUM159  BT549

Mammosphere number

CTL BEZ MLN

**

Mammosphere number

CTL BEZ MLN

**
with a CMV Renilla luciferase reporter. Media were changed and cells were treated with BEZ235 or MLN128 for 17 to 48 hours. Cells were lysed and the Dual Luciferase Reporter Assay (Promega) was performed according to the manufacturer’s instructions. Luciferase readings were determined using the GloMax Multidetection System (Promega), and the ratio of firefly luciferase to Renilla luciferase was calculated.

Transmission electron microscopy
SUM159 cells were treated with vehicle, 250 nmol/L BEZ235, or 100 nmol/L MLN128 for 48 hours, processed for transmission electron microscopy (TEM) and imaged in the Vanderbilt Cell Imaging Shared Resource-Research Electron Microscopy facility.

Embedding. Samples were fixed in 2.5% gluteraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4 at room temperature for 1 hour and then transferred to 4°C overnight. Samples were then washed in 0.1 mol/L cacodylate buffer and incubated for 1 hour in 1% osmium tetroxide at room temperature, followed by another wash with 0.1 mol/L cacodylate buffer. Subsequently, the samples were dehydrated through a graded ethanol series and three exchanges with 100% ethanol, followed by two exchanges with pure propylene oxide (PO). Samples were then serially infiltrated with 25% Epon 812 resin and 75% PO (1:3) for 30 minutes at room temperature, with 50% Epon 812 resin and 50% PO (1:1) for 1 hour at room temperature, and then infiltrated once more with 50% Epon 812 resin and 50% PO (1:1) overnight at room temperature. The samples were subsequently infiltrated with 100% Epon 812 resin for 48 hours and then allowed to polymerize at 60°C for an additional 48 hours.

Sectioning and Imaging. Thick sections (500 nm to 1 μm) of the embedded samples were collected using a Leica Ultracut microtome. Sections were contrast stained with 1% toluidine blue and imaged with a Nikon AZ100 microscope. Ultra-thin sections (70-80 nm) were cut and collected on 300-mesh copper grids and post-stained with 2% uranyl acetate and then with Reynolds’ lead stain. Samples were imaged on the Philips/FEI Tecnai T12 electron microscope at various magnifications.

In vivo studies
All animal experiments and procedures were done in accordance with a protocol approved by the Vanderbilt University Institutional Animal Care and Use Committee. Athymic female mice (Harlan Sprague Dawley) were inoculated with 5 × 10^6 SUM159 or 8 × 10^6 MDA468 cells mixed with Matrigel (1:1) in the #4 mammary gland. Ten days (for SUM159) or 3 weeks (for MDA468) later, mice bearing tumors measuring ≥75 mm^3 were randomized to treatment with vehicle, MLN128 (1 mg/kg 3 times weekly p.o.), GSI-IX (10 mg/kg, 4 days on 5 days off i.p.), or both drugs. Tumor diameters were serially measured with calipers and mouse weight determined 2 times weekly. Tumor volume in mm^3 was calculated by the formula: \( \text{volume} = \frac{\text{length} \times \text{width}^2}{2} \).

Xenograft dissociation
Xenografts were collected and tumor fragments either flash frozen in liquid N2 or fixed in 10% formalin followed by embedding in paraffin. In some cases, xenografts were harvested and rinsed in PBS, mechanically minced with sterile blades in DMEM/F12/5% FBS and antibiotics/antimycotics in C-tubes (Miltenyi Biotech), and then incubated with 1× Collagenase/Hyaluronidase + 0.2 mg/mL DNaseI for 30 minutes at 37°C. Dissociated cells were passed through 70- and 35-μm filters and separated from debris using a Ficol-Paque gradient. Cell viability was verified by trypan blue exclusion before inoculation into tumor-free nude mice.

Statistical analysis
Statistical differences were determined using the Student t test. For the NanoString analysis of RNA from paired tumor biopsies, paired t tests were utilized. For animal studies, the differences between treatment groups were determined by the Mann-Whitney test with Bonferroni post hoc corrections. For the in vivo limiting dilution assay, extreme limiting dilution assay (ELDA) analysis was performed, and the difference between groups was recorded as described previously (20). A P value of <0.05 was considered to be statistically significant.

Results
TNBC cell lines display CSC properties following inhibition of TORC1/2
Treatment of SUM159, BT549, and MDA231 TNBC cells with each the PI3K/mTOR inhibitor BEZ235 and the TORC1/2 inhibitor MLN128 decreased growth and expression of activated mTOR substrates S6 ribosomal protein and 4EBP-1 (Fig. 1A and B). Following a 3-day treatment with BEZ235 and MLN128 but not with the TORC1 inhibitor RAD001, surviving SUM159, BT549, and MDA231 cells were enriched for the following CSC markers as determined by FACS: ALDH^+^, CD44^hi^/PROCR^+, and PROCR^+/ESA^+, respectively (Fig. 1C). These are established markers of tumor-initiating populations in these cell lines (Table 1). Consistent with the induced changes in CSC markers, cells surviving BEZ235 and MLN128 treatment exhibited increased mammosphere formation (Fig. 1D). Treatment with the pan-PI3K inhibitor BKM120 did not increase CSC markers in SUM159 and BT549 cells (Supplementary Fig. S1A). This result coupled with the effects induced by BEZ235 and MLN128 suggested that TORC1/2 inhibition but not PI3K inhibition was causally associated with CSC induction. Another feature of CSCs and tumor-initiating cells is increased nuclear dye/label retention (21, 22). Using a dye retention assay, we observed that 40% and 20% of cells treated 10 days with
TORC1/2 inhibition increases Notch1 expression and activity in TNBC cell lines. A, SUM159 cells were treated with control or 100 nmol/L MLN128 for 48 hours. RNA was extracted and used in the cancer stem cell–specific PCR array. Fold regulation of gene expression changes in MLN128-treated cells versus CTL was determined using the SA Biosciences PCR Array software. B, SUM159, BT549, CAL120, CAL51, MDA468, and MDA231 cells were treated with 250 nmol/L BEZ235 or 100 nmol/L MLN128 for 72 hours. q-PCR analysis was performed for NOTCH1 (*, P < 0.05; **, P < 0.005). C, SUM159 and BT549 cells were treated with 250 nmol/L BEZ235 and 100 nmol/L MLN128 in the presence and absence of 10 μmol/L GSI-IX for 72 hours. Immunoblot analysis for the intracellular domain of Notch1 (NICD) and actin was performed. D, SUM159, BT549, and MDA468 cells were treated with 250 nmol/L BEZ235 and 100 nmol/L MLN128 for 72 hours. Immunoblot analysis for Jagged1 (JAG1), phospho–4EBP1, and actin expression was performed. E, q-PCR for Notch1 targets HES1 and HEY2 was performed for SUM159 and BT549 cells treated with BEZ235 and MLN128 (*, P < 0.04; **, P < 0.01). F, SUM159 and BT549 cells were treated with 100 nmol/L MLN128 for 0.5, 3, 24, 48, and 72 hours, followed by immunoblot analysis for NICD, actin, and phospho–S6. G, SUM159 cells were transfected with control, Rictor, Raptor, or both Rictor and Raptor siRNAs for 72 hours. Immunoblot analysis was performed using the indicated antibodies. FACS analysis for ALDH positivity was also performed at the same time point as shown on the right (*, P = 0.008). Error bars, mean ± SEM.

Figure 2.
BEZ235 and MLN128, respectively, retained the Cell Tracker dye compared with <5% of untreated cells (Supplementary Fig. S1B). These results suggest that pharmacologic inhibition of TORC1/2 in TNBC cells results in the survival of a cell population with CSC-like features.

**Inhibition of TORC1/2 increases Notch1 signaling**

In order to identify genes associated with CSCs surviving TORC1/2 inhibition, we used a Cancer Stem Cell–specific PCR array. SUM159 cells were treated with MLN128 for 48 hours and assessed for changes in CSC gene expression. Notch1, Jagged1 (JAG1), POUF51 (OCT4), MUC1, and MYC expression was increased >2-fold upon MLN128 treatment, with Notch1 being the top hit with a near 4-fold induction (Fig. 2A). Supporting the array data, treatment of six TNBC cell lines with each BEZ235 and MLN128 for 72 hours induced Notch1 mRNA levels anywhere from 2- to >20-fold as measured by qPCR (Fig. 2B). Using a separate Stem Cell–specific PCR array to confirm these findings, both Notch1 and JAG1 were induced >1.5-fold upon BEZ235 treatment of SUM159 cells (Supplementary Fig. S2A). In the Stem Cell PCR array, a 10-fold increase in FGF1 mRNA was observed, which was confirmed in SUM159 and BT549 cells treated with each BEZ235 and MLN128 for 3 to 48 hours (Supplementary Fig. S2B and S2C). A similar induction of FGF1 mRNA was seen in MLN128-treated CAL51, CAL120, and MDA468 but not in MDA231 cells (Supplementary Fig. S2D and S2E). Concurrent with the induction of Notch1 mRNA, BEZ235 and MLN128 treatment increased expression of the active Notch1 intracellular domain (NICD). Drug-induced NICD expression was ablated by the γ-secretase inhibitor GSI-IX, which prevents the cleavage of full-length Notch1 (Fig. 2C). In addition, protein expression of the Notch ligand JAG1 was increased in BEZ235- and MLN128-
treated TNBC cells (Fig. 2D). Drug-induced Notch1 activation was associated with increased expression of the classical Notch target genes HES1 and HEY2 (Fig. 2E). Furthermore, BST49, MDAM468, and SUM159 cells transfected with a NICD-responsive transcriptional reporter displayed increased reporter activity upon 17- to 48-hour treatment with BEZ235 and MLN128 (Supplementary Fig. S2F and S2G). MLN128-induced treatment was time dependent, being detected at 24 hours and increasing up to 72 hours (Fig. 2F). To genetically replicate TORC1/2 inhibition, we downregulated Rictor and Raptor with siRNA. Dual knockdown of Rictor and Raptor augmented NICD expression and the ALDH population in SUM159 cells; these were not observed with Rictor or Raptor siRNA alone (Fig. 2G). These data suggest that TORC1/2 inhibition induces expression of CSC genes Notch1 and Jag1, the stem cell gene FGF1, and activates Notch signaling.

Next, we investigated whether chronic inhibition of TORC1/2 also induces a CSC phenotype and Notch1 activity. SUM159 and BT549 cells were maintained in gradually increasing concentrations (50–300 nmol/L) of BEZ235 and MLN128 for a period of 2 months. BEZ235- and MLN128-resistant (BEZR and MLNR) SUM159 and BT549 cells displayed increased CSC markers (Fig. 3A and B) and mammosphere-forming capacity compared with untreated control cells (Fig. 3C and D). This was associated with increased expression of NICD, Jag1, and the Notch1 transcriptional target c-MYC (Fig. 3E), and HES1 luciferase reporter activity (Fig. 3F) in BEZR and MLNR cells compared with their respective untreated controls. BEZR and MLNR cells withdrawn from their respective inhibitor for 72 hours counted to exhibit higher levels of NICD, Jag1, and c-MYC (Fig. 3G). These results suggest that, similar to acute treatment, chronic inhibition of TORC1/2 in TNBC cells sustains a surviving population of CSCs with activated Notch.

Finally, we examined whether a similar enrichment of Notch-activated CSCs occurred with other anticancer therapies. Other studies with TNBC cells have suggested that cells resistant to chemotherapy exhibit cancer stem-like features (19, 23). RNA extracted from 17 matched primary breast tumor biopsies before and after neoadjuvant chemotherapy was subjected to Nanostring analysis. Notch1 and Jag1 mRNA expression was significantly higher in post-chemotherapy, drug-resistant tumors (Supplementary Fig. S3D and S3E). The combination of paclitaxel and MLN128 induced an increase in NICD expression in SUM159 cells, which was abrogated with a γ-secretase inhibitor (Supplementary Fig. S3F). These data also suggest that TORC1/2 inhibitors and anticancer chemotherapeutics result in a resistant Notch1-driven CSC population.

Blockade of Notch1 decreases the CSC population induced by TORC1/2 inhibition

To establish a causal association between Notch1 activity and the CSCs induced by TORC1/2 inhibition, we blocked Notch1 both genetically and pharmacologically. Knockdown of Notch1 using two siRNAs decreased BEZ235 and MLN128-induced CSCs in both SUM159 and BT549 lines as determined by FACS (Fig. 4A and B). Notch1 downmodulation also decreased the ALDH population in SUM159 and BT549 lines as determined by FACS (Fig. 4A and B). Notch1 downmodulation also decreased the ALDH population in SUM159 and BT549 lines as determined by FACS (Fig. 4A and B). Notch1 downmodulation also decreased the ALDH population in SUM159 and BT549 lines as determined by FACS (Fig. 4A and B).

However, the cells surviving the combination displayed increased ALDH positivity and mammosphere formation (Supplementary Fig. 3D and 3E). The combination of paclitaxel and MLN128 induced an increase in NICD expression in SUM159 cells, which was abrogated with a γ-secretase inhibitor (Supplementary Fig. S3F). These data also suggest that TORC1/2 inhibitors and anticancer chemotherapeutics result in a resistant Notch1-driven CSC population.
MLN128-treated xenografts displayed a >4-fold greater tumorigenic potential in vivo compared with cells from vehicle-treated xenografts (P = 0.002; Fig. 4f). This increase in CSC frequency was markedly reduced in cells from tumors that had been treated with MLN128 + GSI (P = 0.001; Fig. 4f). A similar experiment was performed with MDA468 cells. MLN128 treatment decreased growth of MDA468 xenografts (Supplementary Fig. S4D) but enriched for a tumorigenic CSC population in residual drug-resistant tumors. This population was markedly reduced in MLN128 +GSI-treated tumors (P = 0.00001; Supplementary Fig. S4E). These results suggest TORC1/2 inhibition enriches a Notch1-dependent tumor-initiating population.

Notch1 activity induced upon TORC1/2 is dependent on mitochondrial metabolism

To explore mechanisms by which TORC1/2 inhibitors enrich a Notch1-dependent CSC population, we examined metabolic alterations associated with the induction of Notch1 activity and CSCs. A link with cellular metabolisms is suggested by a recent study in pancreatic cancer showed that cells surviving MEK inhibition or KRAS depletion displayed increased mitochondrial metabolism and tumorigenic potential (24). SUM159 and BT549 cells treated with MLN128 expressed increased levels of the mitochondrial stain Mitotracker Red, which is used as readout of mitochondrial membrane potential (Fig. 5A). To determine whether the increased Mitotracker Red staining was due to increased mitochondrial biogenesis, we performed TEM of MLN128-treated SUM159 cells. In MLN128-treated cells, we observed increased mitochondrial number and increased microstructure (labeled droplet fusions; Supplementary Fig. S5A). Similar results were obtained with BEZ235-treated cells (data not shown). In addition to increased mitochondrial mass, BEZ235 and MLN128 treatment increased the expression of the mitochondrial DNA transcription factor, Transcription factor A mitochondrial (TFAM; Fig. 5B). To extend on this induction of TFAM, we performed a Mitochondrial Metabolism PCR Array using SUM159 and BT549 cells. Polycistrionic mitochondrial transcripts from both the H1 and H2 promoter sites of the heavy (H) strand of the mitochondrial genome were increased >2-fold after 3 and 48 hours of MLN128 treatment compared with vehicle-treated cells (Fig. 5C; Supplementary Fig. S5B and S5C). TFAM has been shown to be critical for the production of transcripts from the mitochondrial H strand promoter (25). ATP synthase complex subunits are critical for utilizing the mitochondrial membrane potential alteration during oxidative phosphorylation to generate ATP (26). Expression of the ATP synthase complex subunits (ATP5G2 and ATP5J2) were also upregulated >2-fold in both MLN128-treated SUM159 and BT549 cells (Fig. 5C; Supplementary Fig. S5B and S5C).

To determine if the induction of TFAM and oxidative phosphorylation played a role on CSC induction, we combined MLN128 with two different TFAM siRNAs or with Oligomycin A (ATP synthase inhibitor). TFAM siRNA and Oligomycin A decreased the ALDH1+ population and NICD induced by MLN128 (Fig. 5D and E). TFAM siRNA also enhanced the induction of SUM159 cell viability induced by each BEZ235 and MLN128 compared with each drug alone (Supplementary Fig. S5D). To further support that the inhibition of mitochondrial activity and of oxidative phosphorylation blocked the induction of CSCs upon treatment with the TORC1/2 inhibitor by trumping Notch activation, we rescued Notch1 activity with a GFP-expressing human NICD vector (Fig. 5F). In SUM159 cells cotreated with MLN128 and Oligomycin A, transfection of GFP-hNICD restored the induction of CD44hi expression and mammosphere formation (Fig. 5G). Next, we treated BT549 cells with MLN128 and TFAM siRNA in the presence or absence of a control vector or hNICD. TFAM knockdown decreased MLN128-induced CD44hi cells, but transfection of hNICD restored the CD44hi fraction (Supplementary Fig. S5E). These results suggest that TORC1/2 inhibition augments mitochondrial biogenesis, transcription, and metabolism, which are important for CSC survival and Notch1 activity.

MLN128-mediated Notch1 activity is dependent on FGFR signaling

With the link between mitochondrial metabolism and Notch activity, we next inquired whether induction of the stem cell gene Fgf1 (Supplementary Fig. S2) participated in the interaction of these pathways. In a panel of five TNBC cell lines, acute treatment with MLN128 increased levels of full-length and/or the cleaved form of Fgffr1 (Fig. 5A). Fgffr2, 3, and 4 were undetectable by immunoblot analysis, with the exception of Cal51 (Fgffr2, Fgffr3). However, MLN128 did not alter Fgffr2 and Fgffr3 expression in Cal51 cells (data not shown). We next determined whether Fgffr1 expression was necessary for MLN128-induced NICD expression and mitochondrial activity. Fgffr1 siRNA decreased NICD and TFAM induction in both MLN128-treated SUM159 and BT549 cells (Fig. 5B). Further, MLN128-treated SUM159 cells displayed increased Mitotracker Red expression, which was significantly decreased by Fgffr1 siRNA (Fig. 5C). Similarly, the induction of NICD and TFAM expression was also reduced by Fgffr1 siRNA. These results suggest that TORC1/2 inhibition is dependent on FGFR signaling.

In this study, we observed that TORC1/2 inhibition resulted in decreased survival/growth of TNBC cancer cells both in vitro and in vivo but spared a population with CSC-like properties and enhanced Notch1 activity. Both genetic and pharmacologic inhibition of Notch1 blunted the CSCs induced upon inhibition of TORC1/2. This CSC-like population that survives TORC1/2 inhibition is driven by a Fgffr1–TFAM–Notch1 signaling axis. Activation of Notch1 was not limited to TNBC as inhibition of TORC1/2 in head and neck cancer and ovarian cancer cells also resulted in activation of Notch1 (data not shown). From a clinical perspective, these results suggest that pharmacologic inhibition of TORC1/2 and FGFR signaling may be an effective approach for targeting CSCs in TNBC.
Induction of NICD and CSCs following TORC1/2 inhibition is dependent on mitochondrial metabolism. A, SUM159 and BT549 cells were treated with control or 100 nmol/L MLN128 for 48 hours. Cells were stained with a marker for mitochondrial membrane potential Mitotracker Red-CMXRos and analyzed by flow cytometry. The percentage of Mitotracker Red-positive cells was determined (\( P < 0.0008 \)). B, SUM159 and BT549 cells were treated with vehicle, 250 nmol/L BEZ235, or 100 nmol/L MLN128 for 72 hours. Immunoblot analysis for TFAM, phospho-4EBP1, and actin was performed. C, SUM159 cells were treated with vehicle or 100 nmol/L MLN128 for 48 hours. RNA was extracted, transcribed to cDNA, and applied to a Mitochondrial Metabolism PCR Array. Genes displaying fold changes greater or lower than 1.5 compared with vehicle-treated cells were illustrated. Red highlighted bars indicate mitochondrial polycistronic transcripts. D, immunoblot analysis of SUM159 cells transfected with control or TFAM siRNA, treated with 500 nmol/L oligomycin A (OLI) or 100 nmol/L MLN128 for 72 hours. FACS analysis for ALDH\(^+\) cells was performed (\( P < 0.006 \)). F, SUM159 cells transfected with empty vector and hNICD vector was analyzed by immunoblotting (NTM–Notch transmembrane). G, SUM159 cells were transfected with control vector or human NICD (hNICD) followed by treatment with vehicle or MLN128 ± 500 nmol/L oligomycin (OLI). After 72 hours, the CD44\(^+\) cells were identified by FACS (\( P < 0.005 \)) or seeded as mammospheres and cultured for 10 days. Mammosphere number was determined using Gelcount (\( P = 0.01; **, P = 0.001 \)). Two representative images of mammospheres from each treatment group are illustrated (magnification, \( \times 40 \)). Error bars, mean ± SEM.
Induction of Notch1 and CSCs upon inhibition of TORC1/2 is FGFR dependent. A, SUM159, BT549, MDA468, CAL51, and CAL120 cells were treated with 100 nmol/L MLN128 for 72 hours. Immunoblot analysis for FGFR1 and actin was performed. B, SUM159 and BT549 cells were transfected with control or FGFR1 siRNA and 100 nmol/L MLN128 for 72 hours. Immunoblot analysis for NICD, FGFR1, TFAM, and actin was performed. C, SUM159 cells were transfected with control or FGFR1 siRNA and 100 nmol/L MLN128 for 48 hours. FACS analysis for Mitotracker Red was performed. Cells were treated with 5 μmol/L menadione as a positive control (*, P = 0.003). D, BT549 cells were transfected with control vector or GFP-hNICD followed by transfection with CTL or FGFR1 siRNA and MLN128. After 72 hours, FACS analysis for CD44hi cells was performed (*, P = 0.002; **, P < 0.001). E, SUM159 and BT549 cells were transiently transfected with CTL and FRS2 siRNA or treated with 2 μmol/L lucitanib for 72 hours. Immunoblot analysis for NICD, phospho-S6, and actin expression was performed. F, SUM159 cells were transfected with CTL and FRS2 siRNA for 48 hours followed by qPCR analysis for FRS2 expression (*, P = 0.0003). G, SUM159 and MDA468 cells were treated with 100 nmol/L MLN128 and/or 2 μmol/L lucitanib for 72 hours. FACS analysis for CSC markers was performed (*, P < 0.02). Error bars, mean ± SEM.

H, proposed mechanistic model of NICD and CSC induction following TORC1/2 inhibition in TNBC.
Targeting TORC1/2 and Notch1 Abrogates Tumor Initiation

perspective, these findings underscore a limitation of TORC1/2 inhibitors in cancer because of their ability to induce a signaling nexus that drives the survival of cells with stem-like and tumor-initiating properties. TORC1 inhibition with rapamycin increases the lifespan of mice by increasing the self-renewal and hematopoiesis of hematopoietic stem cells (29). Chronic rapamycin treatment, which by disrupting the raptor/mTOR complex also inhibits TORC2 (30), has been shown to increase animal lifespan by enhancing stem cell survival (31). These observations combined with our findings suggest that TORC1/2 inhibition results in sustenance of a stem cell-like population. This is also important because the pathways used to prevent aging can be exploited as molecular targets, which, once inhibited, would also limit expansion of a CSC population.

In this study, we provide evidence that TORC1/2 inhibition promotes the survival of cells with increased mitochondrial mass and metabolism, which are required for the maintenance of CSCs. A report in pancreatic cancer illustrated that KRAS loss results in the survival of cells with increased mitochondrial metabolism (24). Additionally, the KRAS-deficient surviving cells were tumorigenic and sensitive to inhibition of oxidative phosphorylation. Another study showed that conditional loss of TFAM in keratinocytes decreased two Notch1 transcriptional targets Hes1 and Hey2 (32). Therefore, a tumorigenic population of cells can utilize a mitochondriald-dependent Notch1 axis to survive anticancer therapies. However, the exact mechanism by which oxidative phosphorylation or mitochondrial biogenesis regulates Notch1 activation warrants further investigation.

FGF1 expression was potently induced in several TNBC cell lines upon inhibition of TORC1/2. This is consistent with a recent report by Wilson and colleagues that showed that FGFR ligands can blunt the efficacy of several kinase inhibitors across multiple oncogene-dependent tumor cell lines (33). Another report showed that treatment of HER2þ breast cancer cells with the HER2 inhibitor lapatinib increased the expression of FGFR1 as a mechanism of lapatinib resistance (30). In the study herein, both genetic and pharmacologic inhibition of FGFR decreased MLN128-induced TFAM expression and mitochondrial metabolism (Fig. 6). The link between FGFR1 and mitochondrial activity is consistent with a report using FGFR1-amplification (34, 35). Thus, activation of Notch1 via increased mitochondrial metabolism may be a key event in resistance to anticancer therapies. The mechanism by which FGFR1 regulates TFAM expression remains to be investigated.

In summary, we have identified a mechanism by which TORC1/2 inhibitors sustain a Notch1-dependent tumorigenic population with CSC traits. We propose that this mechanism explains at least in part the limited clinical activity of these drugs as single agents. As a result, therapeutic targeting of Notch1 may be a promising direction to limit this tumor-initiating population and potentially enhance the antitumor effect of TORC1/2 inhibitors in TNBC. Additionally, we have shown that Notch1 is activated via a FGFR1–TFAM-dependent mechanism introducing additional molecular targets (Fig. 6H) that can be exploited in clinical trials in tumors with alterations in the PI3K/mTOR pathway.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


41. Silva IA, Bai S, McLean K, Yang K, Grif.
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