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A genome-wide CRISPR screen identifies genes critical for resistance to FLT3 inhibitor AC220

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Abstract

Acute myeloid leukemia (AML) is a malignant hematopoietic disease and the most common type of acute leukemia in adults. The mechanisms underlying drug resistance in AML are poorly understood. Activating mutations in FMS-like tyrosine kinase 3 (FLT3) are the most common molecular abnormality in AML. Quizartinib (AC220) is a potent and selective second-generation inhibitor of FLT3. It is in clinical trials for the treatment of relapsed or refractory FLT3-ITD-positive and -negative AML patients and as maintenance therapy. To understand the mechanisms of drug resistance to AC220, we undertook an unbiased approach with a novel CRISPR pooled library to screen new genes whose loss of function confers resistance to AC220. We identified SPRY3, an intracellular inhibitor of FGF signaling, and GSK3, a canonical Wnt signaling antagonist, and demonstrated that re-activation of downstream FGF/Ras/ERK and Wnt signaling as major mechanisms of resistance to AC220. We confirmed these findings in primary AML

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patient samples. Expression of SPRY3 and GSK3A was dramatically reduced in AC220-resistant AML samples, and SPRY3-deleted primary AML cells were resistant to AC220. Intriguingly, expression of SPRY3 was greatly reduced in GSK3 knockout AML cells, which positioned SPRY3 downstream of GSK3 in the resistance pathway. Taken together, our study identified novel genes whose loss of function conferred resistance to a selective FLT3 inhibitor, providing new insight into signaling pathways that contribute to acquired resistance in AML.

Keywords

Drug resistance; genetic screen; signal transduction pathway; acute myeloid leukemia

Introduction

Acute myeloid leukemia (AML) is a progressive malignant disease of the bone marrow and blood. FMS-like tyrosine kinase 3 (FLT3) is a protein kinase receptor that is expressed on the surface of many hematopoietic progenitor cells. *FLT3* gene is one of the most frequently mutated genes in AML (1–3). Internal tandem duplication (ITD) of the *FLT3* gene is a gain-of-function mutation common in AML. It is associated with worse prognosis and adverse disease outcome (4–7). Mechanistically, *FLT3-ITD* mutations result in loss of the auto inhibitory function and subsequent constitutive activation of FLT3 kinase as well as its downstream proliferative signaling pathways, including the Ras/MAPK/ERK pathway, STAT5 and PI3K/Akt/mTOR pathway (8–10). Clinically, *FLT3-ITD* mutations are present in roughly 20% of adult AML cases. In majority of the cases, it is a de novo mutation with patients presenting a high leukocyte count with normal cytogenetics. Numerous clinical trial studies have established that patients with *FLT3-ITD* are far more likely to relapse and do so more rapidly than their *FLT3* wild-type counterparts. The median survival of *FLT3* mutant AML patients after first relapse has been reported to be < 5 months(11–13).

The poor prognosis of patients harboring *FLT3* mutations renders FLT3 as an obvious target of therapy. A number of small-molecule tyrosine kinase inhibitors with activity against FLT3 have now been identified and some are currently in clinical trials (12,14,15). Quizartinib (AC220) is a once-daily, orally administered, potent and selective second-generation inhibitor of FLT3. It is currently under clinical trials for the treatment of relapsed or refractory *FLT3-ITD* positive and negative AML patients and as a maintenance therapy. Importantly, even though no FLT3 inhibitors are approved for clinical use, several resistant mechanisms of FLT3 inhibitors have been reported through the early clinical studies (16,17).

Sprouty proteins were first identified in Drosophila by genetic screens as modulators of tracheal and eye development. Several initial elegant studies have demonstrated that Drosophila Sprouty inhibits receptor tyrosine kinases (RTKs)-mediated Ras signaling. Later, studies in mammalian systems also revealed crucial roles for Sprouty in various developmental and physiological processes as well as cancer development, progression and metastasis (18–20). There are four members in the mammalian Sprouty family, *SPRY1-4*. Previous studies implicated Sprouty 1, 2 and 4 in stem cell maintenance, development and cancers (21–23). However, very little is known about *SPRY3*.

GSK3 is a serine/threonine protein kinase first identified as the kinase that phosphorylates and inhibits glycogen synthase. It was later discovered to regulate multiple substrates and is implicated in many cellular processes including embryo development, cell cycle regulation, cell proliferation, and differentiation (24,25). The mammalian GSK3 is encoded by two known genes, *GSK3A* and *GSK3B*. GSK3 is a negative regulator of several other signaling pathways, including Wnt, Notch and PI3K/Akt/mTOR signaling; inhibition of GSK3 by inhibitors activates these pathways (24–26).

Although great success has been achieved in the last three decades in AML therapy, one major obstacle in the treatment of leukemia is drug resistance (27,28). Studies on the mechanisms of AML drug resistance will yield important information about the signaling pathways of leukemia pathogenesis as well as how to circumvent this resistance and improve efficacy of anti-AML drugs (29,30). Here, we reported a genome-wide CRISPR screen for mutations that confer resistance to a selective FLT3 inhibitor AC220. We found that loss of function mutations in *SPRY3* and *GSK3* cause resistance to AC220 in AML cells and that re-activation of downstream signaling in the Wnt and Ras/MAPK pathways is the major mechanism of AC220 resistance conferred by *GSK3* and *SPRY3* deletions.

Materials and Methods

CRISPR screen and sgRNAs construction

GeCKO library was purchased from Addgene (#1000000048), amplified and packaged as lentivirus based on the instructions on Addgene website. The loss of function screen was carried out as described (31). MV4-11 cells were transduced with lentivirus carrying GeCKO library and puromycin selection was performed for 2 days. Then we treated transduced MV4-11 cells with AC220 for 14 days and the survived cells were harvested. The genomic DNA was extracted and PCR was carried out before deep sequencing of sgRNA sequence in the survived cells genome. All deep sequencing data are available at GEO (series accession number GSE 98612). For data analysis, we calculated the enrichment score as: The enrichment score = (sgRNA number from the reads) / (sgRNA number in the library) X $\log_2$(average abundance). The sgRNAs used for validations were synthesized and constructed as described (31). Primer sequences are shown in Supplementary Table 3.

Cell lines and patient samples

Ba/F3-ITD and Ba/F3 lines were a kind gift from Drs. James D. Griffin and Ellen Weisberg at Dana Farber Cancer Institute and Dr. Stephen Sykes at Fox Chase cancer center in 2015. MV4-11 line was kindly provided by Dr. Martin Carroll at UPenn in 2014. MV4-11 cells were maintained in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. The IL-3–dependent murine pro-B cell line Ba/F3 were cultured in RPMI 1640 supplemented 10% FBS and 10 ng/ml IL-3 and 1% penicillin/streptomycin. All cell lines were analyzed and authenticated by morphologic inspection and biochemical examination of *FLT3-ITD* pathway as well as short tandem repeat (STR) profiling analysis. Mycoplasma testing was also performed to exclude the possibility of mycoplasma contamination of all cell lines. The frozen patient samples were obtained from Xenograft core facility of UPenn and written informed consent was obtained from the patients, the studies were conducted in accordance with recognized ethical guidelines and that the studies were approved by UPenn and Temple review board. For culturing primary AML cells, cells were thawed quickly and re-suspended in 10 mL cold RPMI 1640 with 2% HI FBS and centrifuged at 2000RPM for 5 minutes. Cells were incubated for 4 hours and filtered with 40μm filter, then re-suspended in 12 ml RPMI 1640 supplemented with 2% HI FBS and plated in a 6-well plate.

Lentiviral packaging and transduction

Lentivirus was packaged as previously described (32). For transduction, 0.5million MV4-11 cells were seeded in 12-well plate coated with 8μg/ml polybrene (Sigma-Aldrich) and transduced with the lentivirus at an MOI of 0.3 or 20 and then the cells were centrifuged at 1200 g for 2 hours at room temperature and cultured for another 2 hours in the incubator. After 2 hours, medium was changed (RPMI 1640 supplemented with 10% FBS).

Primary AML cells transfection

For patient primary cells, the cells were pre-stimulated with cytokines for 36 hours and nucleotransfected with P3 Primary Cell 4D-Nucleofector X Kit (V4XP-3012) in antibiotics free medium after pre-stimulation. In brief, 2×10^6 primary cells per sample were washed twice in PBS and resuspended in 100μl nucleofector solution with 4μg of plasmids accordingly. The cell/DNA mixture was transferred into the cuvette and transfected with Lonza 4D-Nucleofector System. After transfection, the cells were cultured in RPMI 1640 medium supplemented with 2% HI FBS. After 24 hours, medium was changed with the complete primary cell culture medium and cultured for another 24 hours.

T7EN1 assays and DNA sequencing

After genomic DNA extraction, the genomic region flanking the sgRNA target site was amplified by PCR and T7EN1 assay was performed. T7EN1 assay was conducted as described in our previous work (32). To identify the mutations, the PCR product was sequenced by Sanger sequencing. The primers used for Sanger sequencing were listed in Supplementary Table 3.

Generation of mutant single clones

About 2000 transduced MV4-11 cells were mixed with 1 ml of methylcellulose (MethoCult H4034 Optimum, Stem Cell Technologies) in a 6-well cell culture plate and cultured at 37 °C in a 5% CO_2 incubator. Two weeks later, single clone colonies were picked and cultured in 96-well plate with the complete medium supplemented with 2% penicillin/streptomycin. The cells were passaged every 2 or 3 days and 1/3 of cells were collected for genomic DNA extraction. Then *SPRY3* target region was PCR amplified and sequenced.

Cell number measurement

0.4×10^6 cells were seeded with 1ml complete medium in 12-well plate and AC220 was added at the indicated amounts to cells. After 3、6 or 8 days, 100ul cell suspension was transferred to a 96-well plate and 10ul CCK-8 solution (DOJINDO) was added in each well. The mixture was incubated for 3 hours in incubator. The absorbance at 450 nm was measured using a microplate reader. Cell number was calculated based on the growth standard curve.

Off-target effect examination

Off-target sites were predicted using an online search tool (http://crispr.mit.edu). 3bp mismatches compared with the target consensus sequence were allowed. The predicted off-target sequences were searched using UCSC browse and 500bp flanking the sites were PCR amplified in primary cells and single mutation clone. The PCR product was subjected to T7EN1 assay to determine the mutation. The PCR product was cloned into a TA vector and Sanger sequenced to identify mutations.

Immunoblot analysis

Immunoblotting was performed using whole-cell lysates of MV4-11and Ba/F3 cells supplemented with protease and phosphatase inhibitors (Roche). Proteins were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore), and incubated with primary antibodies listed in Supplementary Table S4. Bands were visualized using HRP-conjugated secondary mouse (Promega) or rabbit (Sigma) or sheep (R&D Systems) antibodies and quantifications were performed using the MultiGauge software (Fujifilm).

Results

Loss of function CRISPR screen in AML cells identified genes critical for drug resistance to AC220

To identify genes whose loss of function confers drug resistance to the FLT3 inhibitor AC220, we performed a genome-wide CRISPR genetic screen in MV4-11, a human AML line harboring a *FLT3-ITD* mutation. This line was established from blast cells of a 10-year-old male with biphenotypic B-myelomonocytic leukemia. It has been shown to be a *FLT3* mutant cell line expressing the phosphorylated receptor protein, making it an appropriate model for *FLT3-ITD*-related research (33). For CRISPR screening, we transduced MV4-11 cells with lentivirus containing a pooled genome-scale CRISPR-Cas9 knockout (GeCKO) library, targeting 18,080 human genes with 64,751 unique guide sequences (6 sgRNAs per gene) (31). This library has been demonstrated to be a very efficient tool to screen for mutations that confer resistance to a RAF inhibitor in a melanoma line. It is considered superior to an shRNA library because of its ability to knock out genes efficiently (31).

First, we packed the library into lentivirus with optimal titer at a multiplicity of infection (MOI) of 0.3 and transduced MV4-11 cells. After viral transduction, we treated the leukemia cells with 3 nM AC220, an optimal dose chosen based on our preliminary tests (Fig. 1A). Transduction of MV4-11 with the CRISPR library but not the vector conferred resistance to AC220 in a subpopulation of cells (Fig. 1B). After 14 days of treatment, we harvested cells from the drug treated group and extracted genomic DNA for PCR the region containing sgRNAs. Then we conducted next generation sequencing (deep sequencing) to identify sgRNAs enriched in drug resistant cells (Fig. 1C). For a number of genes, we found enrichment of multiple sgRNAs that target a few genes after 14 days of AC220 treatment, suggesting that loss of these genes contributes to AC220 resistance (Fig. 1D). Then we ranked the positive hits by the number of the sgRNAs and enrichment changes per sgRNA. As summarized in Supplementary Table 1, we identified one gene (*SPRY3*) with 6 sgRNAs and 12 genes with 5 sgRNAs/gene recovered. Our highest-ranking genes include *SPRY3*, *SERPINE1*, *NUAK1*, *NDUFS5*, *SULT1A3, HDAC5, DDRGK1*, and several members in proto cadherin alpha cluster. To further understand the pathways in AML cells conferring FLT3 inhibitor resistance, we also performed GO/GSEA/pathway analyses with our top candidates and found that genes regulating cell adhesion process are highly enriched (Supplementary Fig. 1). This result is consistent with the previous reports that components of the extracellular matrix and cell adhesion molecules can confer cell adhesion-mediated drug resistance (CAM-DR) to FLT3 inhibitors (34).

SPRY3 is a member of Sprouty proteins that has been shown to function as an antagonist of receptor tyrosine kinases (RTKs)-mediated Ras signaling. Additionally, we also identified *GSK3* as a positive hit in our screen and it has been well known to play an essential role in several signaling pathways in AML.

Knockouts of *SPRY3* and *GSK3* confer resistance to AC220

After identifying potential positive hits from our screen, we next validated whether loss-of-function of individual genes, *SPRY3* or *GSK3*, can cause drug resistance to AC220. *SPRY3* is our top candidate and the only gene with all 6 sgRNAs recovered in our screen. *GSK3* has two isoforms *GSK3α* and *GSK3β* encoded by *GSK3A* and *GSK3B* genes. For *GSK3A* and *GSK3B*, we recovered 4 sgRNAs and 2 sgRNAs respectively in our screen. We chose *GSK3* because it has been implicated in several important downstream molecular pathways of FLT3, including Wnt and PI3K/Akt, in the pathogenesis of AML. To test whether deletion of *SPRY3* or *GSK3* confers resistance to AC220, we used 5 sgRNAs for *SPRY3* and 2 sgRNAs each for *GSK3A* and *GSK3B* to knock out *SPRY3* and *GSK3* respectively in the MV4-11 AML cell line. We transduced cells with lentivirus carrying sgRNAs for the respective genes at an MOI of 20 and performed T7EN1 assays five days after transduction to determine the knockout efficiency. We found that the efficiency of gene disruption ranged from 30% to 90% for all sgRNAs tested (Fig. 2A).

To test whether deletion of *SPRY3* and *GSK3* can confer resistance to AC220, we infected MV4-11 with lentivirus carrying sgRNA and treated transduced cells with different doses of AC220. We then measured cell viability at day 6 post treatment. Consistent with our screen data, *GSK3* or *SPRY3* knockout cells showed marked resistance to treatment with AC220 compared to the control cells (Fig. 2B). Importantly, in the absence of drug treatment, *SPRY3* and *GSK3* null cells grow as well as control cells (Fig. 2C).

Since the CRISPR/Cas9 system can create a spectrum of insertions/deletions (indels) in a cell population, we also tested drug resistance on cloned cells containing a single mutation. We transduced MV4-11 cells with sgRNA targeting *SPRY3* and performed a methylcellulose based colony forming cell (CFC) assay. We then picked 8 clones from the CFC assay, sequenced *SPRY3* in all 8 clones and found that 6 out of 8 clones contained *SPRY3* mutations (Fig. 2D top panel). We then used *SPRY3*$^{-/-}$ single mutation clones to test the drug resistance to AC220. Consistent with the data from the heterogeneous population of CRISPR-mutated cells, all 6 *SPRY3* mutation clones showed strong resistance to AC220 (Fig. 2D bottom panel). The IC50 of 6 *SPRY3* deleted clones was 2.69–5.39 nM while the IC50 for control cells was 0.7 nM. Importantly, the deletion of *SPRY3* was confirmed by western analysis. Furthermore, we also examined other Sprouty family members including *SPRY1, SPRY2* and *SPRY4*, and found that their expression was not affected in *SPRY3* knockouts (Supplementary Fig. 2A, B). Crenolanib is another potent inhibitor of FLT3 and we also tested whether loss of *SPRY3* confers resistance to Crenolanib. We observed that *SPRY3* knockout AML cells were resistant to Crenolanib (Supplementary Fig. 2C). Notably, both *SPRY1* and *SPRY2* are highly expressed in AMLs. To address the question whether other Sprouty members also play a role in AC220 resistance, we designed sgRNAs targeting *SPRY1* and *SPRY2* to knock out these genes and tested AC220 resistance. Clearly, both *SPRY1* and *SPRY2* knockouts confer the resistance to AC220 in AML cells (Supplementary Fig. 2D).

SPRY3 knockout confers resistance to AC220 in primary AML cells

In order to confirm our findings in patients with AML, we measured *SPRY3* and *GSK3A* expression in four patients with AML who are treated with AC220 and clinically resistant to AC220 compared to 6 control AML patient samples who are *FLT3-ITD*+ AML patients without AC220 treatment (Supplementary Table 2). Consistent with our data from the MV4-11 cell line, *SPRY3* and *GSK3A* expression was greatly reduced in AC220 resistant samples compared to the control samples (Fig. 3A). All four AC220 resistant samples were collected at progression to AC220 therapy in patients who previously responded clinically. Among these four AC220 resistant patient samples, we have pre-treatment samples for three samples and measured the expression of *SPRY3* and *GSK3A* in these three-paired patient samples. Remarkably, we found that the expression of *SPRY3* and *GSK3A* was dramatically reduced in the post-treatment samples compared to their baseline (Fig. 3B), suggesting that the expression of *SPRY3* and *GSK3A* might be correlated with the resistance to AC220 in patients. Furthermore, this is also an indication that the mutations identified by our CRISPR screen are clinically translatable.

To test the function of *SPRY3* in AC220 resistance in primary AML cells, we knocked out *SPRY3* in blasts from patients with *FLT3-ITD*+ AML by transfecting Cas9 mRNA and a plasmid containing sgRNA. In blast from patient #2, we achieved ~50% targeting efficiency based on T7EN1 assay (Fig. 3C). Importantly, *SPRY3* deletion AML cells were resistant to AC220 compared to the parental primary AML cells (Fig. 3D). This result clearly demonstrated that loss function of *SPRY3* results in AC220 resistance in primary AML cells. Taken together, *SPRY3* deletion in both an AML cell line and primary AML cells leads to drug resistance to a specific FLT3 inhibitor AC220.

SPRY3 or *GSK3* deletion/inhibition re-activates downstream signaling pathways of *FLT3-ITD* in the presence of AC220

In order to determine the mechanisms by which *SPRY3* or *GSK3* deletion confers drug resistance to AC220, we explored several different signaling pathways downstream of FLT3-ITD. *FLT3-ITD* mutation leads to constitutive activation of FLT3 kinase, with subsequent constitutive activation of its downstream signaling pathways including Ras/MAPK kinase pathway, STAT5, and PI3K/Akt pathway (9,10). Moreover, it was also reported that crosstalk between FLT3 and Wnt pathways plays a critical role in the pathogenesis of *FLT3-ITD*+ AML (35,36). As a potent and specific inhibitor of FLT3, AC220 can effectively inhibit all signaling downstream of FLT3. Since there is no wild type control line for MV4-11, we used Ba/F3 as an alternative line to explore the pathways downstream of *FLT3-ITD*. Ba/F3 is an IL-3 dependent murine pro-B cell line and stable expression of FLT3 in these cells allows IL-3 to be substituted by FLT3 ligand. Consistent with previously published work, addition of FLT3 ligand augmented phosphorylation of AKT, ERK and S6 and increased total levels of β-catenin, suggesting activation of PI3K/Akt, MAPK, mTOR and Wnt pathways respectively (Supplementary Fig. 3A). Moreover, expression of FLT3-ITD led to constitutive activation of AKT, ERK, and mTOR signaling (Supplementary Fig. 3A), indicating that the presence of *FLT3-ITD* in these cells enables ligand-independent activation of FLT3 downstream pathways.

Importantly, treating MV4-11 cells with AC220 for 2 hours reduced β-catenin levels as well as phosphorylation of STAT5, AKT , ERK, and S6 (Fig. 4A), suggesting inhibition of all downstream signaling pathways. To further confirm the effect of AC220 on FLT3-ITD signaling, we tested the effect of AC220 in BaF3 *FLT3-ITD*+ cells as well. Similar to the data observed in MV4-11, we found that AC220 inhibits PI3K/Akt, MAPK, mTOR, and Wnt pathways that are all downstream pathways of FLT3-ITD (Supplementary Fig. 3B).

In terms of the mechanism of AC220 resistance, we speculated that gene mutations that can rescue signaling downstream of FLT3 kinase may confer resistance to AC220. As SPRY3 and GSK3 are known inhibitors of FGF/Ras/MAPK and Wnt signaling respectively, we hypothesized that their loss of function (deletions) would restore Ras/MAPK and Wnt signaling downstream of FLT3 in the presence of AC220, thereby causing cells to be resistant to AC220. To test this hypothesis, we performed a series of experiments to probe for the signaling molecules that potentially regulate drug resistance due to *SPRY3* and *GSK3* disruptions. As we expected, *SPRY3* deletion led to increased phosphorylation of ERK in AC220 treated group compared to the wild type control and specifically rescued the inhibition of RAS/MAPK signaling by AC220 in MV4-11 cells (Fig. 4B). *GSK3A* or *GSK3B* knockout also led to increased β-catenin in the presence of AC220, suggesting activation of Wnt signaling in the knockouts in the presence of AC220 (Fig. 4C).

Pharmacological inhibition of MAP kinase and Wnt signaling pathway re-sensitizes AML cells to AC220

To further test our hypothesis that re-activation of the signaling downstream of *FLT3-ITD* is the mechanism underlie the resistance to AC220, we used a pharmacological approach to modulate FGF signaling pathway. We treated MV4-11 cells with either FGF1 or FGF inhibitor PD161570 in combination with AC220. Consistent with our hypothesis, we observed that FGF1 treatment confers the cells resistant to AC220 while FGF inhibitor PD161570 sensitizes cells to AC220 (Fig. 5A). Biochemically, we observed that FGF1 treatment also increased phosphorylation of ERK (Supplementary Fig. 3C), suggesting FGF1 rescued the inhibition of MAPK signaling by AC220 in MV4-11.

To test whether activation of Wnt signaling can confer resistance to AC220, we treated MV4-11 FLT3-ITD+ cells with a potent GSK3 inhibitor Chiron that activates Wnt signaling. We found that treatment of Chiron confers AML cells resistant to AC220. Biochemically, AC220 treatment reduced phosphorylation of AKT and ERK as well as the total protein levels of β-catenin in MV4-11. Addition of GSK3 inhibitor Chiron to AC220 treated cells increased total β-catenin levels without affecting other signaling pathways (Supplementary Fig. 3B, D), suggesting that GSK3 inhibition restores Wnt signaling in the presence of AC220.

To prove that MAP kinase is the major downstream effector of SPRY3 to confer resistance to AC220, we treated *SPRY3* KO with U0126, a highly selective inhibitor of both MEK1 and MEK2, and tested resistance to AC220. Supporting our hypothesis that MAP kinase is indeed the major downstream effector of the AC220 resistance pathway, we found that treatment of U0126 re-sensitize AML cells to AC220 (Fig. 5B). Moreover, we also treated AML cells with a β-catenin inhibitor PNU74654 and found that it also re-sensitizes AML cells to AC220 (Fig. 5C). Together, these findings strongly suggest that re-activation of MAP kinase or Wnt signaling is the key mechanism to cause AC220 resistance in AML.

SPRY3 expression is down-regulated in *GSK3* knockout AML cells

The similar resistance phenotype of *GSK3* and *SPRY3* knockouts suggests that these two genes might be functionally linked. To further understand the connection between *GSK3* and *SPRY3*, we measured the expression of *SPRY3* in *GSK3* knockout AML cells and *GSK3* expression in *SPRY3* knockout AML cells by real time PCR. Interestingly, we found that *SPRY3* expression is markedly reduced in *GSK3* knockout cells (Fig. 6A). Conversely, expression of *GSK3* is not significantly altered in *SPRY3* knockout AML cells (Fig. 6B, C). Consistently, the protein level of SPRY3 was diminished in *GSK3* knockout AML cells (Fig. 6D). This data suggests that GSK3 might be the upstream regulator of SPRY3 and *GSK3* regulates transcription of *SPRY3* in the acquired AC220 resistance pathway.

Taken together, our results demonstrate that AC220 inhibits multiple pathways downstream of FLT3-ITD whereas deletion of *SPRY3* or *GSK3* restores RAS/MAPK or Wnt signaling to confer resistance to AC220. *SPRY3* might be a downstream effector of *GSK3* in AC220 resistance signaling pathway.

No off-target mutagenesis was observed in *SPRY3* knockout

CRISPR/Cas9 has been demonstrated to create off-target mutations depending upon cell type and experimental setting (37) (38). To examine potential off-target effects, we predicted the possible off-target sites using an online tool (http://crispr.mit.edu) and identified at least 6 potential off-target sequences with high score for *SPRY3* sgRNA #4 (Supplementary Fig. 4A). Since there is only one predicted off-target sequence is within a coding region (NM-001004439), we examined this site extensively. We PCR amplified genomic DNA from primary AML cells and sequenced the PCR product of the potential off-target locus. When we scrutinized the sequencing data of 4 clones, we found that there are no mutations at this potential off-target site (Supplementary Fig. 4B). Although we cannot exclude the possibility of mutations at other sites, the off target effect are unlikely given the phenotype are observed with multiple sgRNAs.

Discussion

Pooled mutagenesis screen for gene mutations mediating drug resistance

The CRISPR/Cas system, a powerful genome editing approach was initially discovered by several groups as an effective defense mechanism utilized by bacteria against virus infection (37–39). Subsequently, several seminal publications clearly demonstrated the possibility of using this technology to achieve high efficiency genome editing in mammalian systems, both mouse and human (40–42). Different versions of the guide RNA can be used to target Cas9 to specific sequences for genome engineering in cells as well as multicellular organisms (37,39,43). More recently, Dr. Feng Zhang's group at the Broad Institute of MIT and Harvard developed a CRISPR knockout library (GeCKO) and validated this system by performing a genome wide screen for genes conferring drug resistance to a therapeutic RAF inhibitor, Vemurafenib (PLX), in A375 melanoma cells. The screen revealed genes whose loss of function confers melanoma cells resistant to PLX (31). Of note, several other genome-wide CRISPR pooled screens have uncovered mediators of drug resistance, pathogen toxicity as well as defined cell-essential genes of the human genome (44–48). In our screen, for a number of genes, we found enrichment of multiple sgRNAs that target each gene after 14 days of AC220 treatment, suggesting that loss of these particular genes contributes to AC220 resistance. Thus, CRISPR has been proved to be a very useful tool to screen for drug resistant mutants in several types of cancer cells including AML.

Downstream signaling molecules regulating drug resistance to FLT3 inhibitors

Given the importance of *FLT3* mutations in AML, tyrosine kinase inhibitors (TKIs) have been developed to treat patients carrying *FLT3-ITD* mutations. Quizartinib (AC220) a more recent kinase inhibitor of FLT3 that has high selectivity for FLT3 is currently under clinical study. It has been shown to have very selective in-vitro and in-vivo activity and sensitivity against FLT3 (14,49). In comparison with other FLT3 inhibitors, AC220 appears to be 1–2 orders of magnitude more potent *in vivo*. Moreover, it has a very long plasma half-life due to that it has improved pharmacokinetics. Interestingly, it has been reported that 11 of 45 patients (24%) receiving AC220 experienced transient clinical responses, and 4 patients achieved complete remission in a phase I study in relapsed/refractory AML (14,49–51). Based on this promising phase I data, a phase II trial of AC220 in relapsed/refractory patients with *FLT3-ITD* mutations has been carried out. In this Phase 2 study, as a monotherapy, AC220 at multiple doses demonstrated a high response rate in relapsed/refractory FLT3-ITD positive patients. The treatment results in an overall better survival in FLT3-ITD positive AML patients compared to historical survival data reported (14,49–51).

Importantly, even though no FLT3 inhibitors have been approved for clinical use yet, several resistant mechanisms of FLT3 inhibitors have been reported through early clinical studies. Some studies identified the mutations in *FLT3* confer drug resistance (17,52,53). However, the drug resistant mutations in the downstream pathways of FLT3 have not been systematically studied. Our study for the first time has uncovered that loss-of-function of

SPRY3 and *GSK3* cause drug resistance. It also provides new insight into the signaling pathways downstream of FLT3-ITD in AML. Intriguingly, we found that expression of *SPRY3* is dramatically reduced in *GSK3* knockout AML cells. This result raises the possibility that *GSK3* regulates transcription of *SPRY3* directly or indirectly in acquired AC220 resistance pathway. It is tempting to speculate this regulation is through Wnt signaling. It would be important to explore the detailed mechanism by which *GSK3* controls transcription of *SPRY3* in AML cells.

Relevance of identified drug resistant mutations to human leukemia

In our screen, we identified *SPRY3* and *GSK3* as positive hits and loss of function of *SPRY3* or *GSK3* leads to the resistance of AML cells to AC220. Importantly, we confirmed this resistance effect in primary leukemia samples. Furthermore, we also demonstrated that the expression level of *SPRY3* and *GSK3A* correlates with clinical AC220 resistance in human leukemia samples. All these data strongly suggest that the genes we identified in our screen play a critical role in AC220 resistance in primary human AML samples. Importantly, we also showed that treating cells with either FGF signaling inhibitor or MAPK inhibitor or β-catenin inhibitor greatly increases the sensitivity of AML cells to AC220. This could be potentially important for developing future FLT3-ITD+ AML synergistic anti-AML therapy. It would be interesting to test whether treating patients who are clinically resistant to AC220 with FGF inhibitor or MAPK inhibitor would re-sensitize leukemic cells to AC220. Of note, our conclusion is also further supported by a recent report showing that FGF2 promoted resistance to AC220 through activation of FGFR1 and downstream MAPK effectors in AML cells (54). In summary, our study identified and delineated novel functional roles for *SPRY3* and *GSK3* genes whose deletions lead to FLT3 inhibitor resistance and provided new insights into the downstream signaling pathways regulated by FLT3 (Fig. 7).

Supplementary Material

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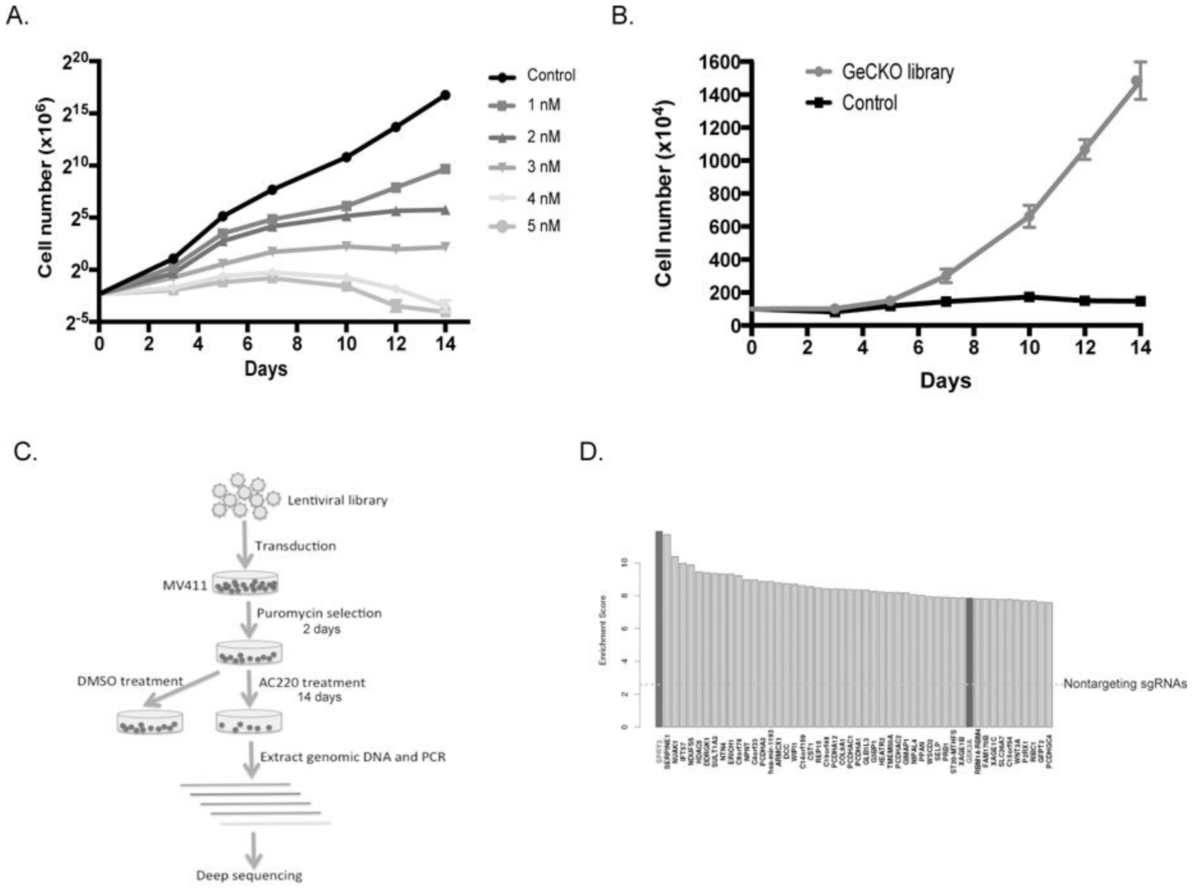

Figure 1. CRISPR screen in MV4-11 AML cells uncovers genes whose loss of function confers AC220 resistance

(A) Cell growth curve of MV4-11 following the treatment with AC220. 0.3 × 10^6 MV4-11 cells were seeded in 12-well plate per well and cultured in complete medium supplemented with 1, 2, 3, 4, 5 nM AC220 or DMSO. Cell numbers were counted using trypan blue at the indicated times.

(B) Cell growth curve of CRISPR GeCKO library transduced MV4-11 following the treatment of DMSO or AC220 over 14 days. 1 × 10^6 transduced cells were seeded in 6-well plate per well and cultured in complete medium supplemented with 3 nM AC220 or DMSO. Cell numbers were counted at indicated times.

(C) A simplified schematic of the AC220 resistance screen with MV4-11 AML cells. The screen condition has been tested for at least three times and the transduction of AML cells and response to AC220 was very reproducible among three replicates.

(D) Enrichment of specific sgRNAs that target each gene after 14 days of AC220 treatment and identification of top candidate genes. The X axis represents enriched genes and Y axis represents sgRNA enrichment score which is calculated using: (sgRNA number from the reads) / (sgRNA number in the library) X log2 (average abundance). The blue line in the plot indicates the enrichment score for the non-targeting sgRNAs. The top 50 ranked genes based on the enrichment score are shown.

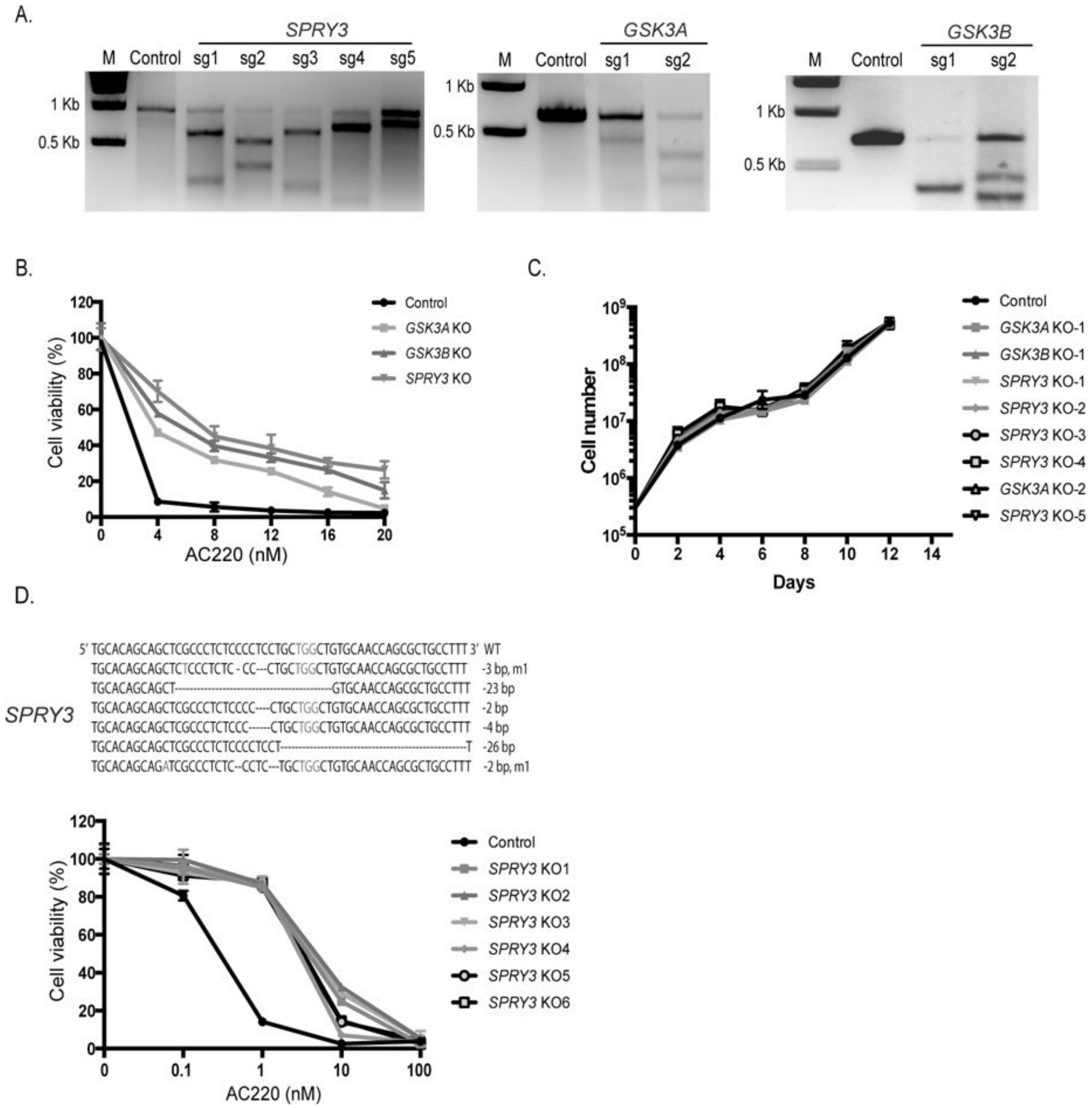

Figure 2. Validation of the screen that disruption of *GSK3* and *SPRY3* in MV4-11 causes resistance to AC220

(A) T7EN1 assay analysis of specific sgRNA-mediated indels at *GSK3* or *SPRY3* locus in MV4-11 cells. The MV4-11 cells were transduced with lentivirus carrying sgRNA targeted *GSK3* or *SPRY3*. The genomic DNA from the cells was extracted and PCR amplified to test efficiency of gene disruptions by T7 endonuclease I assay using a 1.5% agarose gel. The lower migrating bands in lanes indicate the disrupted gene alleles. The left panel is *SPRY3*; middle panel is *GSK3A*; right panel is *GSK3B*.

(B) MV4-11 cells were transduced with lentivirus carrying sgRNAs targeting *GSK3* or *SPRY3*. The drug resistance of *GSK3* or *SPRY3* knockouts and the control cells were measured. 0.4 × 10^6 cells were plated in 12-well plate and treated with indicated amounts of AC220 for 6 days and cell numbers were counted.

(C) Cell growth curve of *SPRY3* and *GSK3* knockout cells in the absence of AC220. 0.4 × 10^6 cells were plated in 12-well plate and cultured in complete medium. Cell numbers were counted at indicated times. The results from one representative experiment of three replicates are shown.

(D) Top panel: The sequence of mutant alleles in six *SPRY3* knockout single clones comparing to wild type on top. PAM sequence is labeled in red.

Bottom panel: Cell growth curve of *SPRY3* knockout single clones following the treatment with AC220. 0.4 × 10^6 cells were plated in 12-well plate and treated with indicated amounts of AC220 for 6 days and cell numbers were counted. The results from one representative experiment of three replicates are shown.

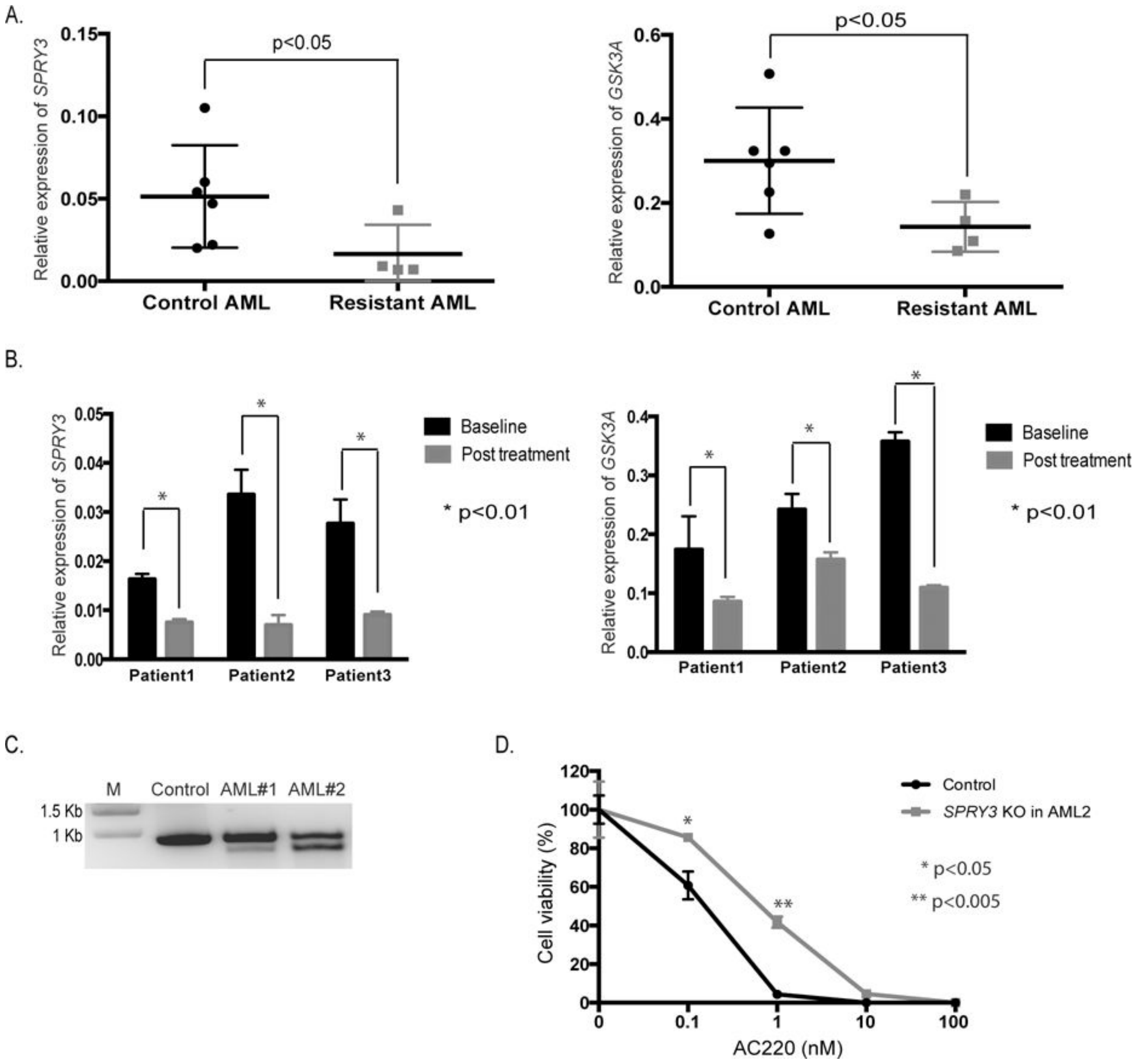

Figure 3. Disruption of *SPRY3* in primary AML leads to resistance to AC220

(A) Left: Expression of *SPRY3* was measured by real time PCR in control and AC220 resistant FLT3-ITD+ AML patient samples. The expression level of *SPRY3* was normalized to housekeeping *PPIB* gene. Right: Expression of *GSK3A* was measured by real time PCR in control and AC220 resistant FLT3-ITD+ AML patient samples. The expression level of *GSK3A* was normalized to housekeeping *PPIB* gene.

(B) Left: Expression of *SPRY3* was measured by real time PCR in three pairs of pre- and post- AC220 treatment FLT3-ITD+ AML patient samples. The expression level of *SPRY3* was normalized to housekeeping *PPIB* gene. Right: Expression of *GSK3A* was measured by real time PCR in three pairs of pre- and post- AC220 treatment FLT3-ITD+ AML patient samples. The expression level of *GSK3A* was normalized to housekeeping *PPIB* gene.

(C) T7EN1 assay analysis of specific sgRNA-mediated indels at *SPRY3* locus in the control and two FLT3-ITD+ AML patient samples.

(D) *SPRY3* was deleted in FLT3-ITD+ AML patient sample (#2) by transient transfection of Cas9 mRNA and a plasmid containing sgRNA. The resistance to AC220 was measured after 6 days treatment. The results from one representative experiment of three replicates are shown.

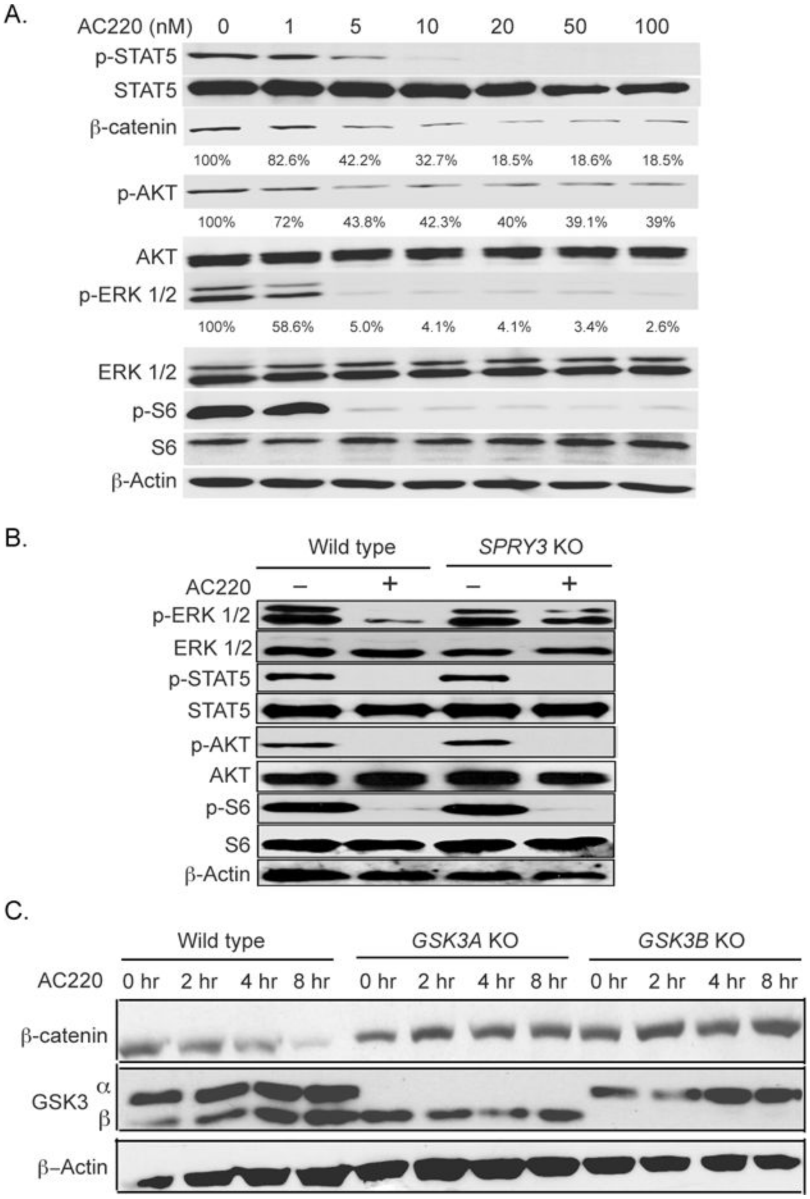

Figure 4. AC220 inhibits multiple FLT3-ITD downstream signaling pathways and knockouts of *SPRY3* or *GSK3* reactivate RAS/ERK and Wnt signaling pathways in the presence of AC220

(A) Western blot analysis of AC220 treated MV4-11 cells. 0.3 × 10^6 cells were starved for overnight and treated with AC220 at indicated concentrations. Cells were harvested after 2 hours treatment and lysed. Then western blots analysis was performed. The quantification of bands are shown below the gel.

(B) Western blot analysis of AC220 treated wild type and *SPRY3* knockout cells. 0.3 × 10^6 wild type and *SPRY3* knockout cells were starved overnight and treated with 10nM AC220 for 6 hours. The cells were harvested and lysed and western blots analysis was carried out.

(C) Western blot analysis of AC220 treated wild type and *GSK3* knockout cells. 0.3 × 10^6 cells were starved overnight and treated with 10nM AC220. Cells were harvested at indicated times and lysed. Western blots analysis was performed.

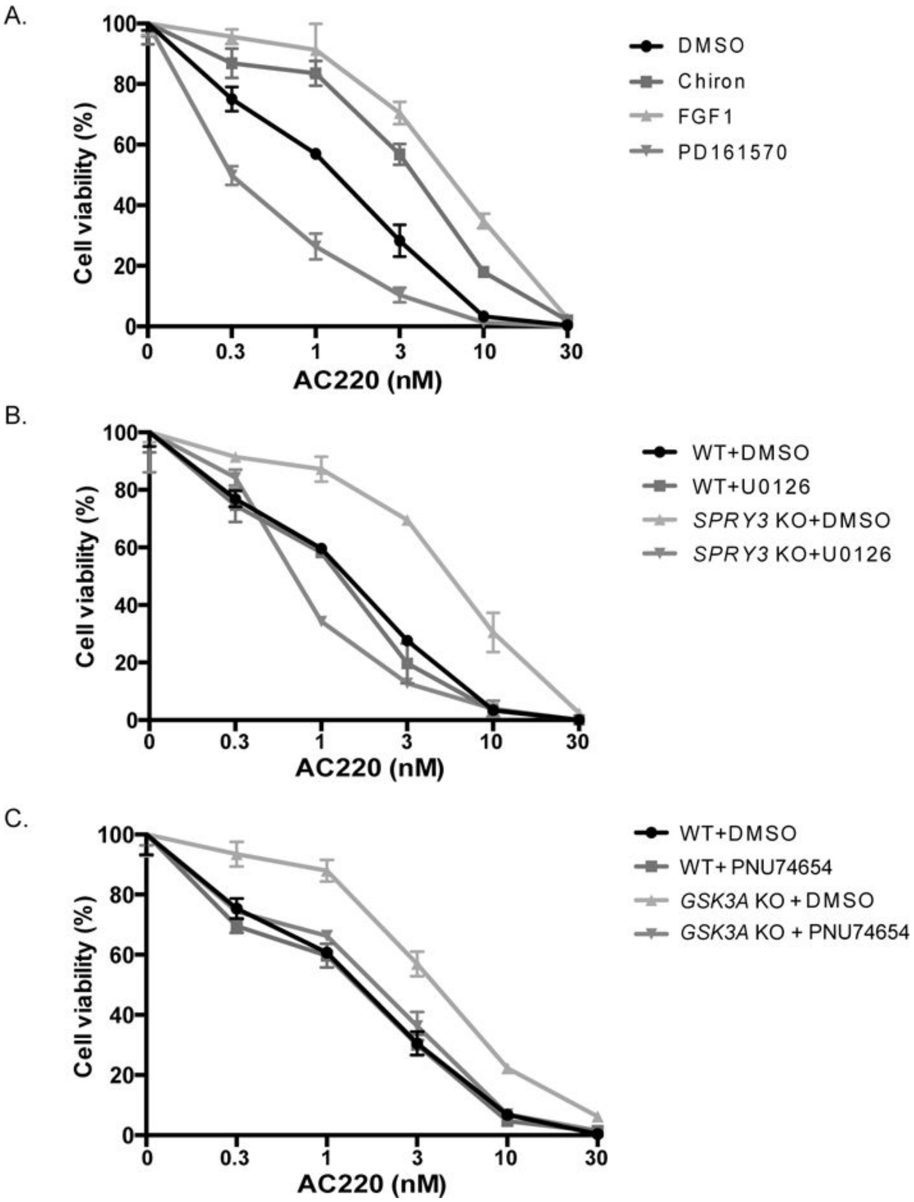

Figure 5. Pharmacological inhibition of MAP kinase and Wnt signaling pathway re-sensitizes AML cells to AC220

(A)Growth curve of MV4-11 cells which were treated with 1ng/ml FGFa or 50nM PD161570 or 1.5uM Chiron in combination with different doses of AC220. Cell numbers were counted after 3 days.

(B) Growth curve of MV4-11 or SPRY3 knockout MV4-11 cells which were treated with either DMSO or ERK inhibitor U0126 at 5uM in combination with different doses of AC220. Cell numbers were counted after 3 days.

(C)Growth curve of MV4-11 or GSK3A knockout MV4-11 cells which were treated with either DMSO or β-catenin inhibitor PNU74654 at 10mM in combination with different doses of AC220. Cell numbers were counted after 3 days.

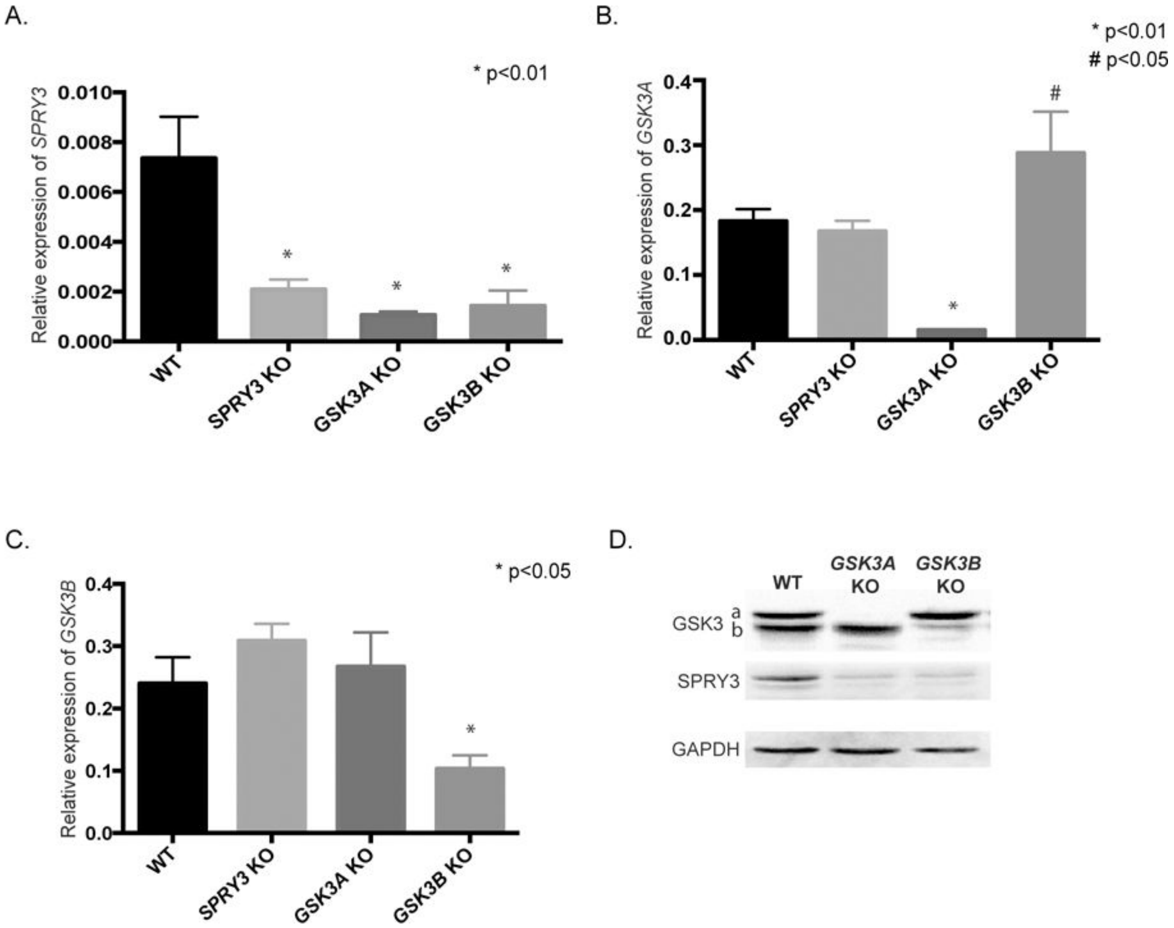

Figure 6. Expression of *SPRY3* is markedly reduced in *GSK3A and GSK3B* knockout AML cells

(A) Expression of *SPRY3* was measured by real time PCR in MV4-11, *SPRY3*, *GSK3A* and *GSK3B* knockout MV4-11 cells. The expression level of *SPRY3* was normalized to housekeeping *PPIB* gene.

(B) Expression of *GSK3A* was measured by real time PCR in MV4-11, *SPRY3*, *GSK3A* and *GSK3B* knockout MV4-11 cells. The expression level of *GSK3A* was normalized to housekeeping *PPIB* gene.

(C) Expression of *GSK3B* was measured by real time PCR in MV4-11, *SPRY3*, *GSK3A* and *GSK3B* knockout MV4-11 cells. The expression level of *GSK3A* was normalized to housekeeping *PPIB* gene.

(D) The protein level of SPRY3 was assessed by western blot in GSK3A and GSK3B knockout MV4-11 cells.

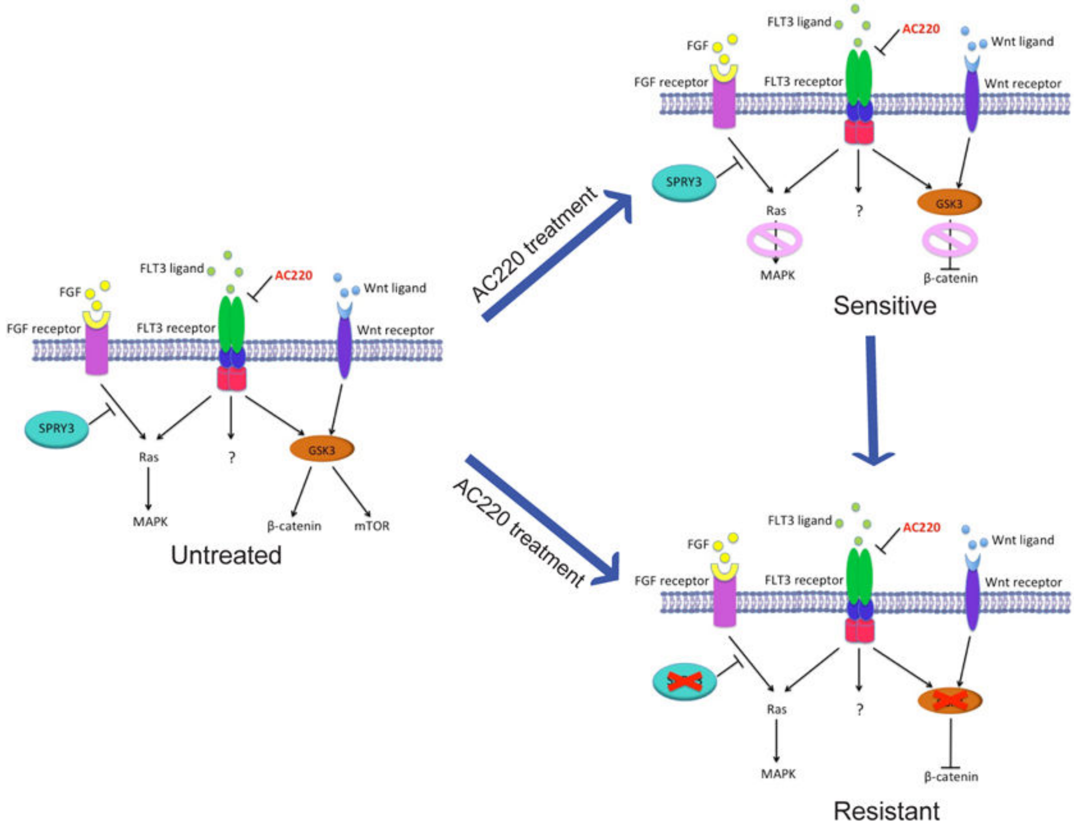

Figure 7. Model of SPRY3 or GSK3 knockouts confer resistance to AC220

FLT3-ITD constitutively activates downstream signaling pathways of FLT3. Treatment of FLT3-ITD+ cells with AC220 inhibits all the pathways. SPRY3 or GSK3 knockouts reactivate downstream signaling pathways of FLT3-ITD and confer resistance to AC220. The untreated or AC220 sensitive AML cells can develop the drug resistance during the therapy because of SPRY3 or GSK3 mutations. The major downstream signaling pathways are Wnt and MAP kinase pathways.