MEF2C Phosphorylation Is Required for Chemotherapy Resistance in Acute Myeloid Leukemia

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ABSTRACT

In acute myeloid leukemia (AML), chemotherapy resistance remains prevalent and poorly understood. Using functional proteomics of patient AML specimens, we identified MEF2C S222 phosphorylation as a specific marker of primary chemoresistance. We found that Mef2c S222A knock-in mutant mice engineered to block MEF2C phosphorylation exhibited normal hematopoiesis, but were resistant to leukemogenesis induced by MLL–AF9. MEF2C phosphorylation was required for leukemia stem cell maintenance and induced by MARK kinases in cells. Treatment with the selective MARK/SIK inhibitor MRT199665 caused apoptosis and conferred chemosensitivity in MEF2C-activated human AML cell lines and primary patient specimens, but not those lacking MEF2C phosphorylation. These findings identify kinase-dependent dysregulation of transcription factor control as a determinant of therapy response in AML, with immediate potential for improved diagnosis and therapy for this disease.

SIGNIFICANCE: Functional proteomics identifies phosphorylation of MEF2C in the majority of primary chemotherapy-resistant AML. Kinase-dependent dysregulation of this transcription factor confers susceptibility to MARK/SIK kinase inhibition in preclinical models, substantiating its clinical investigation for improved diagnosis and therapy of AML. Cancer Discov; 8(4); 1–20. © 2018 AACR.

INTRODUCTION

Acute myeloid leukemias (AML) are cancers of the blood that originate in the hematopoietic progenitor cells as a result of the accumulation of genetic mutations that lead to cell transformation. Recent advances in genomic profiling have revealed distinct genetic subsets of AML, including specific mutational classes of cytogenetically normal and chromosomally rearranged leukemias (1–3). Overall, AML is characterized by a prevalence of mutations of genes encoding regulators of gene expression, such as the MLL–AF9 (KMT2A–MLL3) gene fusion that dysregulates the expression of genes controlling self-renewal, differentiation, and cell survival (4). Recent studies have begun to reveal specific molecular dependencies that can be used for improved targeted therapies in AML (5). In spite of this knowledge, intensive chemotherapy and stem cell transplantation continue to be essential means to achieve cure in the treatment of AML. However, current chemotherapy regimens remain inadequate and fail to induce or sustain remissions in more than 50% of adults and 30% of children with AML (6–8). Thus, improved therapeutic strategies to overcome or block chemotherapy resistance are needed.

Patients with distinct molecularly identifiable types of leukemia have been found to exhibit varying degrees of response to chemotherapy, leading to therapy intensification for patients with high-risk disease (9–12). However, human leukemias also exhibit large heterogeneity of chemotherapy response, and its molecular determinants continue to remain poorly understood. For example, mutations of DNMT3A, as well as gene fusions such as MLL–AF9 and NUP98–NSD1, have been found to contribute to chemotherapy resistance (13–15). However, patients with leukemias with these and other mutations can nonetheless achieve durable remissions (3). Likewise, leukemias that exhibit primary chemotherapy resistance that is refractory to induction chemotherapy have not been found to show significant enrichment for these or other single-gene mutations (9, 11, 16). Thus, additional genetic or molecular mechanisms must cause chemotherapy resistance in AML.

In support of this idea, human leukemias and their genetically engineered mouse models are characterized by distinct cell populations, comprising defined functional compartments such as leukemia stem or initiating cells that exhibit unique phenotypic properties, including self-renewal and enhanced cell survival (17, 18). In part, these behaviors are...
caused by co-option of developmental programs that regulate normal hematopoiesis, such as for example self-renewal and therapy resistance of $MLL−AF9$ leukemias (4, 19). In particular, MEF2C, a member of the MADS family of transcription factors, normally regulates hematopoietic self-renewal and differentiation, supports the proliferation of $MLL−AF9$ leukemias, and is associated with the increased risk of relapse when highly expressed in multiple subtypes of AML in patients (19–25). However, the precise molecular mechanisms by which MEF2C is dysregulated in AML are not known. Recurrent mutations of MEF2 family members, including MEF2B, MEF2C, and MEF2D in refractory lymphoblastic leukemias and lymphomas (26–28), suggest that MEF2C and its orthologs may regulate a general mechanism of therapy resistance.

Here, using recently developed high-accuracy mass spectrometry techniques, we determined phospho-signaling profiles of human AML specimens collected at diagnosis from patients with primary chemotherapy resistance and failure of induction chemotherapy. Analysis of these profiles revealed high levels of phosphorylation of S222 of MEF2C, which was found to be significantly associated with primary chemotherapy resistance in an independent cohort of cytogenetically normal and $MLL$-rearranged leukemias. By integrating genome editing and biochemical and cell biological approaches, we tested the hypothesis that MEF2C phosphorylation promotes chemotherapy resistance and that its blockade can be leveraged for improved AML therapy. These studies have revealed an unexpected dependence on kinase-dependent dysregulation of transcription factor control as a determinant of therapy response in AML, with immediate potential for translation into improved diagnosis and therapy for this disease.

RESULTS

Phosphorylation of S222 in MEF2C Is a Specific Marker of AML Chemotherapy Resistance

Previously, we assembled a cohort of primary AML specimens matched for AML subtypes and therapy and collected at diagnosis from patients with failure of induction chemotherapy and those who achieved remission after two cycles of cytarabine and daunorubicin-based induction chemotherapy (16). In this analysis, we found that defined gene mutations were associated with primary chemotherapy resistance only in a minority of cases. Thus, we sought to investigate alternative molecular mechanisms that may explain primary chemotherapy resistance in AML.

We focused on phospho-signaling because kinase activation is one of the hallmarks of AML pathogenesis (29, 30). Recent advances in quantitative proteomics, particularly in high-efficiency, multidimensional fractionation platforms (31), enable in-depth analysis of signaling molecules from rare cell populations (32). Leukemia cells purified from a discovery cohort of eight diagnostic adult AML bone marrow aspirate specimens with normal karyotypes (Supplementary Table S1) were analyzed by metal affinity chromatography (IMAC; ref. 33) and isobaric tagging (iTRAQ) mass spectrometry (34). This yielded 2,553 unique phosphopeptides, 34 of which were significantly enriched in induction failure specimens (Supplementary Data S1; Supplementary Fig. S1A and S1B). We identified phosphorylation of serine 222 (pS222) in MEF2C among the top 20 most highly abundant phosphoproteins in induction failure specimens as compared with age, therapy, and disease-matched remission specimens ($P = 5.0 \times 10^{-3}$, t test, Fig. 1A and B; Supplementary Fig. S1B).

MEF2C was the preferred candidate to study given its known function as an oncogene in lymphoid malignancies (35) and cooperation with $MLL$-rearranged AML (19). The observed MEF2C pS222 peptide was distinct from the related MEF2A, MEF2B, and MEF2D peptides (Supplementary Fig. S1C). We also observed phosphorylation of S396 MEF2C (Supplementary Data S1), but this modification was present in substoichiometric amounts, as measured using quantitative targeted mass spectrometry (36). In addition, this analysis revealed phosphoproteins previously implicated in therapy resistance, such as HSBP1 pS15 (37), as well as those not previously observed but likely functional, such as HGF pT503 (29).

To investigate the diagnostic significance of MEF2C pS222 in primary AML chemotherapy resistance, we assessed the prevalence of MEF2C pS222 in an independent cohort of 47 pediatric and adult primary AML specimens matched for age, disease biology, and therapy (Supplementary Table S2; ref. 16). As previously observed, this cohort included specimens with gene mutations associated with poor prognosis such as those with cryptic rearrangements of $MLL/KMT2A$ and combined $DNMT3A$ and $NPM1$ mutations (Supplementary Table S2). We developed an affinity-purified antibody against MEF2C pS222 and validated its specificity using synthetic peptide competition and phosphatase assays (Supplementary Fig. S2A–S2C). We found that expression of MEF2C pS222 and total MEF2C were significantly associated with induction failure and primary chemotherapy resistance ($P = 6.5 \times 10^{-4}$ and $6.0 \times 10^{-3}$, respectively; Fig. 1C and D; Supplementary Fig. S2D–S2G), remaining statistically significant in multivariate analyses (Supplementary Table S3). Overall, pS222 and total MEF2C expression levels were high in the induction failure as compared with complete remission specimens (Supplementary Fig. S2H and S2I) and significantly associated with induction failure as compared with disease relapse ($P = 2.7 \times 10^{-3}$; Fig. 1E). In addition, presence of MEF2C pS222 above its median expression level correlated with reduced overall event-free survival (EFS; $P = 3.8 \times 10^{-2}$; Fig. 1F) and was a significant binary predictor of poor outcome as assessed by the ROC analysis ($P = 3.2 \times 10^{-2}$; Fig. 1G). In addition, we analyzed MEF2C expression data from the ECOG E1900 cohort of young adult AML and found that high MEF2C expression correlated with reduced EFS ($P = 3.8 \times 10^{-2}$; Supplementary Fig. S2K). In contrast, the highly homologous MEF2D did not exhibit this association ($P = 0.98$; Fig. 1D; Supplementary Fig. S2J). Post hoc review of the top 10 specimens with moderate MEF2C pS222 expression from remission cases at diagnosis revealed that 6 of these cases had in fact developed early relapse after initial induction therapy treatment (Fig. 1C), corroborating the specific association of MEF2C pS222 with AML chemotherapy resistance and suggesting that MEF2C phosphorylation is necessary but not sufficient to cause chemotherapy resistance. Overall, these findings indicate that MEF2C pS222 is a specific...
Figure 1. Phosphorylation of MEF2C at serine 222 is associated with primary AML chemoresistance. A, Phosphoproteomic screen for differentially abundant protein phosphorylation sites detected in diagnostic AML specimens in patients with primary chemotherapy resistance and induction failure, as compared with patients who achieved complete induction remission, with pS222 marked in red (Supplementary Data S1, Supplementary Fig. S1A and S1B). B, Volcano plot of protein phosphorylation sites detected in induction failure versus complete remission specimens, with candidate phosphoproteins marked, including pMEF2C (red). C, Heat map of MEF2C expression and S222 phosphorylation in a matched cohort of 47 specimens, as measured using quantitative fluorescence immunoblotting, and normalized to actin. #, Specimens from patients with high pS222 expression who achieved complete remission but experienced AML relapse. ^, \( P = 6.0 \times 10^{-3} \) and ^^, \( 6.5 \times 10^{-4} \) for remission versus failure for MEF2C and pS222 MEF2C, respectively (\( t \) test). D, Representative western immunoblot analysis for MEF2C, pS222 MEF2C, and MEF2D in a cohort of age, disease, and therapy-matched AML patient specimens with induction failure and complete remission. The human AML cell lines OCI-AML2 and U937 serve as positive and negative controls for MEF2C expression and S222 phosphorylation, respectively. E, Normalized log2 expression of pS222 MEF2C compared with actin in induction failure, relapse and complete remission AML patient specimens. *, \( P = 2.7 \times 10^{-2} \) and **, \( 5.5 \times 10^{-3} \) for induction failure versus relapse and remission, respectively (\( t \) test). F, Event-free survival analysis of 47 AML patient specimens assessed in C–E, separated above or below median pS222 MEF2C expression levels. \( P = 4.6 \times 10^{-2} \) (log-rank test). G, ROC curve analysis for pS222 MEF2C in this cohort. \( P = 3.2 \times 10^{-2} \) (Wilcoxon test).
marker of primary chemoresistance and failure of induction therapy in AML.

**MEF2C pS222 Is Dispensable for Normal Hematopoiesis**

The activity of MEF2C is known to be regulated by post-translational modifications, including acetylation, sumoylation, and phosphorylation, that can affect the recruitment of transcriptional corepressors and coactivators (38–40). Analysis of existing phospho-signaling data revealed the presence of MEF2C S222 phosphorylation in human K562 leukemia cells and cytokine-stimulated hematopoietic progenitor cells (41, 42). The specific association of MEF2C pS222 with failure of induction chemotherapy raises the possibility that MEF2C S222 phosphorylation promotes chemoresistance in AML. To test this hypothesis, we first sought to investigate the potential function of MEF2C S222 phosphorylation in normal hematopoiesis. Thus, we engineered knock-in mice harboring a loss-of-function Mef2c S222A allele that cannot be phosphorylated, and a gain-of-function S222D allele that mimics phospho-serine by using CRISPR/Cas9 genome editing (Fig. 2A; Supplementary Fig. S3A). Genotyping of founder animals identified Mef2cS222A/S222A and Mef2cS222D/S222D mutant alleles in 10% and 7% of the mice born, respectively. We confirmed the absence of apparent off-target mutations of the Mef2c locus by genomic DNA sequencing of each founder animal. To control for possible off-target effects, we obtained two independent founder strains for both Mef2cS222A and Mef2cS222D alleles, and back-crossed them to wild-type C57BL/6J mice. Subsequently, Mef2cS222A/S222A and Mef2cS222D/S222D mice obtained from heterozygous intercrosses were detected at expected Mendelian ratios (Supplementary Fig. S3B) and obtained from heterozygous intercrosses were detected at expected Mendelian ratios (Supplementary Fig. S3B) and.

**MEF2C Phosphorylation Cooperates with Distinct Leukemia Oncogenes and Is Required for Leukemia Stem Cell Maintenance**

Protection of Mef2cS222A/S222A hematopoietic progenitor cells from MLL–AF9-induced leukemogenesis suggests that MEF2C S222 phosphorylation may be necessary for the survival of MLL–AF9-transformed leukemia cells. To investigate the function of MEF2C S222 phosphorylation in
Figure 2. A therapeutic window for targeting MEF2C phosphorylation in AML. A, Sequencing electropherograms of tail genomic DNA from Mef2c\textsuperscript{S222A/S222A} and Mef2c\textsuperscript{S222D/S222D} mice, demonstrating specific CRISPR/Cas9-induced c.TCA>GCG and c.TCA>GAT mutations, as underlined red and green, respectively. WT, wild-type. B, Western immunoblot of bone marrow B220\textsuperscript{+} and Gr-1\textsuperscript{+}/CD11b\textsuperscript{+} cells from Mef2c\textsuperscript{S222A/S222A} and Mef2c\textsuperscript{S222D/S222D} mice. C, Total numbers of myeloid (Gr-1\textsuperscript{+}/CD11b\textsuperscript{+}), B-cell (B220\textsuperscript{+}), and T-cell (CD4\textsuperscript{+}/CD8\textsuperscript{+}) bone marrow cells from Mef2c\textsuperscript{S222A/S222A} and Mef2c\textsuperscript{S222D/S222D} mice as assessed by FACS analysis. Error bars, SD of the mean from 3 mice. E, Peripheral blood chimerism of CD45.2\textsuperscript{+} cells at 4 weeks following competitive transplantation. Error bars, SD of the mean of 10 animals per group. F, Peripheral blood engraftment of CD45.2\textsuperscript{+} cells as a function of time posttransplant as in E. Bars, mean. G, Serial replating clonogenic efficiencies of bone marrow GMP cells from Mef2c\textsuperscript{S222A/S222A}, Mef2c\textsuperscript{S222D/S222D}, and wild-type littermates transduced with MLL-AF9. Error bars, SD of the mean of 3 biological replicates (additional data in Supplementary Fig. S6). *, P = 3.3 x 10\textsuperscript{-3} of WT; MLL-AF9 versus S222A/S222A; MLL-AF9 (t test). H, Kaplan-Meier survival curves of mice transplanted with MLL-AF9-transformed bone marrow GMP cells from Mef2c\textsuperscript{S222A/S222A}, Mef2c\textsuperscript{S222D/S222D} and wild-type littermates. P = 6.8 x 10\textsuperscript{-4} for Mef2c\textsuperscript{S222A/S222A} vs. wild-type littermates, log-rank test for 10 animals per group. Solid and dashed black lines denote wild-type littermates for Mef2c\textsuperscript{S222A/S222A} and Mef2c\textsuperscript{S222D/S222D}, respectively.
MLL–AF9-induced leukemia maintenance, we transformed wild-type bone marrow GMP cells by retroviral transduction of MSCV-IRES-GFP MLL–AF9 and coexpressed wild-type MEF2C, S222A, or S222D mutants using the MSCV-IRES-tdTomato (MIT) retrovirus (Supplementary Fig. S4A). This allowed us to purify GFP/tdTomato double-positive MLL–AF9-transformed cells using FACS where MEF2C transgenes can function as dominant mutants because of their dimerization with endogenous MEF2C (46, 47). We confirmed that all MEF2C transgenes were expressed at approximately equal levels by western immunoblotting, with MEF2C S222A-transduced cells exhibiting substantially reduced but not completely eliminated levels of pS222 as compared with wild-type controls (Supplementary Fig. S4B). We found that MLL–AF9 leukemia cells expressing MEF2C S222A exhibited transient reduction in clonogenic efficiency in vitro (Supplementary Fig. S4C) and displayed increased apoptosis (Supplementary Fig. S4D) as compared with cells expressing wild-type MEF2C. MEF2C S222D-expressing cells behaved similarly to MEF2C wild-type–expressing cells, similar to the phenotype of MLL–AF9-transduced Mef2cS222D/S222D leukemias, indicating that MEF2C phosphorylation is necessary but insufficient to cause enhanced leukemia cell survival. Notably, when allowed to develop leukemia (Supplementary Fig. S4E) and secondarily transplanted into sublethally irradiated mice to assess leukemia maintenance in vivo, MLL–AF9;MEF2C S222A leukemias were significantly impaired in leukemia development as compared with MLL–AF9;MEF2C wild-type leukemias (P = 2.2 × 10−10, log-rank test, Fig. 3A). Using limiting dilution analysis, we found that the expression of wild-type phosphorylated MEF2C increased the leukemia initiating cell frequency as compared with MIT control (1/16 vs. 1/85) and consistent with previous reports (48), whereas the expression of MEF2C S222A significantly blocked this effect (1/139, P = 6.7 × 10−8, χ² test, Fig. 3B; Supplementary Fig. S4F–S4H). These results indicate that MEF2C S222 phosphorylation is required for the enhanced maintenance of leukemia stem cells and its cooperation with MLL–AF9 leukemogenesis in vivo.

To investigate MEF2C S222 phosphorylation in non-MLL-rearranged leukemias, we assessed its function in leukemias with inactivating mutations of RUNX1 and internal tandem duplication (ITD) mutations of FLT3, a leukemia

![Figure 3](https://example.com/figure3.png)

**Figure 3.** MEF2C phosphorylation is required for leukemia stem cell survival and maintenance. A, Kaplan–Meier survival curves of secondary recipient mice transplanted with 100 cells of wild-type MLL–AF9;MEF2C, dominant-negative MLL–AF9;MEF2C S222A, or control MLL–AF9;MIT-transformed leukemias. P = 2.2 × 10−10 for MLL–AF9;MEF2C S222A versus MLL–AF9;MEF2C, log-rank test for 20 animals per group. B, Limiting dilution analysis of frequency of leukemia-initiating cells in secondary MLL–AF9 transplants. Solid and dashed lines represent the calculated stem cell frequencies and their 95% confidence intervals, respectively. P = 6.7 × 10−8 for S222A versus MEF2C (χ² test). C, Colony formation of primary Runx1+;Flt3ITD MEF2C leukemia cells. Below, representative micrographs of colonies at day 7. *, P = 1.9 × 10−5 S222A versus MIT. D, Kaplan–Meier survival curves of tertiary recipient mice transplanted with 50,000 cells of wild-type Runx1+;Flt3ITD;MEF2C, dominant-negative Runx1+;Flt3ITD;MEF2C S222A and Runx1+;Flt3ITD;MEF2C S222D or control Runx1+;Flt3ITD;MIT-transduced leukemias. P = 3.1 × 10−3 for MLL–AF9;MEF2C S222A versus MLL–AF9;MEF2C, log-rank test for 5 animals per group. (continued on following page)
MEF2C Phosphorylation in Chemoresistant AML

subtype associated with high rates of therapy failure that also exhibits high levels of pS222 MEF2C. First, we obtained genetically engineered mouse Runx1−/−;Flt3ITD leukemias (49), which we transduced with wild-type MEF2C and transgenes modeling loss and gain of S222 phosphorylation using the MIT retrovirus (Supplementary Fig. S5A–S5C). We found that Runx1−/−;Flt3ITD leukemias expressing MEF2C S222A displayed significantly reduced clonogenic efficiency in serial replating as compared with cells transduced with wild-type or S222D MEF2C (P = 1.9 × 10−4, t test, Fig. 3C). Similar to the MLL−Af9 leukemia model, we observed that mice transplanted with secondary Runx1−/−;Flt3ITD;MEF2C wild-type leukemia cells exhibited accelerated leukemia development as compared with Runx1−/−;Flt3ITD;MIT control leukemias, which was blocked in Runx1−/−;Flt3ITD;MEF2C S222A–expressing leukemias (P = 3.1 × 10−3, log-rank test; Fig. 3D). In contrast, we found that MEF2C S222 phosphorylation was dispensable for leukemias induced by ectopic retroviral expression of Hoxa9 and Meis1 (Supplementary Fig. SSD–SSH), consistent with the epistatic function of aberrant Hox gene expression in AML (50, 51). Thus, MEF2C S222 phosphorylation is required for maintenance of Runx1−/−;Flt3ITD leukemias.

MEF2C Phosphorylation Is Required for Leukemia Maintenance in MLL−Rearranged Human AML Cells

To assess the function of MEF2C S222 phosphorylation in human AML, we analyzed its expression in a panel of human AML cell lines and identified both MLL−rearranged and nonrearranged leukemias with activated levels of MEF2C pS222, including OCI-AML2, MOLM-13, K562, and Kasumi-1 (Supplementary Fig. S6A and S6G). Thus, we used a doxycycline-inducible lentivirus vector to express wild-type and dominant-negative MEF2C S222A and S222D mutants and confirmed near-physiologic transgene expression and pS222 levels by western immunoblotting (Fig. 3E and F; Supplementary Fig. S6B). We observed that OCI-AML2 and MOLM-13 AML cell lines with endogenous MEF2C S222 phosphorylation had significantly reduced viability upon the doxycycline-induced expression of MEF2C S222A as compared with wild-type MEF2C (P = 8.4 × 10−3 and 3.4 × 10−3, t test, respectively; Fig. 3G) and S222D MEF2C, at least in part due to the induction of apoptosis (Supplementary Fig. S6C and S6D) and reduction of cells in S phase (Supplementary Fig. S6E and S6F), in agreement with prior reports of cell

**Figure 3.** (Continued) E, Western immunoblot analysis for MEF2C and pS222 MEF2C in OCI-AML2 cells lentivirally transduced with wild-type MEF2C or dominant-negative MEF2C S222A transgenes and treated for 48 hours with 600 ng/mL of doxycycline to induce transgene expression. F, Quantitative analysis of MEF2C and pS222 MEF2C, as measured using quantitative fluorescence immunoblotting, and normalized to actin, demonstrating equal expression of MEF2C and MEF2C S222A protein (*) and significantly reduced abundance of pS222 MEF2C (**) P = 1.1 × 10−3 for MEF2C S222A vs. MEF2C (t test). Error bars, SD of the mean for 3 biological replicates. G, Growth of human AML cell lines lentivirally transduced with wild-type MEF2C or dominant-negative MEF2C S222A and MEF2C S222D transgenes and treated for 72 hours with 600 ng/mL doxycycline to induce transgene expression. Error bars, SD of the mean for 3 biological replicates. *, P = 3.4 × 10−3 and **, 8.4 × 10−3 for MEF2C versus MEF2C S222A, respectively (t test). H, Kaplan-Meier survival curves of NSG mice transplanted with OCI-AML2 cells transduced with wild-type MEF2C and dominant-negative MEF2C S222A transgenes, and treated with doxycycline in chow 3 days following transplantation continuously in vivo. P = 3.5 × 10−6 MEF2C versus MEF2C S222A, log-rank test for 10 animals per group.
AML2 cells (expressing wild-type MEF2C or nontransduced control OCI-AML2) xenografted into NOD-SCID-IL2Rc−/− mice by tail-vein injection in immunodeficient mice (Fig. 3H). Cells were transplanted into NOD-SCID-IL2Rc−/− mice for the survival of human AML cells in vitro and in mouse xenografts.

Figure 4. MEF2C phosphorylation is required for MEF2C-mediated gene expression program. A, Activity of luciferase transcriptional MEF2 reporter in HEK293T cells tertivally transduced with wild-type MEF2C or mutant MEF2C S222A, as compared with vector control. Error bars, SD of the mean for 3 biological replicates. *, P = 4.0 × 10⁻² for MEF2C S222A versus MEF2C, t test. B, Western immunoblot analysis for MEF2C and pS222 MEF2C in transcriptional reporter cells, demonstrating equal protein expression of MEF2C transgenes, and reduced pS222 in MEF2C S222A transduced cells.

To investigate the function of MEF2C phosphorylation in human leukemias in vivo, we next assessed the effects of inhibiting MEF2C S222 phosphorylation by expression of MEF2C S222A in OCI-AML2 cells transplanted orthotypically by tail-vein injection in immunodeficient mice (Fig. 3H). MEF2C functions as a transcription factor by sequence-specific recognition of MEF2 response elements (54), through regulated recruitment of transcriptional coactivators and corepressors via its transactivation (residues 118–473) domain (38, 55). To test the hypothesis that MEF2C S222 phosphorylation regulates MEF2C transcriptional activity, we assessed the transactivation of MEF2 response element–driven firefly luciferase as compared with a cytomegalovirus promoter–driven Renilla luciferase control introduced by electroporation in K562 cells. To model the effects of MEF2C S222 phosphorylation, we used lentivirus transduction to generate K562 cells expressing wild-type or dominant-negative S222A MEF2C upon doxycycline treatment (Supplementary Fig. S6G–S6H). As expected, expression of wild-type MEF2C led to an 8-fold increase in MEF2C transcriptional activity as compared with unmodified control K562 cells, which was rescued by mutant S222A protein expression (P = 3.1 × 10⁻³, t test; Supplementary Fig. S6I). We confirmed these findings in HEK 293T cells stably expressing wild-type and mutant MEF2C S222A (Fig. 4A). Expression of the wild-type MEF2C
in HEK293T cells that lack endogenous MEF2C led to an 18-fold increase in MEF2C transcriptional activity as compared with the vector control ($P = 6.3 \times 10^{-1}$, t test, Fig. 4A).

Despite equal levels of wild-type MEF2C and mutant S222A protein expression (Fig. 4B), MEF2C transcriptional activity was significantly reduced due to the effects of MEF2C S222 phosphorylation in the S222A mutant ($P = 4.0 \times 10^{-2}$, t test; Fig. 4A and B). This suggests that MEF2C S222 phosphorylation is required for maximal activation of MEF2C-dependent gene transcription.

To elucidate the gene expression program controlled by MEF2C S222 phosphorylation in AML cell survival, we analyzed gene expression profiles of OCI-AML2 cells expressing wild-type as compared with dominant-negative MEF2C S222A using RNA sequencing (RNA-seq; Fig. 4C). Consistent with the near-physiologic expression of MEF2C transgenes (Fig. 3E), we found essentially no significant differences in gene expression profiles of cells expressing wild-type MEF2C upon doxycycline-induced transgene expression (Fig. 4C). In contrast, we observed a significant change in gene expression of cells upon doxycycline-induced expression of MEF2C S222A, as compared with both uninduced cells and cells expressing wild-type MEF2C (Fig. 4C). In particular, we found 276 genes that were significantly altered in expression in cells expressing MEF2C S222A as compared with uninduced cells (Fig. 4C; Supplementary Data S2). Using qRT-PCR, we found that none of the previously reported canonical MEF2C target genes, such as Nr4a1/Nur77, Hdac7, Jun, and Cebpa, were significantly changed in expression upon expression of MEF2C S222A (Supplementary Fig. S4I–S4L). Instead, gene set enrichment analyses (GSEA) revealed that loss of MEF2C S222 phosphorylation led to downregulation of distinct gene expression programs, including E2F and MYC target genes (Fig. 4D; Supplementary Data S2). In addition, we observed altered expression of genes regulating apoptosis and cell cycle (Fig. 4D and E; Supplementary Data S2), consistent with the phenotypic induction of apoptosis and cell-cycle defects upon the blockade of MEF2C S222 phosphorylation in mouse and human leukemia cells (Supplementary Fig. S4D and S6C–S6F).

To identify genes specifically regulated by S222 phosphorylated MEF2C, we profiled chromatin accessibility using the assay for transposase accessible chromatin with sequencing (ATAC-seq), annotated for the presence of canonical MEF2 binding sites, in OCI-AML2 cells expressing wild-type and S222A-mutant MEF2C upon 48 hours of doxycycline-induced

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**Figure 4.** (Continued) F, Combined analysis of differentially expressed genes identified in RNA sequencing (RNA-seq) shown in C versus differentially accessible genes identified in the assay for transposase accessible chromatin with sequencing (ATAC-seq) containing canonical MEF2 sequence motifs. S222A-induced and S222A-repressed genes are highlighted in red and blue, respectively. G, Western immunoblot analysis for LYL1 in OCI-AML2 cells lentivirally transduced with wild-type MEF2C or dominant-negative MEF2C S222A transgenes and treated for 48 hours with 600 ng/mL of doxycycline to induce transgene expression. Below, quantitative analysis of LYL1 expression normalized to actin. *, $P = 1.3 \times 10^{-2}$; **, $P = 1.2 \times 10^{-2}$; ***, $P = 2.9 \times 10^{-2}$ for MEF2C S222A versus MEF2C respectively. H, BH3 profiling of OCI-AML2 cells transduced with wild-type MEF2C or dominant-negative MEF2C S222A transgenes, and treated with 600 ng/mL doxycycline for 48 hours to induce transgene expression. Error bars, SD of the mean for 3 biological replicates. *,$ P = 1.3 \times 10^{-2}$; **, $P = 2.9 \times 10^{-2}$ for MEF2C S222A versus MEF2C respectively. I, Western immunoblot analysis for MEF2C in DSS-treated OCI-AML2 cells lentivirally transduced with wild-type MEF2C or dominant-negative MEF2C S222A transgenes and treated for 48 hours with 600 ng/mL of doxycycline to induce transgene expression.
transgene expression. This approach was necessary, because mapping MEF2C genome localization using chromatin immunoprecipitation with sequencing (ChIP-seq) was not possible due to the inadequate chromatin enrichment achieved with currently available MEF2C-specific antibodies. Using ATAC-seq analysis annotated for the presence of MEF2 binding sites as a surrogate of MEF2C binding, we identified genes that exhibited significant changes in chromatin accessibility of MEF2 binding sites and concomitantly had significant changes in their expression, as measured using RNA-seq (Fig. 4F; Supplementary Fig. S6J and Data S2). This analysis identified a total of 262 genes, including 18 genes repressed by the expression of MEF2C S222A, consistent with their aberrant induction by S222 phosphorylated MEF2C, such as MYC and LYL1 (marked in blue, Fig. 4F). Observed gene expression changes in LYL1 repression were verified by protein immunoblotting (Fig. 4G), potentially explaining previous findings of MEF2C-dependent LYL1 expression and its aberrant expression in AML (56–58). Similarly, we also observed 159 genes that were upregulated by MEF2C S222A expression, consistent with their repression by phosphorylated MEF2C in AML cells. These genes included BCOR, BCL6, and NCOA3, as well as apoptotic regulators BIM, CASP8, and FOXO3 (marked in red, Fig. 4F). In agreement with the MEF2C S222 phosphorylation–induced suppression of these proapoptotic factors, we found that OCI-AML2 cells expressing MEF2C S222A exhibited enhanced apoptotic priming compared to MEF2C wild-type cells as assessed by BH3 profiling flow cytometry, with particular sensitivity to BIM and BID (Fig. 4H), consistent with their reported interactions with BIM, CASP3, and FOXO3 (59–62).

Homodimerization or heterodimerization of the MEF2 family of transcription factors is a key mechanism of their regulation, which can be modulated by posttranslational modifications (46, 63). To investigate whether MEF2C S222 phosphorylation can regulate its oligomerization, we treated OCI-AML2 cells induced to express MEF2C wild-type or S222A with disuccinimidyl suberate (DSS), a cell-permeable cross-linker, and assessed MEF2C S222 phosphorylation by western immunoblotting (Fig. 4G), potentially explaining previous findings of S222 phosphorylation (Supplementary Fig. S7D), consistent with dimerization of MEF2C, which was significantly increased levels of pS222, which was blocked by the coexpression of the enzymatically impaired MARK3 T211A activation loop mutant (refs. 64, 65; Fig. 5C). Importantly, expression of MARK3 failed to induce S222 phosphorylation or activate the transcriptional activity of the MEF2C S222A mutant that cannot be phosphorylated (Fig. 5C). Taken together, these data indicate that MARK kinase family members can specifically phosphorylate S222 MEF2C in vitro.

To evaluate whether MARK3 could phosphorylate MEF2C S222 and regulate its transcriptional activity in cells, we coexpressed MEF2C and MARK3 in HEK293T cells and assessed MEF2C phosphorylation and transcriptional activity by western immunoblotting and transcriptional reporter assays, respectively (Fig. 5C). We found that MEF2C transcriptional activity was significantly increased upon coexpression of MEF2C and MARK3 as compared with cells expressing MEF2C alone (P = 3.4 × 10−5, t test; Fig. 5C). This increased transcriptional reporter activity was associated with increased levels of pS222, which was blocked by the coexpression of the enzymatically impaired MARK3 T211A activation loop mutant (refs. 64, 65; P = 5.0 × 10−5, Fig. 5C). Importantly, expression of MARK3 failed to induce S222 phosphorylation or activate the transcriptional activity of the MEF2C S222A mutant that cannot be phosphorylated (Fig. 5C). Taken together, these data indicate that MARK kinase signaling can regulate MEF2C transcriptional activity specifically in an S222 phosphorylation–dependent manner. Next, we tested the hypothesis that inhibition of MARK-mediated phosphorylation of MEF2C may have antileukemic efficacy. Genetic depletion of MARK3 using shRNA interference was not sufficient to reduce MEF2C pS222 phosphorylation (Supplementary Fig. S7D), consistent with potentially redundant functions of MARK3, MARK2, and MARK4. Therefore, we identified the ATP-competitive kinase inhibitor MRT199665, which exhibits high selectivity and potency against MARK and the structurally similar SIK and MELK kinases as compared with other AMP kinase family members (66). We found that SIK, NUAK, and MELK kinases, which can be inhibited by MRT199665 at nanomolar concentrations, scored in the bottom 45% of the recombinant kinase screen for MEF2C phosphorylation (Supplementary Data S3). Thus, we reasoned that MRT199665 can be used as a selective inhibitor to block MEF2C S222 phosphorylation.
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phosphorylation. To test this directly, we first investigated whether MRT199665 treatment can phenocopy the suppression of MEF2C phosphorylation–induced gene expression program by the dominant-negative MEF2C S222A mutant. Transcriptome profiling of OCI-AML2 cells treated with 100 nmol/L MRT199665 for 48 hours revealed significant gene expression changes as compared with vehicle control–treated cells, where 191 and 128 genes were upregulated and downregulated, respectively (Supplementary Fig. S7E and Data S4). GSEA revealed that MRT199665-treated cells and MEF2C S222A–expressing cells exhibit similar changes in gene expression (r = 0.64, Fig. 5D), including downregulation of cell-cycle and MYC target genes and upregulation of genes mediating apoptosis (Fig. 5E; Supplementary Data S4). Furthermore, treatment of OCI-AML2 and MOLM-13 cells with increasing concentrations of MRT199665 led to a dose-dependent reduction in total and pS222 MEF2C (Fig. 5F; Supplementary Fig. S7F), causing more than 40% reduction in MEF2C phosphorylation at 10 nmol/L as compared with untreated cells, as assessed by quantitative fluorescent western immunoblotting (Fig. 5G; Supplementary Fig. S7G). MRT199665 treatment also caused a decrease of total MEF2C protein expression, suggesting that its phosphorylation can affect MEF2C stability or degradation, similar to previous reports (53). Notably, we observed that MRT199665 treatment led to dose-dependent loss of MEF2C S222 phosphorylation in OCI-AML2 cells expressing wild-type MEF2C, but not MEF2C S222A, consistent with its specific effects on MEF2C S222 phosphorylation (Supplementary Fig. S7H–S7I). Thus, MRT199665 treatment can block MEF2C S222 phosphorylation in AML cells.

Consistent with this mechanism, we found that human AML cell lines with endogenous MEF2C phosphorylation (OCI-AML2, MV-4;11, MOLM-13, and Kasumi-1) were more sensitive to MRT199665 as compared with cell lines lacking MEF2C (NB-4, HEL, HL-60, and U937), with mean 50% inhibitory concentrations (IC50) of 26 ± 13 versus 990 ± 29 nmol/L, respectively (P = 5.6 × 10−5; t test, Fig. 5H), with untreated cells.

Figure 5. Chemical inhibition of MARK-induced MEF2C phosphorylation exhibits selective toxicity against MEF2C-activated human AML cells. A, Recombinant screen for serine kinases that phosphorylate MEF2C S222, as assayed by significant pS222 MEF2C product inhibition marked in red. B, Phosphorylation activity of recombinant MARK3 (red) as compared with control CAMK1a (black) on model substrate as product inhibited by synthetic pS222 MEF2C peptide. Staurosporine serves as positive control. Error bars, SD of the mean for 3 biological replicates. * P = 4.1 × 10−4 for MARK3 activity with and without MEF2C; t test. MSA, mobility shift assay. C, Activity of luciferase transcriptional MEF2 reporter in HEK293T cells lentivirally test. MSA, mobility shift assay. ** P = 5.0 × 10−5; **** P = 1.5 × 10−7 for MEF2C S222A vector versus MEF2C vector; MEF2C MARK3 versus MEF2C vector; MEF2C; MARK3 versus MEF2C MARK3; and MEF2C S222A; MARK3 versus MEF2C; MARK3, respectively (t test). Below, western immunoblot analysis demonstrating equal protein expression of MEF2C and MARK3 transgenes, with reduced S222 phosphorylation by expression of MEF2C S222A and MARK3 T211A mutants. D, Correlation analysis of differentially expressed gene sets between S222A–expressing OCI-AML2 cells and MRT199665–treated OCI-AML2 cells. r = 0.64, Pearson correlation coefficient. E, GSEA of significantly upregulated and downregulated gene sets. [continued on next page]
and displayed reduced leukemia growth in clonogenic assays (Supplementary Fig. S7J). Importantly, expression of MEF2C in OCI-AML2 cells conferred enhanced susceptibility to MRT199665, which was partially rescued by the expression of MEF2C S222D that models constitutive MEF2C S222 phosphorylation (Fig. S1). Likewise, we found that primary patient AML specimens with MEF2C activation including MLL-rearranged and non-MLL-rearranged leukemias (Supplementary Table S4 and Supplementary Fig. S8A) treated in short-term cultures ex vivo exhibited enhanced susceptibility and apoptosis in response to MRT199665 treatment as compared with AML cell lines lacking MEF2C expression (IC50 = 280 ± 136 vs. 1300 ± 360 nmol/L, respectively, \( P = 1.8 \times 10^{-5} \), \( t \) test, Fig. S5; Supplementary Fig. S8B–S8D). Thus, activation of MEF2C in human AML confers susceptibility to MARK/SIK kinase inhibition.

### MEF2C Phosphorylation Is Required for Chemotherapy Resistance in AML

Because MEF2C phosphorylation was specifically observed in diagnostic AML specimens in patients with failure of induction therapy (Fig. 1), we hypothesized that MEF2C S222 phosphorylation induces chemotherapy resistance. In agreement with this prediction, OCI-AML2 cells induced to express dominant-negative MEF2C S222A were significantly more sensitive to both cytarabine and doxorubicin treatment as compared with cells expressing wild-type MEF2C, MEF2C S222D, or untransduced control OCI-AML2 cells (\( P = 8.8 \times 10^{-8} \) and \( P = 3.4 \times 10^{-5} \), respectively, \( \chi^2 \) test; Fig. 6A and B).

To investigate this therapeutic effect in vivo, we transplanted OCI-AML2 cells induced to express MEF2C S222A as compared with wild-type MEF2C into NSG mice, induced expression of transgenes by doxycycline chow, and treated leukemic mice with cytarabine or PBS control (Fig. 6C and D). We found that mice with leukemias expressing MEF2C S222A were significantly sensitized to cytarabine treatment in vivo as compared with MEF2C wild-type expressing controls (\( P = 6.8 \times 10^{-3} \), log-rank test; Fig. 6D).

Because MEF2C phosphorylation is required for AML chemotherapy resistance, we investigated the antileukemic efficacy of MARK/SIK kinase inhibition in combination with chemotherapy. Because MRT199665 exhibits rapid metabolism and elimination in mice and consequently is not suitable for animal studies (67), its effects on chemotherapy resistance could only be assessed \textit{in vitro}. We found that human AML cell lines with endogenous MEF2C phosphorylation (OCI-AML2, MV4-11, MOLM-13, and Kasumi-1) were significantly sensitized to cytarabine in the presence of 100 nmol/L MRT199665, as compared with cell lines lacking MEF2C (NB-4, HEL, HL-60, and U937) that remained unaffected by MRT199665 (\( P = 0.024 \), paired \( t \) test; Fig. 6E).

Similarly, patient-derived primary AML specimens with MEF2C...
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Figure 6. MARK kinase inhibition overcomes chemotherapy resistance of MEF2C-activated AML cell lines and patient cells. Growth of OCI-AML2 cells lentivirally transduced with wild-type MEF2C or dominant-negative MEF2C S222A or MEF2C S222D transgenes and treated with 600 ng/mL doxycycline to induce transgene expression and increasing concentrations of cytarabine (A) and doxorubicin (B) for 72 hours. Error bars, SD of the mean for 3 biological replicates. P = 8.8 × 10−8 and 3.4 × 10−9 for MEF2C versus MEF2C S222A by nonlinear regression for cytarabine and doxorubicin, respectively. C, Kaplan–Meier survival curves of NSG mice transplanted with OCI-AML2 cells transduced with wild-type MEF2C and dominant-negative MEF2C S222A transgenes, and (D) treated with doxycycline in chow 3 days following transplantation continuously in vivo. One week following transplantation, animals were treated with vehicle or cytarabine (blue arrow) intraperitoneally for 5 days. P = 6.8 × 10−5 MEF2C versus S222A cytarabine treated, log-rank test for 10 animals per group. E, Cytarabine IC50 values for human AML cell lines with MEF2C activation (MEF2C +) as compared with those lacking MEF2C (MEF2C −) after 48 hours of drug treatment in the absence (−MRT) or presence (+MRT) of 100 nmol/L MRT199665. Each data point represents the mean of biological triplicates of an individual sample. *, P = 0.024 for +MRT versus −MRT for MEF2C-activated cells by paired t test. F, Cytarabine IC50 values for primary patient AML specimens or normal CD34 cells with MEF2C after 48 hours of drug treatment in the absence (−MRT) or presence (+MRT) of 100 nmol/L MRT199665 in vitro. Each data point represents the mean of biological triplicates of an individual sample.
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activation (2 MLL-rearranged and 2 non-MLL-rearranged) exhibited enhanced susceptibility to cytarabine treatment in the presence of MRT199665 in vitro (IC50 = 58 ± 76 vs. 230 ± 280 nmol/L in the presence vs. absence of MRT199665, respectively, P = 0.036, t test, Fig. 6F), in contrast to healthy CD34+ human blood umbilical cord progenitor cells, which showed no sensitization (Fig. 6F). Taken together, these results indicate that MEF2C phosphorylation is required for AML chemotherapy resistance, which can be blocked by MARK/SIK kinase inhibition.

DISCUSSION

Our current findings indicate that MEF2C S222 phosphorylation is a specific marker of primary chemotherapy resistance and failure of induction chemotherapy in patients with both cytogenetically normal and chromosomally rearranged AMLs (Fig. 1). Activity of S222-phosphorylated MEF2C appears to be aberrant, insofar as mice genetically engineered to block its phosphorylation exhibit normal steady-state and stress hematopoiesis, but are resistant to leukemogenesis induced by the MLL–AP9 oncogene in vivo (Fig. 2). At least in part, this effect is due to the requirement for MEF2C phosphorylation for the survival of leukemia stem or initiating cells (Fig. 3) and enhanced transcriptional activity and induction of gene expression programs regulating cell survival and apoptosis (Fig. 4). Finally, MARK kinases can specifically phosphorylate MEF2C, potentiating its transcriptional activity (Fig. 5), and inhibition of MEF2C phosphorylation can overcome chemotherapy resistance of MEF2C-activated human AML cell lines and patient leukemias (Fig. 6).

Resistance to chemotherapy remains the major barrier to improving clinical outcomes for patients with AML, yet our current concepts of chemoresistance lack sufficient explanatory power. For example, inactivation of TP53 and overexpression of xenobiotic transporters have been found to cause chemoresistance, but the majority of patients with primary refractory or relapsed AML lack identifiable mutations of TP53 or known components of TP53-dependent DNA damage response, and do not exhibit xenobiotic transporter overexpression (1–3). Likewise, we do not yet understand how recently identified groups of genetic mutations associated with inferior clinical outcomes cause chemoresistance (3). Recently, impaired susceptibility to mitochondrial apoptosis has been associated with chemoresistance in AML (68, 69). However, the molecular mechanisms responsible for these differences also remain poorly understood. Our findings identify kinase-dependent dysregulation of transcription factor control as a determinant of therapy response in AML, at least in part mediated by aberrant survival and apoptosis resistance of leukemia stem cells (Fig. 7). This suggests that varied mechanisms of chemotherapy resistance and disease relapse in patients may be ultimately caused by aberrant gene expression programming of privileged leukemia cell subsets that provide a chemoresistant disease reservoir (70).

The observation of MEF2C S222 phosphorylation in genetically diverse AML subtypes, including cytogenetically normal and MLL-rearranged leukemias, raises the possibility that common gene expression programs and regulatory mechanisms may be engaged by distinct genetic and molecular classes of AML, such as for example distinct groups of mutations and/or their induction in specific leukemia-initiating cell populations. Likewise, recurrent mutations of MEF2B, MEF2C, and MEF2D in refractory lymphoid cancers (26–28) suggest that MEF2 family members may regulate essential survival or homeostatic mechanisms in hematopoietic cells, which cause therapy resistance in myeloid and lymphoid malignancies. However, whereas mutational activation of MEF2C in refractory T-cell acute lymphoblastic leukemias appears to inhibit apoptosis via NR4A1/NUR77-mediated effects on BCL2 (35), apoptosis resistance induced by MEF2C phosphorylation in AML appears to involve Bmf, Casp8, and FoxO3 dysregulation of Bim and BID (Fig. 4). Additionally, MEF2C phosphorylation–dependent regulation of Lyl1 expression suggests that its oncogenic functions in AML may contribute to the co-option of hematopoietic stem cell programs (71), at least in part mediated by aberrant transcription factor complexes (72), which remains an important question for future studies.

We observe that MEF2C is both phosphorylated and highly expressed in AML, and its leukemogenic activities may therefore be due to both its high abundance and S222 phosphorylation. Changes in MEF2C abundance and S222 phosphorylation may affect its activity by (i) recruitment of distinct coactivators or corepressors, (ii) altered dimerization with endogenous MEF2 family members, and/or (iii) altered target gene localization due to secondary changes in the posttranslational modifications and sequence binding preferences of its DNA binding domain (47, 73–75). We found that MEF2C S222 phosphorylation can regulate the assembly of its transcriptionally active complex, which is associated with its induction of both activating and repressive gene expression programs. This leads to the dysregulation of genes controlling cell survival such as Lyl1, Myc, Bmf, Casp8, and FoxO3 (Fig. 4), consistent with the previously reported activities of MEF2C as both transcriptional activator and repressor (76). This dysregulation is likely important for both AML pathogenesis and chemotherapy resistance, and it will be important to determine whether shared mechanisms regulate leukemia-initiating cell populations and those resistant to chemotherapy. Because our functional studies were largely based on MLL–AP9-rearranged and Runx1-mutant leukemias, it is possible that MEF2C phosphorylation may involve distinct molecular mechanisms in different leukemia subtypes. It is also possible that the marked phenotype of nonphosphorylatable MEF2C mutants may be due to dominant-negative effects on other MEF2 family members, given their apparent dimerization. Similarly, although MARK3 can specifically phosphorylate MEF2C S222, additional serine kinases inhibited by MRT199665, or those which were not included in our screen, such as mTOR, may induce MEF2C phosphorylation in distinct types of AML.

The MARK family was originally discovered based on its functions in controlling cell polarity and microtubule dynamics as part of the basal lateral polarity complex (77, 78), but their functions in hematopoietic cells are not yet defined (79). Our finding that MARKs can regulate MEF2C suggests that the basal lateral polarity complex may have distinct
functions in normal hematopoiesis, which may be dysregulated in leukemia cells. Similarly, the mechanisms of MARK3-mediated phosphorylation of MEF2C in chemotherapy-resistant AML remain to be defined. For example, MARK kinases can be phosphorylated and activated by GSK-3β (80), which can in turn be activated by integrin signaling, including reports of its activation in AML (81). Thus, activation of MARK signaling and MEF2C phosphorylation may be linked to autocrine or paracrine signaling by AML cells and the bone marrow niche (29, 82, 83).

Finally, the lack of apparent phenotypes in knock-in mutant mice homozygous for \(\text{Mef2c}^{S222A/S222A}\) or \(\text{Mef2c}^{S222D/S222D}\) suggests that phosphorylation of MEF2C S222 is dispensable for normal development, establishing a compelling therapeutic window and substantiating its therapeutic targeting. However, additional studies of MEF2C phosphorylation in specific physiologic states (24, 25), and further preclinical development of selective non-pyrolypyrimidinone MARK inhibitors, will be needed to advance their use as MEF2C-targeted therapeutics. Future functional profiling studies of
large AML cohorts should establish the prevalence of MEF2C phosphorylation and other mechanisms of primary chemotherapy resistance.

METHODS

Collection of Patient Samples

Written informed consent and approval by the Institutional Review Boards of participating institutions was obtained in accordance with the Declaration of Helsinki for all subjects. Primary chemotherapy resistance was defined based on the presence of at least 5% of abnormal blasts by morphologic and immunophenotypic assessment of bone marrow aspirates obtained after two cycles of induction chemotherapy, as assessed by the respective institutional or central pathologic reviews. Specimens were collected and leukemia cells purified as previously described (16). Mononuclear cells were purified using Ficoll gradient centrifugation, and leukemia cells were purified by negative immunomagnetic selection against CD3, CD14, CD19, and CD235a, based on the absence of their expression by the majority of AML specimens.

Production and Purification of pS222 MEF2C Antibody

A phospho-specific antibody against MEF2C S222 (RefSeq ID: NM_002397.4) was generated by PhosphoSolutions (Catalog p1208-222, RRID:AB_2572427; PhosphoSolutions). Rabbits were immunized with a synthetic phosphopeptide corresponding to amino acids surrounding S222 of human MEF2C, conjugated with Keyhole limpet hemocyanin. Serum isolated from peripheral blood of immunized rabbits was screened for phospho-specificity using enzyme-linked immunosorbent assay (ELISA). ELISA-positive sera were pooled and sequentially affinity purified using both the phosphopeptide and non-phosphopeptide columns to isolate the affinity-purified pS222 MEF2C antibody.

Phosphoproteomics Screen

Phosphoproteomic profiling was performed as described previously (31). Briefly, purified leukemia cells were lysed using guanidine hydrochloride, and proteins were reduced with dithiothreitol, alkylated with iodoacetamide, and digested using trypsin. Tryptic peptides were purified by solid phase extraction, and purified peptides were labeled using iTRAQ reagents. Phosphoproteins were enriched using iron affinity chromatography, and separated using three-dimensional RP-SAX-RP chromatography coupled to nanoelectrospray ion source. Spectra were recorded using Orbitrap Velos mass spectrometer (ThermoFisher Scientific) in data-dependent mode. Data files were analyzed using multiplexier (84). Analysis of phosphoproteomics data is detailed in Supplementary Methods.

Cell Lines

Human AML cell lines were obtained from DSMZ and HEK293T cells from ATCC in 2013. Cell line authentication testing by STR genotyping (Genetics DNA Laboratories) for all cell lines was performed in May 2014 and February 2017. The absence of Mycoplasma sp. contamination was verified most recently in August 2017 using Lonza MycoAlert (Lonza Walkersville, Inc.). Cell lines were used for experiments in this article within 20 passages from thawing.

Mouse Studies

All mouse studies were conducted with approval from the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee. C57BL/6J, NOD-SCID IL2R−/−null (NSG), and B6.SJL-Ptprc Peep/Boj mice were all obtained from The Jackson Laboratories. Knock-in Me2CδS222A and Me2CεS222D mutant mice were generated using CRISPR/Cas9 genome editing and homologous recombination. A CRISPR gRNA targeting exon 5 of the Me2c gene was designed (GGAATGGATACGGCAACCC) and expressed in vitro using the approach previously described (85). The donor templates for recombination were designed according to Wefers and colleagues (86), which produced in-frame substitutions and the addition of restriction sites for genotyping. Mouse zygotes from C57BL/6J:CBA F2 hybrids were injected with 100 ng/μL of gRNA, 50 ng/μL of Cas9 mRNA, and 50 ng/μL of donor oligonucleotide into the pronucleus. Founder mice were first screened using restriction endonuclease digestion of genomic DNA isolated from tail tissues and positive mice analyzed by PCR. For additional details, see the Supplementary Methods. For all experiments using Me2c knock-in mutant animals, wild-type littermates were used as controls.

Murine Bone Marrow Transplantation Models

For primary MLL–AF9 and Hoxa9/Meis1 mouse leukemia transplants, 150,000 and 500,000 transduced cells, respectively, were transplanted by intravenous injection into lethally irradiated (900 rad) C57BL/6J animals with 125,000 support wild-type bone marrow cells and monitored for the development of leukemia. For Runx1−/−;Fbe3−/− mouse leukemia transplants, 750,000 and 50,000 secondary leukemia bone marrow cells harvested from moribund primary recipients were transplanted by intravenous injection into sublethally irradiated (600 rad) C57BL/6J mice. For competitive bone marrow transplantation studies, 1 million CD45.2 mononuclear bone marrow cells from Me2c transgenic mice were mixed with 1 million CD45.1 mononuclear bone marrow cells from B6.SJL-Ptprc Peep/Boj mice and transplanted via tail-vein injection into lethally irradiated C57BL/6J recipient animals. Transplanted mice were monitored by CD45.1/CD45.2 peripheral blood chimerism using FACS with the surface markers CD45.2 and CD45.1. Antibodies used in flow cytometry are detailed in Supplementary Table S5.

Human AML Xenograft Model

NSG mice (8–10 weeks old) were sublethally irradiated (200 rad) and transplanted with 500,000 OCI-AML2 cells via tail-vein injection. Doxycycline-inducible transgene expression was induced using doxycycline chow 3 days after transplantation (Harlan). In xenotransplants involving cytarabine treatment, 1.2 million OCI-AML2 cells were transplanted into nonirradiated NSG mice to account for the toxicity effects of cytarabine. Cytarabine in PBS (30 mg/kg/day) was administered 7 days after transplantation intraperitoneally for a total of 5 doses.

Recombinant Kinase Screen

The protein kinase screen was performed using a recombinant serine kinase library as previously described (87). Briefly, 172 serine/threonine kinases (Supplementary Data S3) were individually expressed as N-terminal GST-fusion proteins in insect cells, and purified using glutathione sepharose chromatography. A synthetic peptide corresponding to phosphoserine 222 for human MEF2C (RefSeq ID: NM_002397.4) Ac-GNPRN[pS]PGLLVC-NH2 was synthesized (Tufts University), purified on chromatography coupled to nanoelectrospray ion source. Spectra were recorded using Orbitrap Velos mass spectrometer (ThermoFisher Scientific) in data-dependent mode. Data files were analyzed using multiplexier (84). Analysis of phosphoproteomics data is detailed in Supplementary Methods.

Data Accession

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Statistical Analysis

All in vitro experiments were performed a minimum of three independent times with a minimum of three biological replicates. Statistical significance was determined using a two-tailed Student t test for continuous variables, Pearson χ2 test for limiting dilution assays, and log-rank test for survival analysis. For ROC curve analysis, MEF2C S222 phosphorylation expression above the median was considered to be positive. Data were plotted as mean values with error bars representing standard deviation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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