***Project 0:***

*A subset of lung adenocarcinoma tumors harbor a genomic alteration in the ALK receptor tyrosine kinase gene, by which the ALK gene is fused to another gene, leading to constitutive activation of ALK, which is the driver of these tumors. ALK targeted therapy is super effective in these tumors. However, most tumors develop resistance to treatment. Through the performance of a CRISPR screen in ALK-fusion lung adenocarcinoma cell lines, you have identified that inactivation of the STK11 gene may confer resistance to ALK-targeted inhibitors. How would you design a project to validate this and try to come up with a therapeutic approach to prevent or revert resistance driven by inactivation of STK11?*

**Targeting STK11 to overcome resistance to ALK inhibition in lung adenocarcinoma**

Lung cancer is the leading cause of cancer-related death worldwide. *ALK* gene rearrangements occur in ~1-2% of lung adenocarcinomas, and small molecule inhibitors targeting this oncogene have significantly improved the outcomes of patients with *ALK-*rearranged lung cancer. However, responses are often short-lived, and the vast majority of patients ultimately progress to a treatment refractory state for which no effective therapies exist. Identifying mechanisms of resistance may uncover therapeutic targets to reverse, or even prevent, adaptive resistance to ALK inhibition.

To identify drivers of resistance to ALK inhibition, we performed a positive selection genome-wide CRISPR screen in two independent ALK-rearranged lung cancer cell lines. We used the GeCKO v2 library (123,411 gRNAs, six gRNAs per gene) and transduced each of the two cell lines at a multiplicity of infection (MOI) of 0.3, maintaining a library representation of 1000x. Cells were treated with the IC90 of the ALK inhibitor crizotinib for 72 hours. Following treatment, surviving clones were allowed to recover and were expanded off drug for 7 days. Cells were harvested for gDNA extraction, and library amplicons were submitted for sequencing. We identified for further analysis genes for which ≥4 gRNAs were present at a frequency of >5% in each of the two cell lines following selection. Using this stringent criteria, 25 hits were identified for further analysis.

To further narrow the candidate list, we performed a secondary CRISPR screen targeting the 25 hits of the genome-wide screen. We cloned a secondary CRISPR library containing 6 gRNAs per target (total 150 gRNAs) into a lentiviral vector. Four independent ALK-rearranged cell lines were transduced with the secondary library (MOI 0.3, representation 1000) and treated with the IC90 of crizotinib for 3 days. Following expansion of surviving clones, gDNA was submitted for amplicon sequencing. STK11 was identified as the top hit of the secondary screen: 6/6 gRNAs targeting STK11 were enriched in all four cell lines tested, at a final frequency >10%.

The STK11 gene encodes a serine/threonine kinase that serves as a metabolic checkpoint and tumor suppressor gene. It is inactivated in approximately 20% of lung adenocarcinomas. In low nutrient conditions, STK11 phosphorylates the AMP-activated protein kinase (AMPK) and other related kinases to arrest cell growth. Inactivation of STK11 has been associated with resistance to PD1/PDL1 inhibition. However, its role in therapy resistance to ALK inhibition is unknown. We hypothesize that STK11 inactivation drives resistance to ALK inhibition via metabolic adaptations and downstream activation of mitogenic signaling.

**1. Aim 1: Determine whether STK11 inactivation bypasses ALK inhibition via reactivation of mitogenic signaling**

Our genome-wide and secondary focused screens indicate that STK11 inactivation renders ALK-rearranged cells resistant to ALK inhibition. To validate these findings via a targeted approach, we will knockout (KO) STK11 using CRISPR in four independent ALK-rearranged lines (using 2 independent gRNAs to decrease the probability of off-target effects). Single cell derived clones will be expanded and sequenced to derive STK11-deleted lines. As a negative control, cell lines will be transduced with gRNAs targeting a non-coding region of the genome (gRNA control). STK11-KO and control cell lines will be treated with the ALK inhibitors crizotinib or alectinib. Cell viability across a range of drug concentrations will be analyzed using a luminescent viability assay, and the IC50 will be calculated for each line and drug. We predict that STK11-KO cells will exhibit decreased response to ALK inhibition, consistent with the CRISPR screen data.

For in vivo validation experiments, STK11-KO and gRNA control cells will be engrafted subcutaneously into NSG mice, and treated with crizotinib or vehicle (15 mice per group). We hypothesize that STK11 deletion will confer resistance to ALK-inhibition in vivo, leading to increased tumor volume and decreased overall survival in the STK11-KO relative to the gRNA-control group.

We will perform bulk RNA-sequencing to define the molecular mechanisms that drive resistance to ALK inhibition downstream of STK11. STK11-KO and gRNA control cell lines will be treated with crizotinib or DMSO for 48 hours, and RNA will be collected before and after treatment. We will perform differential gene expression analysis in DMSO versus crizotinib groups, and STK11-KO versus gRNA control cells. We hypothesize that STK11-KO cells treated with crizotinib will exhibit increased activation of mitogenic pathways (such as mTOR or MAPK), relative to gRNA control cells. Candidate hits will be validated in Aim 2 by cloning gRNAs into a focused CRISPR screen library, and via pharmacologic interventions (for example, mTOR or MAPK inhibitors).

To validate these findings in a clinically-relevant context, we will prospectively collect ctDNA data of patients currently enrolled in a phase III trial of the novel ALK inhibitor panalkitinib. We will first confirm that STK11-KO lines are resistant to panalkitinib, as shown for other ALK inhibitors. For clinical validation, ctDNA data will be collected pre-treatment (day 0), on-treatment (day 1 of every 28-day cycle) and at progression. We hypothesize that STK11 mutations on pre-treatment ctDNA samples will correlate with decreased progression free survival on panalkitinib. Furthermore, we hypothesize that a subset of patients with secondary resistance to panalkitinib will exhibit acquired STK11 mutations.

**2. Determine whether metabolic adaptations drive resistance to ALK inhibition downstream of STK11 loss**

Clinical strategies to overcome resistance to ALK inhibition are urgently needed. In low-nutrient conditions, STK11 exerts its tumor suppressive effects via phosphorylation (and activation) of AMPK and other related kinases. AMPK in turn regulates metabolic pathways to arrest cell growth until nutrient conditions improve. Given that metabolic adaptations are at least partially responsible for tumor growth in the context of STK11 mutations, we hypothesize that targeting metabolic pathways (to simulate nutrient low conditions) may overcome the phenotypic changes associated with STK11 loss.

To identify metabolic targets that may overcome resistance to ALK inhibition in the context of STK11 loss, we will perform a focused dropout CRISPR screen targeting ~3,000 metabolic genes, including enzymes, transporters and metabolic transcription factors (30,000 total gRNAs) (Birsoy K. et al. Cell 2015). In addition, we will clone into the library gRNAs targeting candidate hits derived from the RNA sequencing data in Aim 1. The metabolic library will be transduced into ALK-rearranged STK11-KO cells (generated in Aim 1), at a MOI of 0.3 and representation of 1000x. To identify metabolic targets that restore sensitivity to ALK inhibition, transduced cells will be treated with the IC50 of crizotinib for 7 days. Cell will be harvested for gDNA extraction and amplicon sequencing. ALK-rearranged cell lines with intact STK11 will be used a control, enabling us to identify drivers of resistance directly linked to STK11 loss. Hits for which gRNAs are depleted in crizotinib relative to DMSO conditions with adjusted p-value < 0.01 (in STK11-KO but not in STK11-intact lines) will be validated using targeted CRISPR knockouts.

To expedite clinical translation, we will identify drugs targeting metabolic hits of the CRISPR screen. Several drugs targeting metabolic pathways are approved for the treatment of diabetes, obesity, and other conditions. Metformin, for instance, inhibits the mitochondrial respiratory chain, resulting in low ATP synthesis and AMPK activation. We hypothesize that inhibition of metabolic targets that result in low ATP synthesis (or that are sensed by cancer cells as low-nutrient conditions, even when ATP levels are normal) may overcome the phenotypic effects of SKT11 loss. Candidate drugs will be first tested in vitro, using four independent ALK-rearranged STK11-KO cells. Drugs that overcome resistance to ALK inhibition will be validated in a subcutaneous mouse xenograft. Mice will be randomized to vehicle, crizotinib, metabolic drug, or crizotinib plus metabolic drug (15 mice per group). Mice will be treated for 4 weeks, and tumor volume and overall survival will be recorded for 3 months. Lastly, we will follow the same approach to validate these findings in a patient-derived xenograt model.

Through these experiments, we aim to understand the molecular dynamics of resistance to ALK inhibition driven by STK11 loss, and to develop therapeutic approaches that directly overcome metabolic adaptations, restrain the expansion of resistant clones, and lengthen treatment responses in patients with ALK-rearranged lung cancer.