Identifying Novel Vulnerabilities in Clear Cell Sarcoma Through Functional and Genomic Screening

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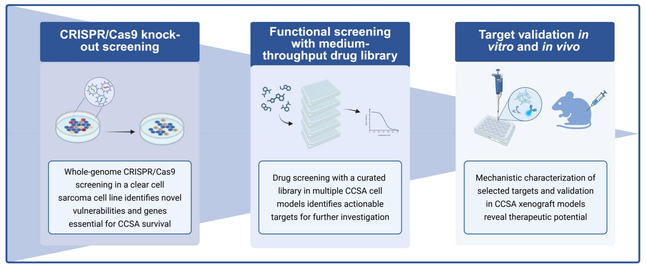
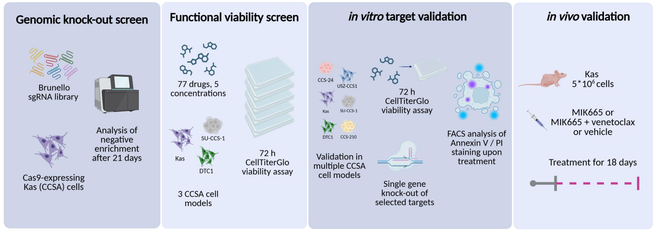


Introduction

Clear cell sarcoma (CCSA) is a rare and highly malignant soft tissue sarcoma, characterized by two recurrent translocations t(12;22)(q13;q12) or t(2;22)(q34;q12), leading to expression of EWSR1-ATF1 or EWSR1-CREB1 fusion genes, respectively. CCSA has a high propensity to relapse locally (up to 50%) and metastasize distantly. Metastasized CCSA is resistant to conventional chemotherapy, targeted therapy and immunotherapy approaches. Furthermore, oncogenic gene fusions involving transcription factors are notoriously difficult to target. Novel approaches to treat CCSA have focused on deranged signaling pathways, however, effective and curative treatments are still lacking. This study aims to uncover new therapeutic strategies for CCSA by utilizing a whole-genome CRISPR/Cas9 knock-out screen combined with functional drug screening.

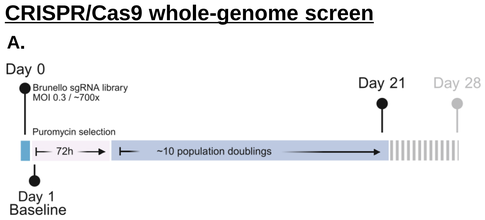
Methods

We performed a genome-wide knock-out screen in the CCSA cell line Kas using the pooled Brunello sgRNA library (Addgene #73178, ~4 guides/gene, 19'114 genes). Based on the results of the CRISPR/Cas9 screening, three CCSA cell lines (SU-CCS-1, DTC-1, KAS) were treated with a custom drug library tailored to the hits of the CRISPR screen (77 compounds). Compounds included targeted top hits, pathways linked to the top hits and pathways identified in a GSEA analysis. Genes identified as critical vulnerabilities in CCSA were validated through single-gene knockouts, and promising therapeutic targets were further mechanistically interrogated and evaluated in a CCSA xenograft model.



Results

CRISPR/Cas9 whole-genome screen



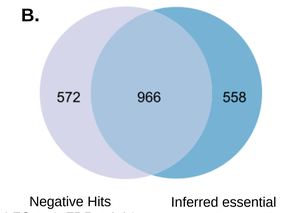


Figure 1. **A.** Timeline and schematic of CRISPR/Cas9 screen using the Brunello sgRNA library. At the start of the screen, cells were transduced with an MOI 0.3, in order to introduce only one sgRNA per cell and achieve a coverage of ~725x. Cells were collected for DNA extraction and analysis at baseline (day 1) and day 21. Analysis of negative enrichment was done using the MaGeck pipeline. **B.** A negative log-fold change (LFC) of ≤ -1 and a false discovery rate (FDR) of ≤ 0.01 was found in 1552 genes. Using publicly available data from DepMap, 965 of these genes can be classified as essential.

Negative enrichment of genes essential for clear cell sarcoma survival

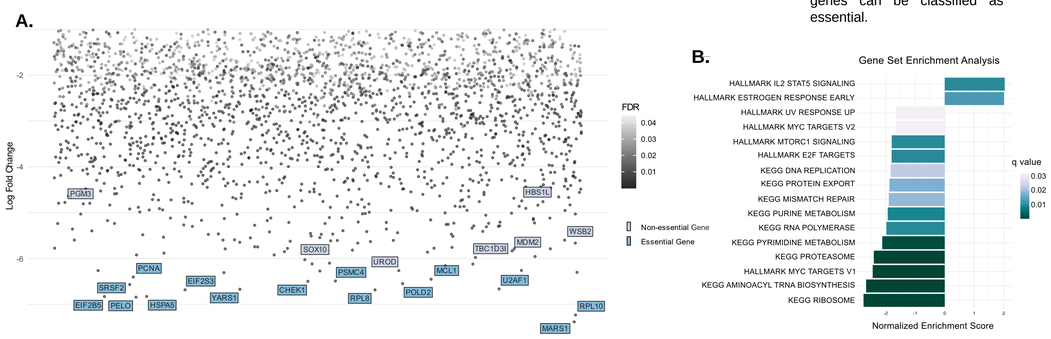


Figure 2. **A.** Distribution of genes with a negative log-fold change (LFC) of ≤ -1 and a false discovery rate (FDR) of ≤ 0.05 shown distributed along a random index. Distribution of genes with a negative LFC of ≤-1 and a FDR of ≤ 0.05 shown along a random index. Labelled are the top 15 genes and other selected negatively enriched genesFor example, SOX10, a well-known CCSA marker. **B.** Gene set enrichment analysis was performed on a subset of genes, FDR ≤ -0.01 and LFC ≤ -1, to elucidate the presence of overreaching pathways. MSigDB Hallmark pathways and KEGG pathways were interrogated.

Functional screening of CCSA cell models with curated medium-throughput drug library

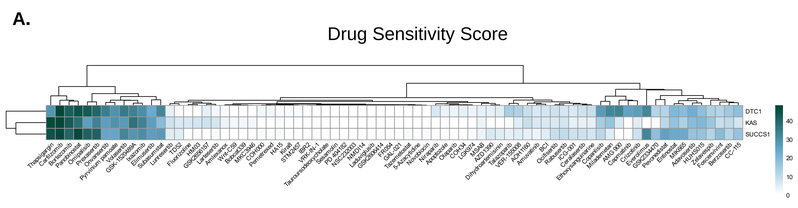


Figure 3. A manually curated drug library, based on the CRISPR/Cas9 whole-genome screen results, of 77 drugs in 5 concentrations was tested on 3 CCSA cell models. **A.** Heatmap showing the Drug Sensitivity Score (DSS), as calculated by the Breeze pipeline, depicts the effect of the tested drugs. In this screen, we identified PI3K/mTOR inhibitors, proteasome inhibitors, DNA repair inhibitors and an MCL1 inhibitor among others to reduce the viability of CCSA cells. **B.** Dose-response curves of MIK665 (MCL1i) indicating a high DSS across all three CCSA cell models. **C.** Validation of MIK665 inhibition in a panel of 6 CCSA cell models and one fibroblast cell line after 72h.

Selected single gene knock-out experiments confirm CRISPR screen results

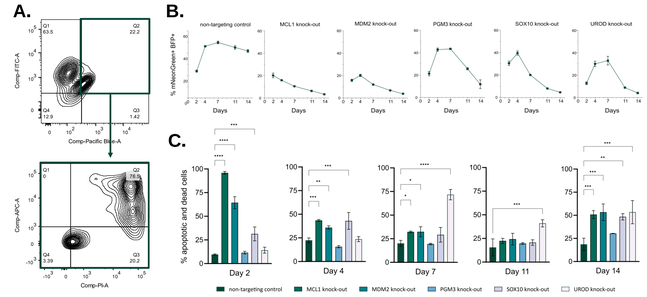


Figure 4. Single-gene knock-out of 5 CRISPR/Cas9 screen hits. Cas9(mNeonGreen)-expressing Kas cells were transduced with sgRNAs (BFP) targeting selected genes and analyzed on days 2, 4, 7, 11, and 14 for presence of knock-out and apoptosis/cell death markers. **A.** Representative FACS plots showing gating strategy. **B.** Percentage of cells containing Cas9 and sgRNA **C.** Percentage of GFP+/BFP+ cells undergoing apoptosis and/or cell death.

Combination of MCL1 and BCL2 inhibition in clear cell sarcoma

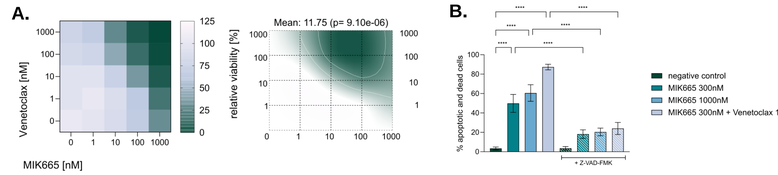


Figure 5. Investigation of combination of MCL1 and BCL2 inhibition *in vitro*: **A.** Relative viability of Kas upon combination treatment with MIK665 and venetoclax (BCL2i) for 72h. **C.** ZIP synergy score (SynergyFinder) indicates synergy upon treatment with MIK665 and venetoclax. **B.** Annexin V / PI staining reveals apoptosis upon treatment with MIK665 and upon combination treatment, which can be mitigated with apoptosis inhibitor Z-VAD-FMK.

MCL1 inhibition in clear cell sarcoma

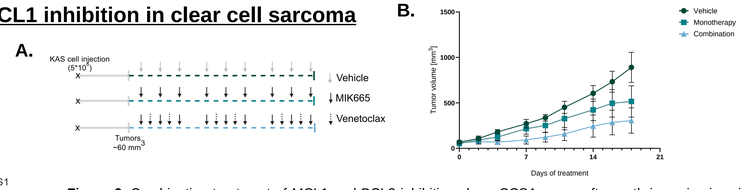


Figure 6. Combination treatment of MCL1 and BCL2 inhibition slows CCSA xenograft growth in an *in vivo* pilot experiment. **A.** Schematic of *in vivo* experiment and treatment schedule. 25 mg/kg MIK665 and 50 mg/kg venetoclax were administered as depicted intraperitoneally and via oral gavage respectively. **B.** Measured tumor over a treatment period of 18 days until humane endpoint was reached for the first vehicle-treated animal. Data are presented as mean ± SEM

Conclusions

- Our dual screening approach leveraging genomic and functional screening offers novel insights into actionable vulnerabilities in CCSA.
- Pharmacological inhibition of MCL1 significantly reduced viability in CCSA cell lines and patient-derived models and synergized with the BCL2 inhibitor venetoclax to enhance apoptosis and suppress xenograft tumor growth.

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- Illustrations created in https://BioRender.com

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