Identifying drug target candidates to treat pediatric rhabdomyosarcoma tumors

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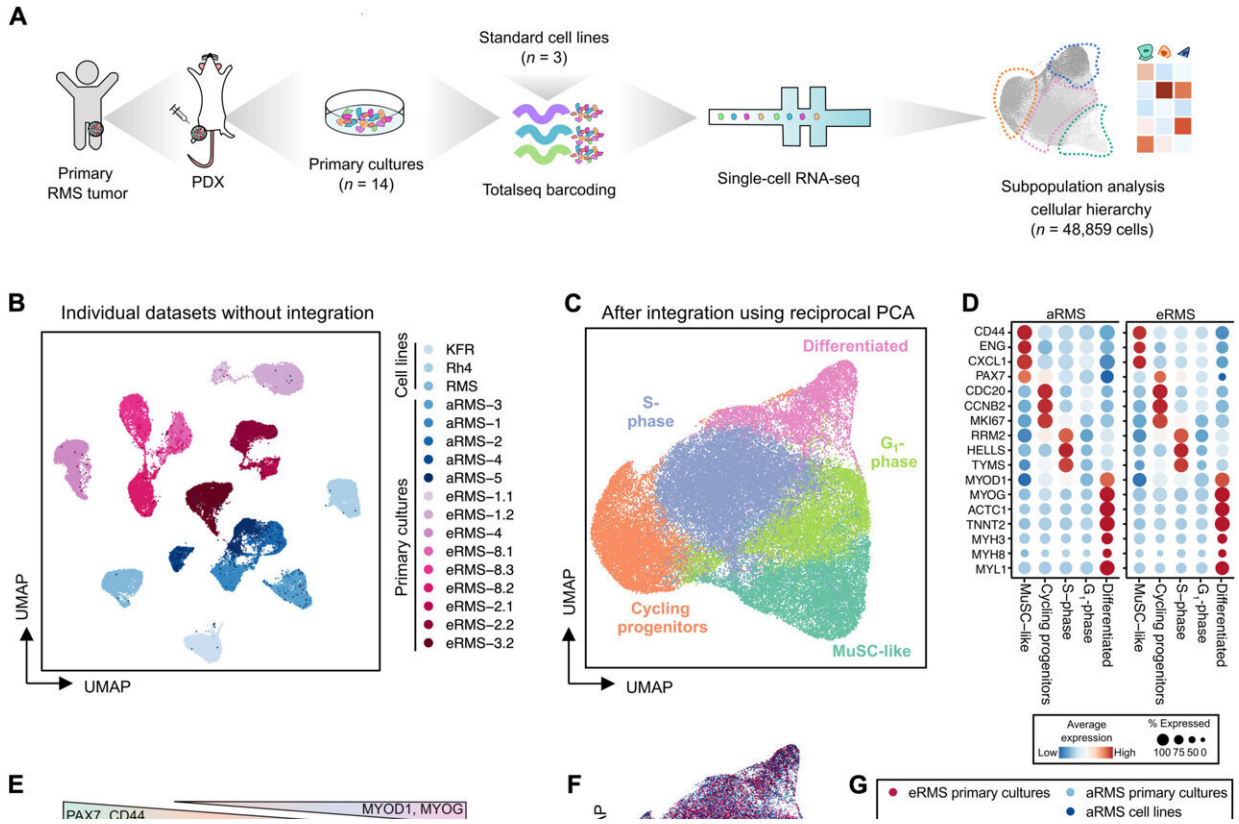


scRNAseq of RMS identifies heterogeneity recapitulating muscle developmental programs. (A) Experimental workflow. (B) UMAP plot of 48,859 RMS cells after regressing the number of count RNA, the percentage of mitochondrial genes, and the run batch effect. Cells are color-coded based on the corresponding sample of origin. (C) UMAP plot of 48,859 RMS cells after integration. Populations identified by Louvain clustering are shown. (D) Dot plot showing expression of lineage-specific marker genes across the different Louvain clusters in aRMS and eRMS samples. (E) Model of skeletal myogenesis with the

populations identified in RMS. UMAP plots are colored on the basis of the expression of markers delineating a myogenic lineage progression. (F) UMAP plot of RMS cells after integration and color-coded based on the sample of origin. (G) Relative proportion of Louvain clusters. Data are represented as means ± SEM; ordinary two-way analysis of variance (ANOVA) with uncorrected Fisher's least significant difference (LSD). *P Science Advances (2023). DOI: 10.1126/sciadv.ade9238

Cancer biologists are yet to understand the mechanisms and cellular hierarchy leading to the developmental arrest in rhabdomyosarcoma (RMS)—a group of pediatric cancers, which remain enigmatic.

In a new report in *Science Advances*, Sara G. Danielli and an international, interdisciplinary team of scientists at the department of Oncology and Children's Research, Stem Cell Biology, and Translational Research in Child and Adolescent Cancer, in the U.S., Switzerland, France, Germany and Spain, combined a myriad of biological methods to understand the etiology and cellular basis of RMS oncogenesis.

The team incorporated single-cell RNA sequencing, mass cytometry and high-content imaging methods to understand the intra-tumoral diversity of patient-derived biopsies. The aggressive alveolar subtype (aRMS) contained plastic muscle stem-like cells and precursors of tumor growth alongside a subpopulation of differentiated cells without proliferative potential that led to better outcomes.

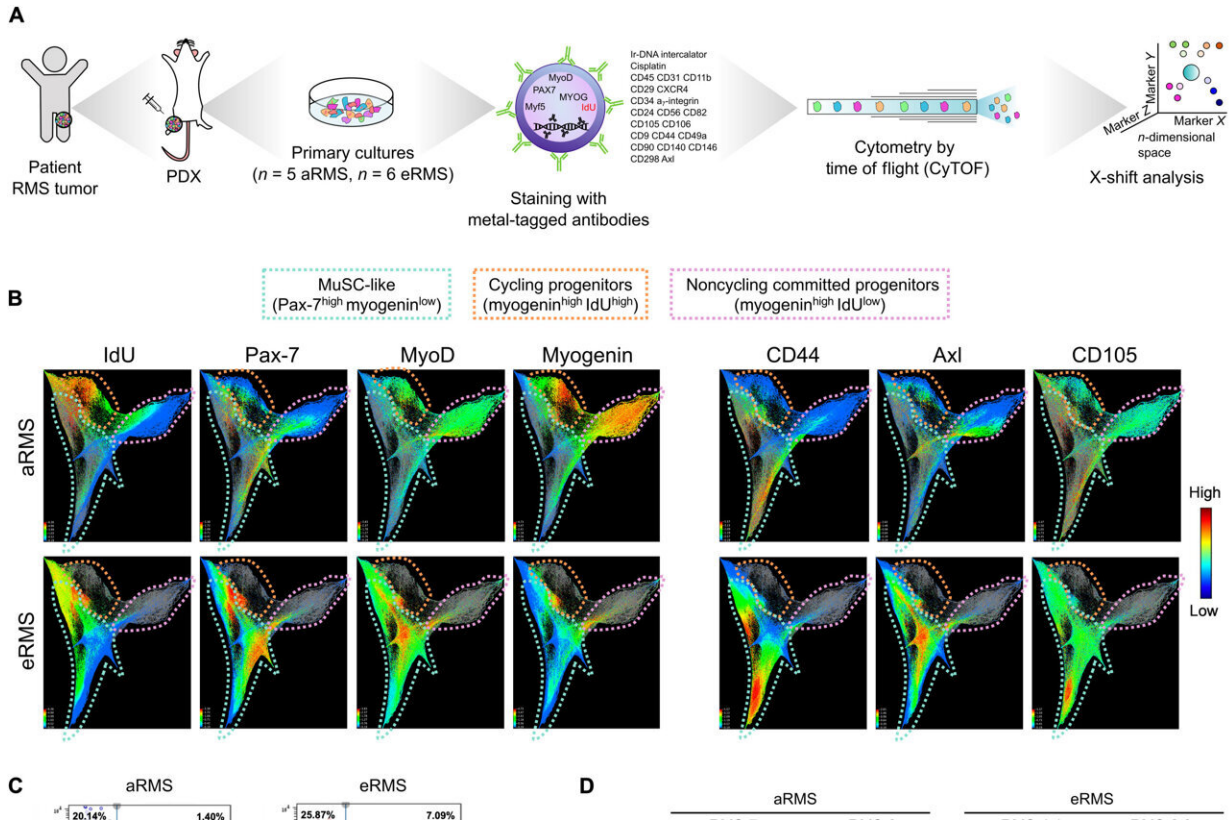
The scientists implemented chemotherapy and observed the dynamics of the aggressive alveolar version of the disease. They then screened the drug candidates and their capacity to propagate the disease towards clinically favorable sub-populations and identified a combination of rapidly accelerated fibrosarcoma (RAF) and mitogen activated protein kinase (MEK) inhibitors to induce muscle differentiation and inhibit

tumor growth. These outcomes provide insights to develop biological states underlying the disease, including aggressiveness, chemoresistance and cell growth, while identifying the Ras pathway as a promising therapeutic target.

Rhabdomyosarcoma (RMS)

Childhood cancer leads to dysregulated human development and is a leading cause of disease-related morbidity and mortality in children and adolescents. Recent advances in single-cell technologies can assist the characterization of intratumoral diversity and shed light to the phenotypic plasticity across several cancer types to highlight their role as emerging hallmarks of oncogenesis.

Researchers are studying the developmental hierarchies of childhood disease to identify their origin and develop effective treatment strategies that target the cellular components. Rhabdomyosarcoma is a common pediatric soft tissue sarcoma occurring as embryonic and alveolar (aRMS) subtypes, of which the latter is more aggressive due to its underlying genetic basis.

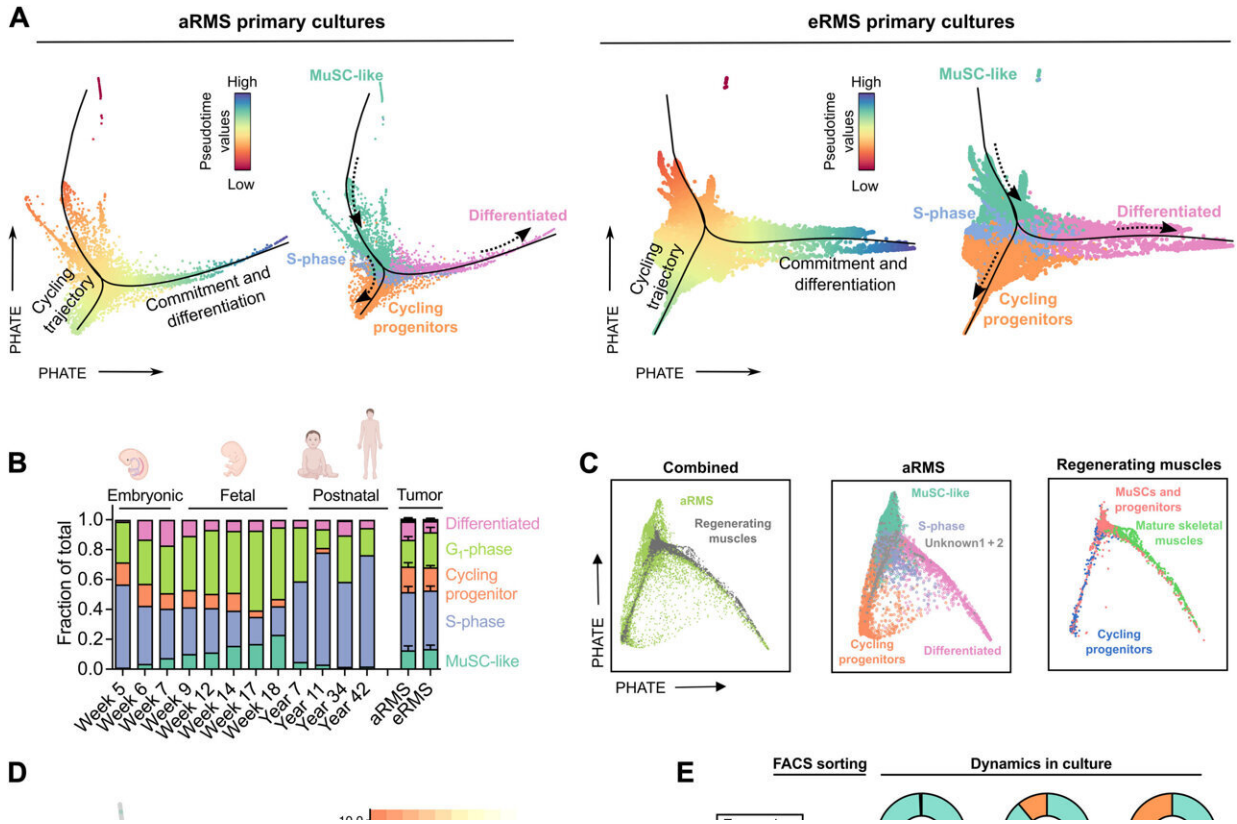


CyTOF defines RMS subpopulations with high resolution. (A) Mass cytometry workflow. (B) Integrated CyTOF dataset from RMS primary cultures clustered with the X-shift algorithm and visualized using single-cell force-directed layout. (C) Biaxial dot plots of Pax-7 or myogenin by IdU across aRMS and eRMS primary cultures. Plots are colored by CD44 expression. (D) Expression of Pax-7, myogenin, and MyHC markers as determined by immunohistochemistry in PDX tumors. Credit: *Science Advances* (2023). DOI: 10.1126/sciadv.ade9238

In the current study, Danielli and colleagues combined scRNA sequencing, mass cytometry, and high-content imaging analysis to examine the intratumoral heterogeneity of the cancer cell lines and of primary cultures obtained from patient-derived xenografts.

The team then screened regulators of the alveolar rhabdomyosarcoma

(aRMS) cell fate via a library of pharmaceutical compounds, including RAS pathway inhibitors trametinib with dabrafenib or regorafenib to direct these cancer cells to differentiate. The combinatorial therapies potentially suppressed the growth of the tumor in patient-derived xenografts to provide a strong rationale to clinically manipulate the RAS pathway to counter oncogenesis in patients.



RMS primary cultures recapitulate a branched myogenic trajectory. (A) PHATE dimensionality reduction (t = 30, knn = 20) plots of aRMS (left) or eRMS (right) primary cultures. Black lines represent pseudotime trajectories calculated using Slingshot (starting cluster: MuSC-like); dashed arrows represent the trajectory direction. Cells are colored on the basis of pseudotime values calculated by Slingshot (left) or on the identified Louvain clusters (right). (B) Clustering distribution of the integrated RMS/human developing skeletal muscle (42) dataset across developmental time points or RMS subtype. (C) PHATE

dimensionality reduction (t = 30, knn = 20) plot of the integrated aRMS primary culture/mouse regenerating skeletal muscle (32) dataset. aRMS cells are color-coded on the basis of Louvain clusters; muscle cells are colored on the basis of the clusters identified in the original publication. (D) PHATE dimensionality reduction (t = 30, knn = 20) plot of aRMS primary cultures colored on the basis of CD44 (green) or MYOG (orange) expression (left plot). The two markers are mutually exclusive (right plot). (E) Flow cytometry analysis of sorted CD44+ and CD44− subpopulations in aRMS-3 cells. Unsorted reference is also shown. Data are represented as means of n ≥ 2 biological replicates. (F) Relative proportion of Louvain clusters across aRMS-1 and aRMS-3 cells before sorting. The percentage of differentiated cells in the CD44− subpopulation is shown. (G) Immunofluorescence analysis of MyHC expression 7 days after sorting of CD44+ and CD44− subpopulations in aRMS-1 cells. The percentage of MyHC+ cells is indicated on the top right of each panel. (H) Proposed model of aRMS hierarchical structure compared to developing or regenerating MuSCs. Credit: *Science Advances* (2023). DOI: 10.1126/sciadv.ade9238

Using single-cell RNA sequencing to identify the distinct muscle development states

The researchers used droplet-based single cell RNA sequencing to understand the intratumoral diversity of cancer cells by profiling 14 patient-derived xenografts and three conventional alveolar rhabdomyosarcoma cell lines in comparison with preceding studies. The team increased the interpatient variability by choosing disease models that originated from diverse oncogenic subtypes, including primary and metastatic sites among diagnostic and recurrent patients who had or had not undergone pretreatment.

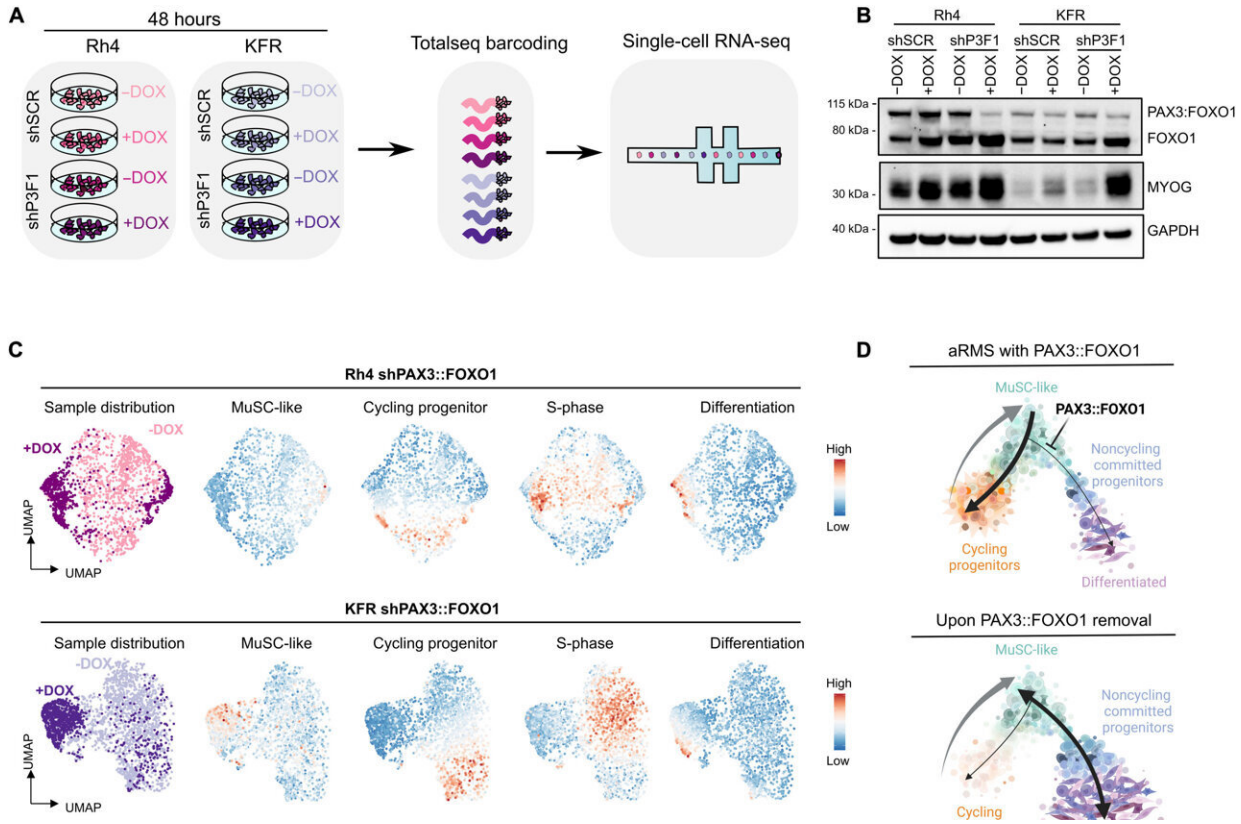
The outcomes revealed how the RMS tumors contained myogenic cells stalled in an immature transcriptional state to ultimately form a minority of differentiated cells. The scientists confirmed the presence of the

subpopulations at the protein level by staining the primary cell cultures with an isotope-conjugated antibody panel to isolate muscle stem-like cells, which they identified using single-cell RNA sequencing to define the distinct states of myogenesis.

Next, they profiled the cells by using cytometry via time-of-flight analyses, where the research outcomes indicated the aRMS cell subset to be of specific interest. The single-cell protein analysis outcomes aligned with the transcriptomic analysis.

Mechanism-of-action of the cancer phenotype and therapeutic intervention in the lab

The team demonstrated the mechanism-of-action of the RMS cancer phenotypes that mirrored the cell-fate decisions of developing, healthy and regenerating skeletal muscle stem-like cells. They studied this relationship via a trajectory inference model based on Slingshot and PHATE data visualization tools, to envision the structure and transitions in high-dimensional biological data. When the scientists projected the single-cell RNA sequencing atlas of the developing human skeletal muscles to the oncogenic single-cell transcriptome; the tumor cells mostly mapped onto muscle cells that were transiting from the embryonic to the fetal stages.



PAX3::FOXO1 down-regulation leads to MuSC-like and differentiated subpopulations. (A) Schematic workflow. (B) Representative WB of Rh4 and KFR cells cultured with (+DOX) or without (–DOX) DOX for 48 hours. (C) UMAP plot of 1978 Rh4 and 2589 KFR cells following KD of PAX3::FOXO1. Lines with shP3F1 were cultured with DOX for 48 hours (+DOX) to induce protein down-regulation and profiled by scRNAseq. Control lines that were not exposed to DOX are also shown (–DOX). UMAP plots are colored by DOX exposure (left) or the overall expression (color scale) of the identified signatures (right). (D) Proposed model of PAX3::FOXO1 heterogeneity across aRMS cell lines. Upon PAX3::FOXO1 removal, the differentiation block is released and the oncogenic loop is disrupted. The cycling progenitor subpopulation disappears, and the remaining cells display MuSC-like or differentiated features. Credit: *Science Advances* (2023). DOI: 10.1126/sciadv.ade9238

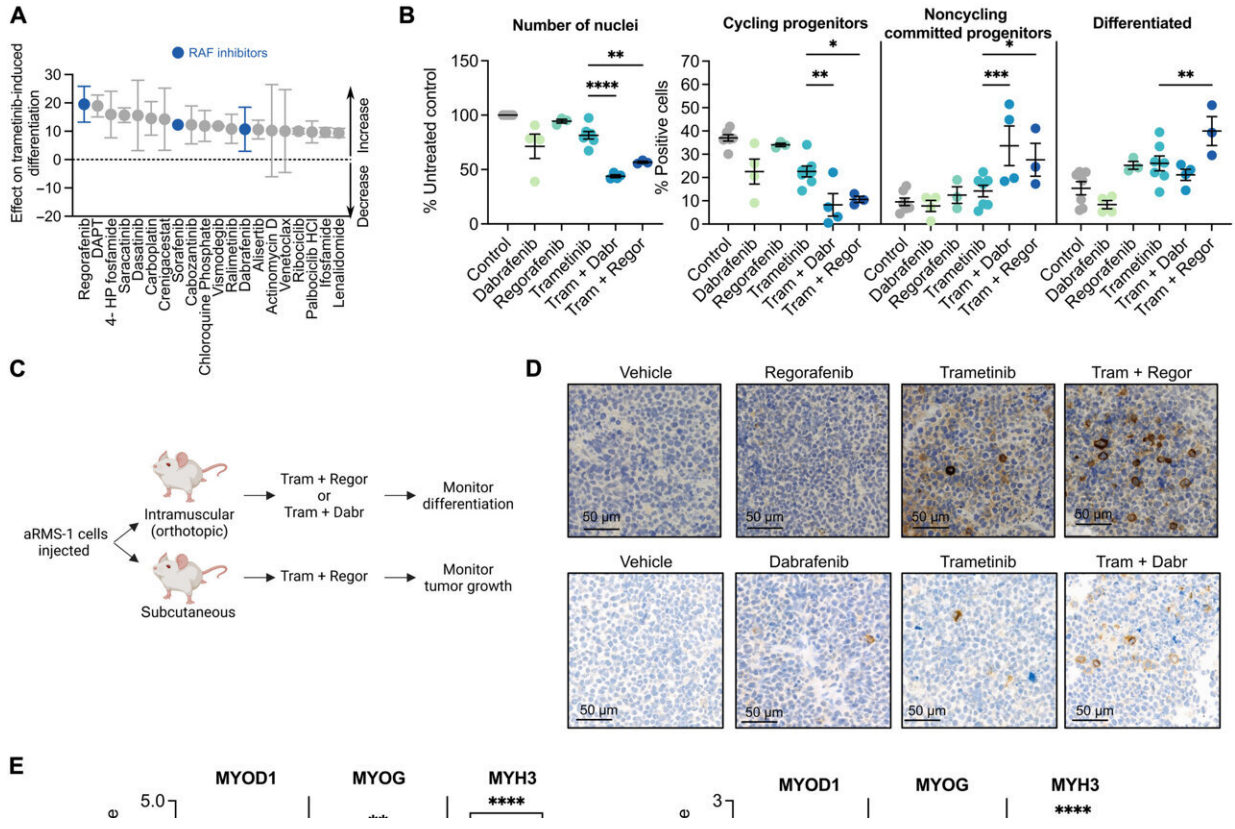
The scientists noted the major driver of the aRMS subtype to be the

PAX3::FOX01 fusion gene; known to repress myogenic differentiation. To study the genes, the team used two knockdown aRMS cell lines against the fusion genes; these treatment measures resulted in decreased levels of the fusion gene to highlight the gene knockdown capacity of the cell lines to either undergo differentiation or halt in a muscle stem-like state, allowing continued targeted fusion-gene therapy. It also appeared that some cell lines that intrinsically resisted treatment underwent cellular trajectory rewiring.

Differentiation therapy with drug combinations

The researchers observed the mechanism-of-action of Trametinib, which hijacked the cell fate of the treatment resistant aRMS cell line and redirected these cells toward differentiation. Of the screened drug moieties Trametinib, Cobimetinib and Erdafitinib induced robust myogenic differentiation.

Since monotherapy remains to succeed in clinical trials, the team identified the more effective combinatorial drug screening to demonstrate the trametinib-induced suppression of the RAS pathway in combination with RAF inhibition as a feasible treatment strategy for aRMS.



Vertical inhibition of the RAF-MEK-ERK cascade potentiates trametinib-induced differentiation and inhibits aRMS tumor growth. (A) Top 20 trametinib-potentiating drugs ranked on the basis of their effect on trametinib-induced differentiation. A score of zero represents the baseline score of trametinib alone; drugs with a positive score potentiate trametinib-induced differentiation. n = 2 biological replicates. (B) Quantification of immunofluorescence analysis of aRMS-1 cells exposed to vehicle controls, 10 µM dabrafenib (Dabr), 1 µM regorafenib (Regor), 10 nM trametinib (Tram), or the indicated combinations for 72 hours; ordinary two-way ANOVA with uncorrected Fisher's LSD. (C) Schematic of in vivo validation experiment. (D) Expression of MyHC as determined by immunohistochemistry in aRMS-1 PDX tumors following in vivo treatment with trametinib (5 mg/kg), regorafenib (15 mg/kg), or their combination (top row) or with trametinib (1 mg/kg), dabrafenib (15 mg/kg), or their combination (bottom row). (E) qRT-PCR analysis of aRMS-1 PDX tumors; ordinary two-way ANOVA with uncorrected Fisher's LSD. (F) Monitoring of tumor growth in mice that were injected with aRMS-1 cells and treated with vehicle, trametinib (5 mg/kg), regorafenib (15 mg/kg), or their combination for

two cycles (gray bars); ordinary two-way ANOVA with Dunnett's multiple comparison correction. (G) Waterfall plot showing the change in tumor volume in mice treated with vehicle, trametinib (5 mg/kg), regorafenib (15 mg/kg), or their combination, at the end of the treatment period (day 12). Mice marked with "*" had to be euthanized before the treatment end point due to toxicity. (H) Proposed model of trajectory rewiring in aRMS following treatment with the MEK inhibitor (MEKi) trametinib in combination with the RAF inhibitor (RAFi) regorafenib or dabrafenib. *P Science Advances (2023). DOI: 10.1126/sciadv.ade9238

Outlook

In this way, Sara G. Danielli and colleagues developed a comprehensive single-cell transcriptomic and proteomic atlas of rhabdomyosarcoma (RMS). The atlas detailed cellular and functional diversity of the disease and revealed key cellular and molecular signatures suited for therapeutic intervention to overcome chemoresistance and tumor relapse. The researchers described the mechanism-of-action of the cell fate underlying impaired differentiation in the aggressive alveolar rhabdomyosarcoma (aRMS) cancer subtype. The work sheds light on how to therapeutically restore myogenic differentiation and block tumor growth.

More information: Sara G. Danielli et al, Single-cell profiling of alveolar rhabdomyosarcoma reveals RAS pathway inhibitors as cell-fate hijackers with therapeutic relevance, *Science Advances* (2023). DOI: 10.1126/sciadv.ade9238

Simone Hettmer et al, Muscling in: Uncovering the origins of rhabdomyosarcoma, *Nature Medicine* (2010). DOI: 10.1038/nm0210-171