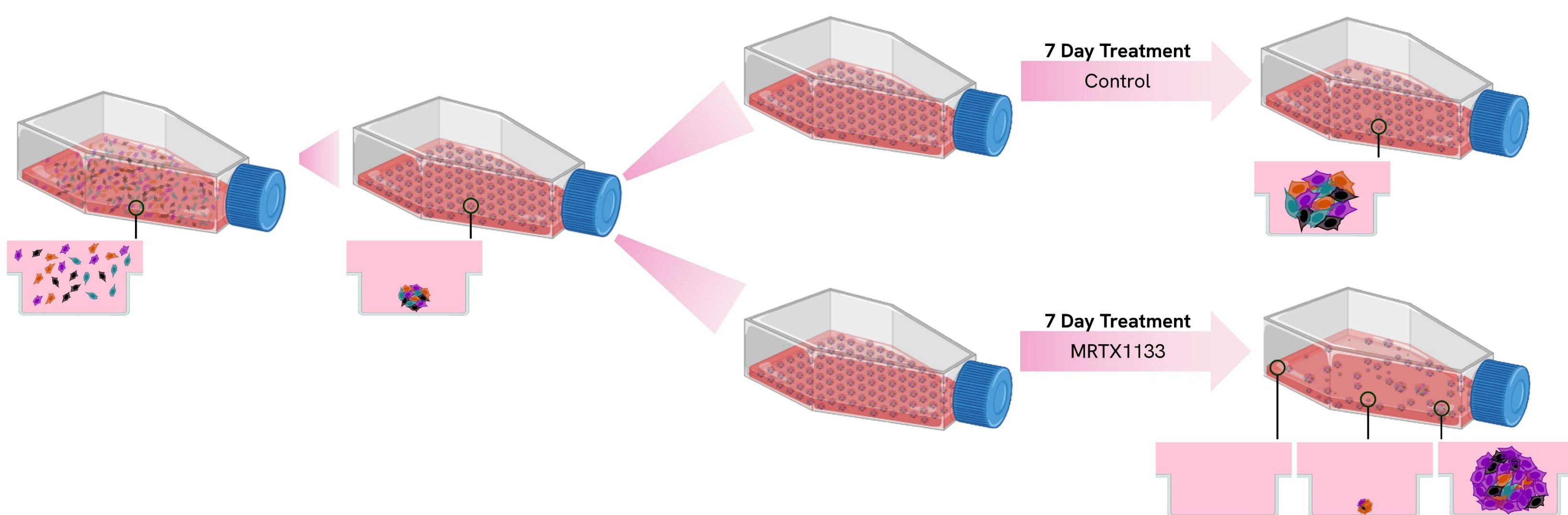


1 Introduction

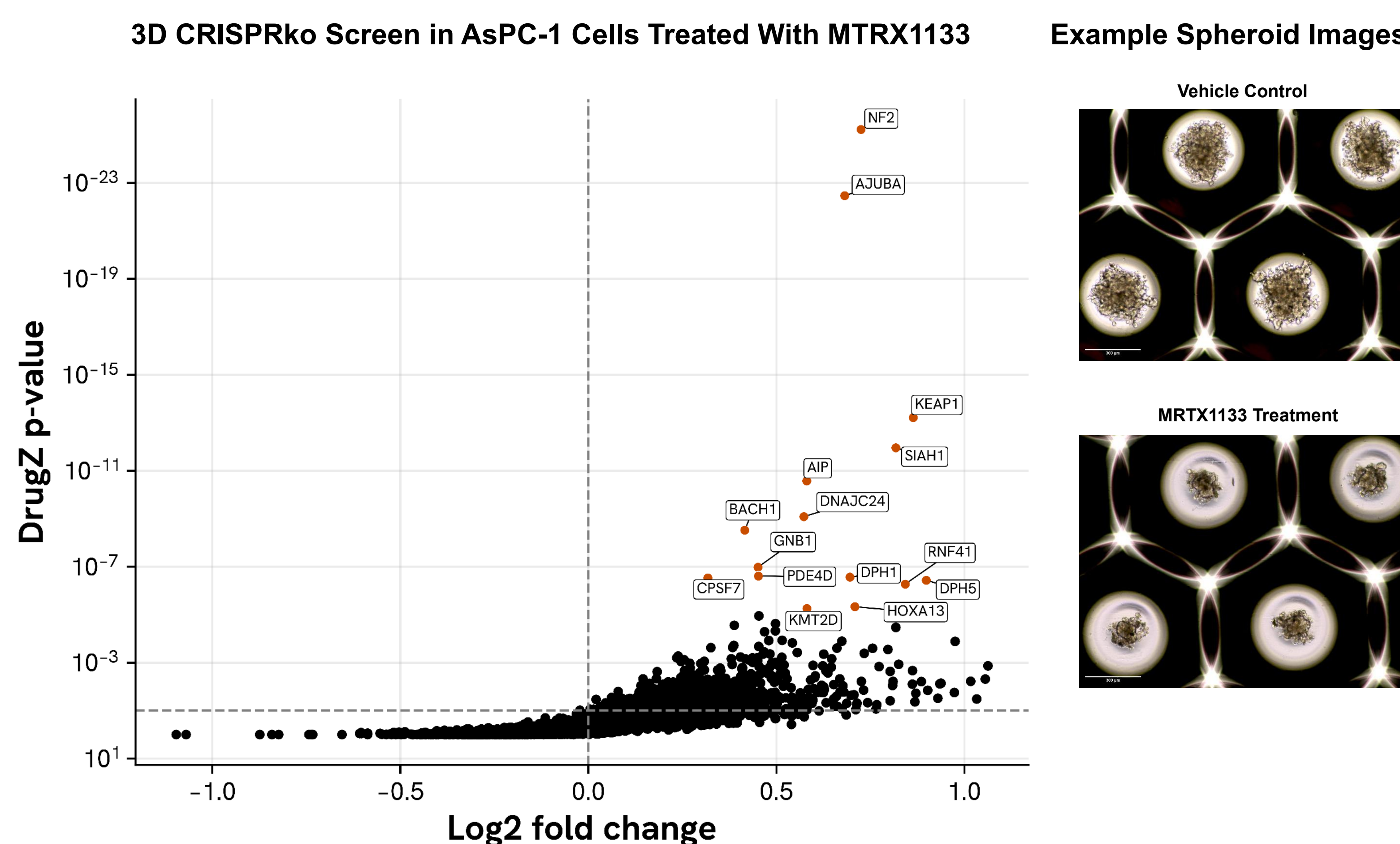
- The advent of targeted RAS inhibitors represents a major breakthrough in oncology, particularly for lung, colon, and pancreatic cancers.
- Clinical efficacy is frequently undermined by the rapid onset of drug resistance.
- This resistance is multifaceted, driven by both genetic alterations and non-genetic mechanisms, including epigenetic reprogramming and signalling pathway rewiring.
- A comprehensive understanding of inhibitor Mechanism Of Action (MOA) and resistance mechanisms can provide valuable insight into the biological processes underlying response to RAS inhibition.
- To address this challenge, we utilised Revvity Preclinical Services Functional Genomic Screening (FGS), Cell Panel Screening (CPS), MERCURIUS™ DRUG-seq with PROGENy pathway analysis and acquired resistance cell models to build a comprehensive data set to holistically understand cellular responses to RAS inhibition.

2 3D functional genomic CRISPR screen identifies mechanisms of resistance to the KRAS G12D mutation-specific inhibitor, MTRX1133



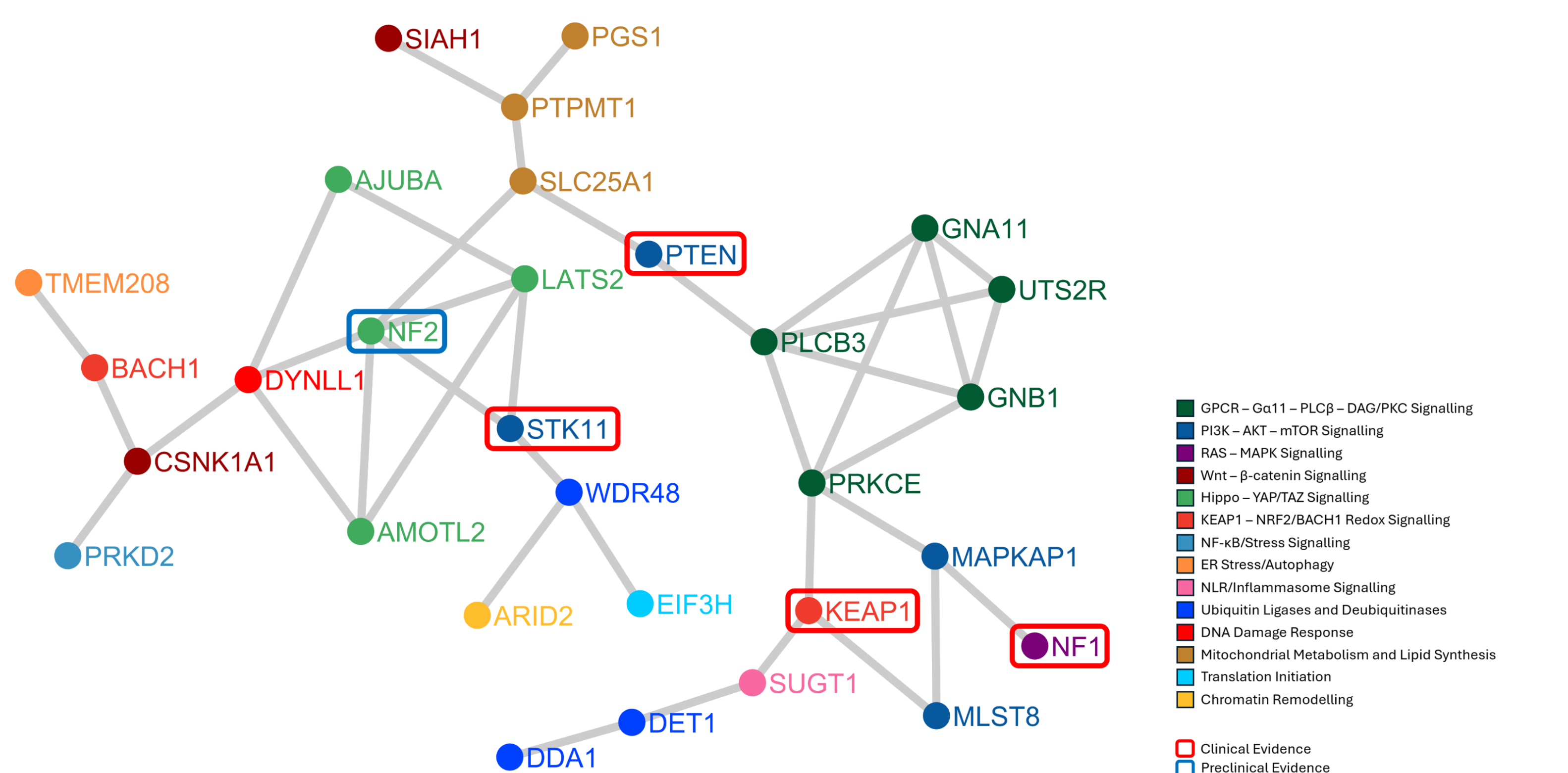
Schematic Diagram of 3D RASi Functional Genomic Screen Methodology - Created with BioRender.com

- AsPC-1 (KRAS^{G12D}) cells were transduced with a whole-genome CRISPRko sgRNA library and cultured in Corning Elplasia® flasks to form 3D spheroids in the presence or absence of MTRX1133 for 7 days to select for resistance inducing perturbations.
- Corning Elplasia® flasks enable the formation of individual, uniform, spheroids, improving consistency across the screen conditions versus traditional ultra-low attachment flasks.



Volcano Plot of MTRX1133 DrugZ Resistance Hits and Representative Brightfield Photomicrographs of Spheroids following 7-day Vehicle Control or MTRX1133 Treatment.

- The DrugZ algorithm¹ was used to identify resistance hits following MTRX-1133 treatment.
- Hits from the 3D screen overlapped with a parallel 2D screen (data not shown) while identifying additional unique hits, enabling deeper network insight into possible RASi resistance mechanisms.
- Unique 3D hits included **AJUBA**, **PTEN** and **STK11**.



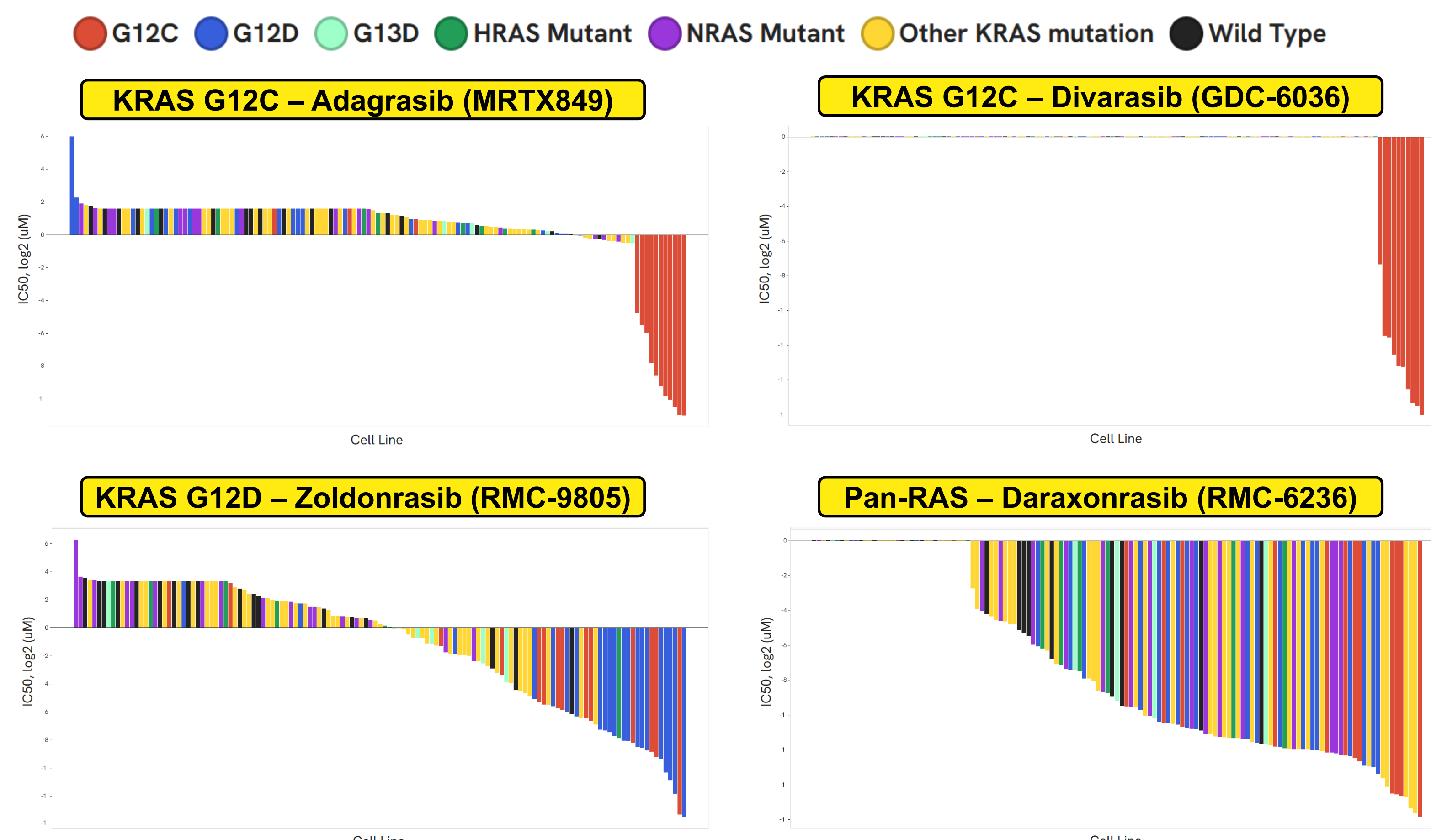
Physical Interaction Network Analysis for Top 100 DrugZ Resistance Hits

- Physical interaction network analysis of the top 100 DrugZ resistance hits mapped genes across 14 RAS-connected signalling pathways.
- Four hits highlighted with red boxes (**PTEN**², **NF1**², **STK11**³, **KEAP1**⁴) are supported by clinical evidence linking their mutation status to poor RAS inhibitor response, acquired resistance mechanisms, and adverse patient prognosis. One additional highlighted hit (blue box) **NF2**⁵ has preclinical evidence for similar associations.

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- Fuse et al, 2019, Preclinical assessment of MEK1/2 inhibitors for neurofibromatosis type 2-associated schwannomas reveals differences in efficacy and drug resistance development. *Neuro Oncol* 21(4):486-497.
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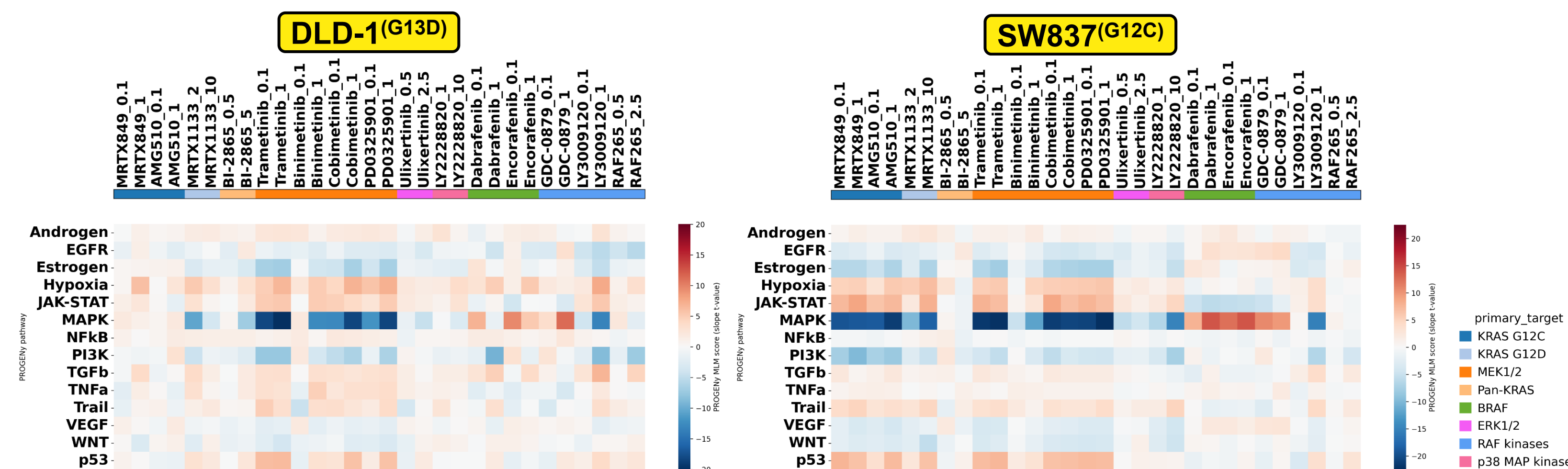
3 3D RAS Cell Panel Screening enables mutation-specific characterization of emerging RAS-targeted therapeutics



Inhibition (IC50) was determined in 5-day spheroid assay from Revvity RAS cell panel. Waterfall plots show rank-order sensitivity.

- G12C inhibitors (MRTX-849, GDC-6036):** Clear selectivity for G12C-mutant cell lines
- G12D inhibitor (RMC-9805):** Primary activity in G12D lines as expected
- Pan-RAS inhibitor (RMC-6236):** Broad activity across all RAS mutants, demonstrating expected lack of allele selectivity.

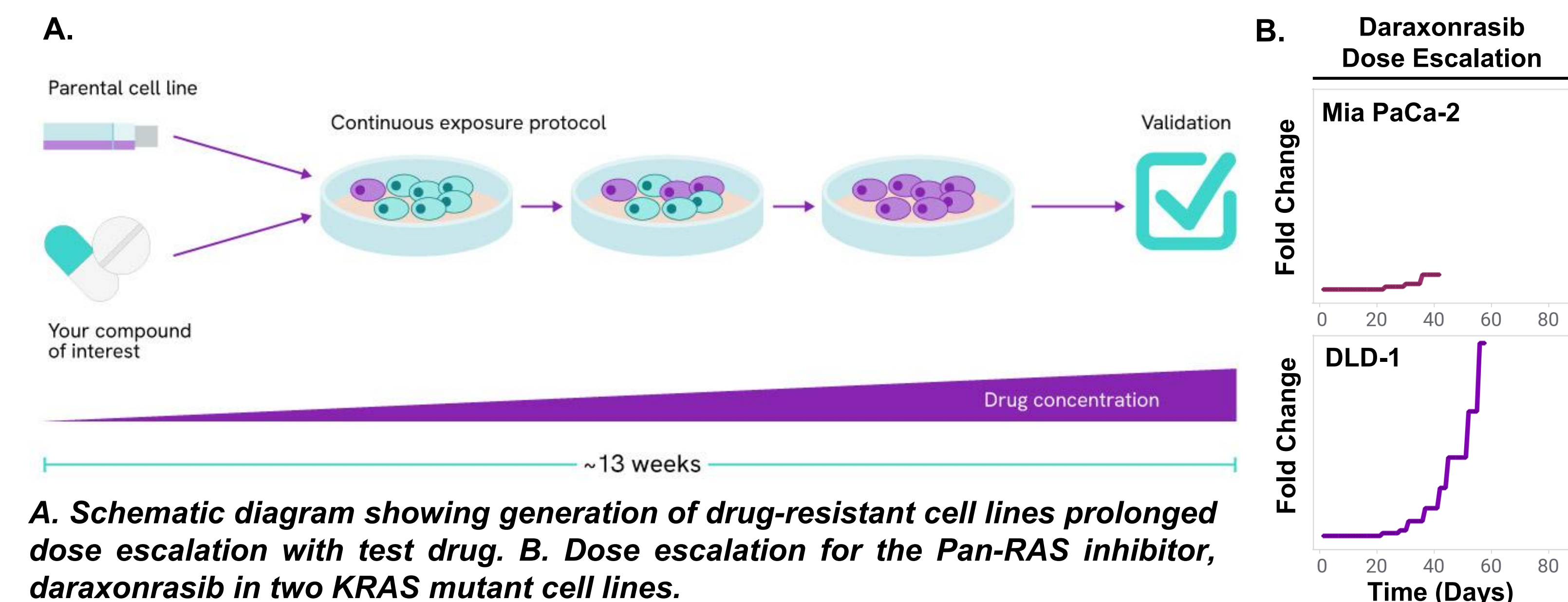
4 MERCURIUS™ DRUG-seq and PROGENy pathway analysis reveals drug class-specific transcriptomic responses to RASi therapeutics



Heatmap showing inferred pathway activity scores (PROGENy Model Likelihood Method, MLM) across 14 RAS-connected signalling pathways in response to 24-hour treatment with seven distinct RAS-targeted therapeutic modalities in two KRAS mutant cell lines.

- KRAS^{G12C} inhibitors show strong MAPK suppression in mutation-matched SW837 (KRAS^{G12C}) but not in DLD-1 (KRAS^{G13D}), confirming allele-selectivity. KRAS^{G12D} inhibitors show weaker MAPK suppression in both cell lines, suggesting modest cross-reactivity of some treatment modalities.
- Paradoxical MAPK activation is observed in both cell lines across BRAF/RAF inhibitors, consistent with well-characterised RAF inhibitor feedback mechanisms in RAS-mutant colorectal cancer contexts⁶.

5 Generating drug-resistance cell line models is a complementary tool to identify mechanisms of resistance



A. Schematic diagram showing generation of drug-resistant cell lines prolonged dose escalation with test drug. B. Dose escalation for the Pan-RAS inhibitor, daraxonrasib in two KRAS mutant cell lines.

6 Conclusions

- 3D spheroid culture in microwell flasks provides a scalable, consistent platform to identify putative resistance mechanisms for RAS inhibitors using pooled CRISPR screening.
- Comparison between 2D and 3D CRISPRko screens revealed resistance hits uniquely enriched in a 3D-specific context, that are supported by clinical evidence for roles in RASi resistance.
- The Revvity Preclinical Services 3D RAS cell panel enables rapid characterization of RAS-targeted therapeutic selectivity and identifies resistance-prone cell line contexts.
- MERCURIUS™ DRUG-seq transcriptomic profiling combined with PROGENy pathway analysis enables rapid characterization of RASi MOA, confirms allele-selective on-target MAPK suppression and identifies off-target cross-reactivity.
- A prolonged dose escalation methodology can generate specific RASi-resistant cell line models, providing a complementary tool for resistance investigation and enables temporal characterisation of resistance emergence.

Combining unbiased genetic screens with deep phenotypic profiling provides a powerful framework for deciphering RASi resistance. This integrated approach elucidates mechanism of action while uncovering genetic and pharmacological vulnerabilities to support ongoing resistance research.