Introduction

We appreciate the exceedingly positive and helpful comments from the previous study section and agree that overcoming bypass mechanisms in cancer will be critical for targeted therapy efficacy. An important contributor to this form of resistance is the AXL receptor, and a better understanding of how, when and where to target this and the other RTKs has the potential for considerable impact. For this, we have assembled a team with extensive computational, proteomic, and clinical expertise that would fit well into and benefit the CSBC. The data assembled here will provide a valuable and unique proteomic dataset that will be made immediately available for further analysis. As noted, this proposal is highly innovative on both the technical and conceptual frameworks. The use of a variety of different assays in cell lines and PDX models should provide a comprehensive dataset of immense value to the consortium. The reviewers included a number of minor concerns we wish to address here and with targeted changes throughout our proposal; we appreciate these constructive points and agree these are minor in the context of the overall proposal:

Reliance on cell lines in the experimental approach We recognize that experiments in established cancer cell lines raise concerns about their applicability to human cancers. Although not perfect, cell lines are necessary for the functional data generated by Aims 1 & 2 which aids prioritization of RTK-adapter interactions prior to our PDX studies. **In addition to our cell line functional studies we have proposed critical validation experiments using PDX models, meant to ensure the relevance of our findings.** Through our collaboration, we can also examine human tumor tissues.

Minor concern—use of overexpression In our work with AP-MS we use retroviral vectors, and we find these express receptors to frequently observed endogenous levels. Overexpression is a common misconception since a frequent approach is to use transient transfection of irrelevant cell lines like H293 which produce a huge amount of overexpressed protein and likely induce spurious interactions. In Dr. Haura's recent EGFR interactome paper, western blots show exogenous baited protein was expressed at comparable levels to that observed endogenously[1,2]. We will also verify the expression level we obtain matches endogenous levels observed.

Minor concern—use of RNAi We have adjusted our approach to use CRISPR-mediated knockdown and knockout in parallel, with RNAi as a backup strategy. We also wish to point out that the conclusions of our study are not directly based on the effects of these reagents on cell response. Where these reagents are used for validation of RTK-adapter interactions, we are additionally measuring the molecular consequences of these interventions and ensuring the paired molecular/cellular effects are consistent with our model.

A minor weakness—adequate but not particularly innovative modeling While new algorithm development is not the central focus of this proposal, we note that innovative application of modeling techniques is an essential component. Non-identifiability is a problem throughout high-dimensional data-driven analysis. Too often only the maximum likelihood or average model is explored. As noted in §1.3, we plan to use Monte Carlo sampling of our model's posterior distribution, and then to interpret and validate the predictions of families of models. Particularly for high-dimensional data as collected here, this is an innovative approach that will be critical to the reproducibility and subsequent impact on the field. We have provided greater detail for the modeling throughout.

Dependence on RTK inhibition may be problematic if resistance occurs via multi-RTK co-activation. Dynamics of resistance development & RTK network evolution is not clearly addressed. We concur that multi-RTK dependence may present a challenge. Importantly, in Aim 3, while we will only select pairs of inhibitor treatments to test experimentally, we will have quantitation of RTK activation for a wider panel of receptors. We will therefore be able to identify if, for example, activation of >2 RTKs predicts poor response. The framework presented here will be critical for identifying approaches for targeting RTK co-activation (Fig. 3). Lastly, the studies here lay groundwork for understanding the dynamic nature of resistance development in a more mechanistic fashion, but we also plan to examine each PDX model before and after treatment to understand the PDX evolution.

Unclear how invasion will be worked into the existing dataset We agree that incorporating invasion previously was ancillary to the core goals of this study and have focused our proposal.

Additional preliminary data would help assess power of approach Due to space constraints, much of the preliminary data was included in a manuscript included as an appendix. This study, now published, shows the power of our overall approach for finding key molecular events governing bypass resistance development[3]. We have added some additional data to reduce the risk of this study (Figs. 3, 6, 2A). This data, in combination with the expertise this collaboration provides, gives us confidence in the impact this study will have on our understanding and ability to overcome bypass resistance.

Specific Aims

Combination therapy holds considerable promise for overcoming intrinsic and acquired resistance to targeted therapies but relies on our ability to precisely identify the best drug combination for particular tumors. While immense focus exists on using genomic information to direct therapeutic approach, many resistance mechanisms can also arise from entirely tumor-extrinsic factors within the microenvironment. The receptor tyrosine kinase (RTK) AXL is widely implicated in resistance to targeted therapies such as those directed against EGFR. Regulation of AXL by phosphatidylserine (PS), as opposed to mutation, amplification or autocrine ligand, make identifying the tumors that will respond to AXL-targeted therapy especially challenging[4].

We propose to study both downstream and receptor-proximal signaling during bypass resistance mediated by AXL, and then across a wider panel of RTKs. Integrating these measurements with quantitative modeling will identify the connectivity between receptors, interacting adapters, and downstream signaling events, thereby defining the essential set of signaling network changes required for tumor cell survival in response to targeted therapeutics. We will then apply this understanding by measuring RTK-adapter interaction using proximity ligation (PLA) to predict RTKs driving bypass resistance and test these predictions in a panel of PDX tumors.

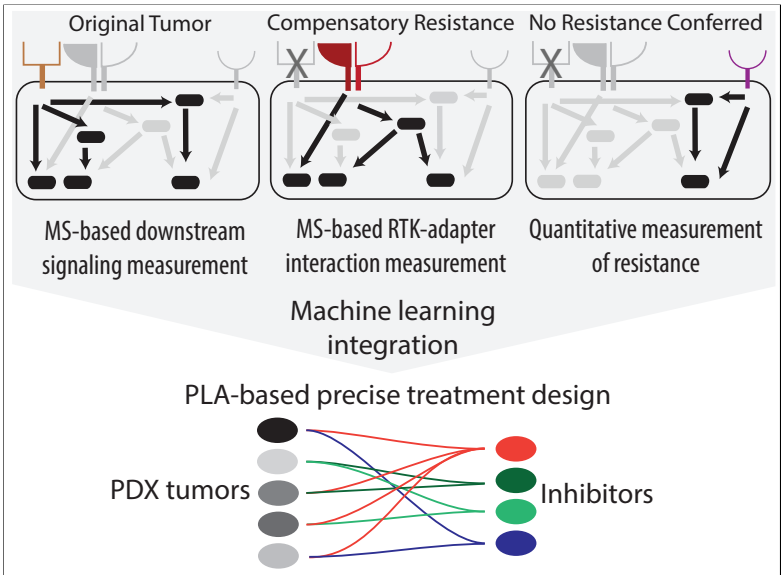

Figure 1: Integrated strategy to map RTK-adapter interactions to promoting resistance. mass spectrometry (MS)-based global phosphorylation and RTK-adapter interaction will be assessed across conditions with variable RTK-mediated bypass resistance. Machine learning will be used to integrate this data and identify critical interactions for measurement to predict combination treatments. These measurements will be assessed for precisely identifying combination therapies in individual patient-derived xenograft (PDX) tumors.

Aim 1: Map the global signaling state changes during switched RTK activation to identify the essential features of bypass resistance *Hypothesis: Context-dependent differences in the resistance-promoting capacity of RTKs can be explained by their downstream pathway activation potential.*

- Functionally evaluate the ability of each RTK and AXL mutant to promote resistance in each cell line model
- Measure the global signaling network state of cells with RTK-induced bypass resistance
- Identify the conserved elements promoting resistance by multivariate statistical modeling
- Validate model predictions of the relationship between signaling network state and resistance

Aim 2: Quantify the corresponding RTK interaction profiles to identify the requisite receptor-level interactions promoting resistance *Hypothesis: Specific RTK-proximal interactions drive bypass resistance-mediated survival.*

- Quantify the complement of receptor-interacting proteins during bypass-mediated resistance
- Correlate adapter interactions and downstream pathway activation to map the downstream consequences of receptor-proximal signaling
- Validate the role of particular adapter interactions in driving therapeutic resistance

Aim 3: Evaluate multiplexed protein interaction measurement as an effective method to predict resistance mechanism *Hypothesis: Coordinate measurement of RTK-adapter protein interactions are more predictive of effective drug combinations than receptor expression or activation levels alone.*

- Validate proximity ligation-mediated detection of selected interactions
- Evaluate the ability of RTK-adapter interaction measurement to identify rare resistant subpopulations
- Determine the predictive capacity of multiplexed interaction measurement for evaluating acquired therapeutic resistance *in vivo* using patient-derived xenografts

This work will considerably improve our ability to identify efficacious drug combinations by: (a) developing a mechanism-based assay for identifying which RTKs are driving bypass resistance, (b) improving our basic understanding of exactly how network-level bypass resistance arises due to activation of non-targeted RTKs both at the receptor-proximal and downstream signaling layer, and (c) expanding our understanding of the RTK AXL with links to resistance, tumor spread, and immune avoidance.

Significance

We propose to study both downstream and receptor-proximal signaling during bypass resistance mediated by AXL, and then across a wider panel of RTKs. Integrating these measurements with quantitative modeling will identify the connectivity between receptors, adapters, and downstream signaling events, thereby defining the essential set of signaling network changes required for tumor cell survival in response to targeted therapeutics. We will then apply this understanding by measuring RTK-adapter interaction by PLA to predict RTKs driving bypass resistance. This information will identify novel points of therapeutic intervention and simultaneously enable predictive resistance mechanism identification in a precise manner.

Overcoming bypass resistance RTKs play a central role regulating cell response to environmental cues during development and homeostasis[5]. RTK dysregulation contributes to a variety of diseases including cancer[6]. RTK-targeted therapies have been successfully applied in cancer treatment to extend and improve patient's lives and are approved for a subset of lung cancer patients. However, the effectiveness of these therapies is always limited by resistance. How resistance occurs varies widely, including through mutation of the drug target to block the effect of therapy, amplification of the drug target to overcome inhibition, pharmacokinetic barriers that block trafficking of the drug to tumor cells, and "bypass" switching to alternative pathways not targeted by therapy[7,8]. In the case of RTK-targeted therapies, many non-targeted RTKs may become activated to provide bypass resistance[9]. Two well-studied combinations are the ability of HER3 to provide resistance to HER2-targeted therapy in breast carcinoma, and the ability of Met to provide resistance to EGFR-targeted therapies in lung carcinoma[10–14]. In each case, the resistance-conferring receptors may contribute to intrinsic or acquired resistance, can become activated by multiple means including ligand-mediated autocrine or paracrine induction, amplification, or mutation, and can effectively be blocked by combination therapy[12,15,16].

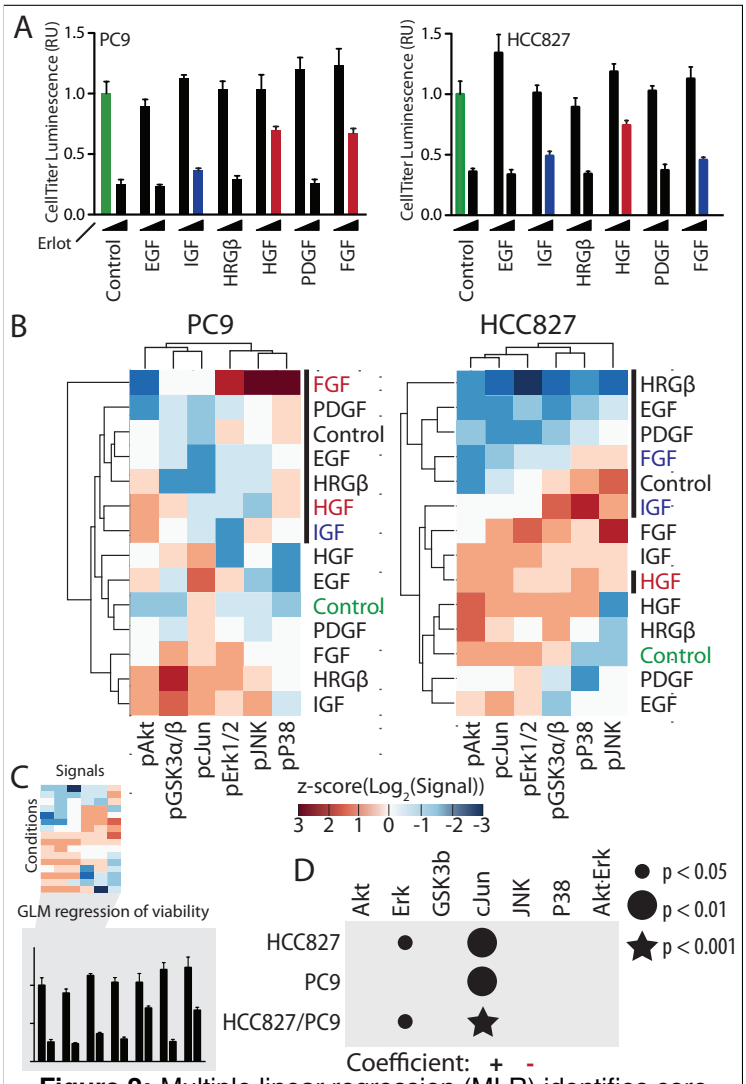

Figure 2: Multiple linear regression (MLR) identifies core bypass resistance pathway components. A) Luminescence-based measurement of PC9 & HCC827 cell viability 72 hrs after treatment with 0 or 1 μM erlotinib and 50 ng/mL of the indicated growth factors (GFs). Bar colors indicate conditions with partial (blue) or full (red) resistance. Error bars indicate SEM of biological replicates (N = 5). B) Phosphorylation measurement 4 hrs after same conditions. C) Schematic of the regression modeling. In order to capture dependencies on coordinate pathway activation, we additionally included an interaction term between pAkt & pErk. Thus, from 6 measurements and a constant term, we regressed 8 input measurements to against viability at 72 hrs. D) MLR models of signaling and viability in each and combinations of the cell lines.

In addition to driving *bona fide* resistance, bypass RTKs can promote "persister" populations of cells for long periods of time[17,18]. These persister populations provide an opportunity for *de novo* resistance mechanisms to arise over time[19]. Importantly, many diverse resistance mechanisms can arise from persister populations, and so targeting these cells upon initial treatment is critical to improving survival outcomes[20].

While bypass signaling is now appreciated as a common mechanism of acquired and intrinsic resistance, exactly what pathway reactivation is essential, and whether it is conserved or varies across cancers and the driving RTK, has not been addressed[10,12,21–24]. RTKs lead to a common set of downstream signals, but in vastly different quantitative combinations. Due to these differences, RTKs differ in their ability to confer resistance[10]. Bypass resistance is generally considered to be the reactivation of some essential set of signaling, often centered around

the Erk and/or Akt pathways, but examples of reactivation not leading to resistance suggest other pathways are also important[10,25]. While appropriate combination therapies will likely provide considerable benefits relative to existing single agents, a more thorough understanding of the signaling networks mediating resistance may reveal targets less susceptible to bypass signaling[26].

Many RTKs can drive bypass resistance, necessitating approaches to identify which RTK is implicated. RTKs work by auto- and trans-phosphorylation, recruitment of adapter proteins, and then phosphorylation of those adapters and other associated proteins. Systems biology approaches have generally concentrated on easily measurable factors such as phosphorylation of downstream pathway components. However, downstream pathway activation measurement lacks information about which upstream receptors are drivers. A recent study from the Haura lab revealed that measuring EGFR interaction with a receptor-proximal adapter protein Grb2 via PLA could predict EGFR inhibitor response better than abundance or activation of the receptor[27]. Such an approach could be applied across receptors to identify which combination is driving tumor cell survival signaling with a better understanding of receptor-proximal RTK signaling and its relation to tumor cell resistance.

Uncovering the contribution of TAM RTK signaling Tyro3, AXL, MerTK (TAM) receptors are a family of RTKs with widespread roles in tumor cell resistance, immune avoidance, and metastasis[15,21,28,29]. The receptor AXL in particular drives resistance to targeted therapies in a wide range of cancers[15,25,30,31]. Resistance through expression and activation of AXL is especially dire as the receptor additionally sustains cells that have undergone epithelial-to-mesenchymal transition and directs tumor cell migration, resulting in potently increased metastatic capacity[15,28,32–34]. Therapies targeting AXL are now in human clinical trials, yet we have a poor understanding of where and when AXL is activated, as well as the molecular pathways engaged to enact phenotypic outcomes[4,35]. TAM receptors are unique among RTKs in that their activity is potently regulated by interaction with phospholipid moieties within the extracellular environment[4,36,37]. Thus, measurements of ligand and/or receptor abundance are unlikely to effectively capture the activation state of these receptors. At the same time, these receptors uniquely include ITIM and ITIM-like domains, and so may have a unique signaling response relative to other RTK families[38]. AXL additionally mediates resistance to PI3k inhibitors in squamous cell carcinomas, and Mek inhibitors in breast carcinoma, so the relevant resistance pathways are unlikely to be simply Erk and Akt[17,39]. A better understanding of AXL signaling in detail will direct approaches to block these deleterious effects.

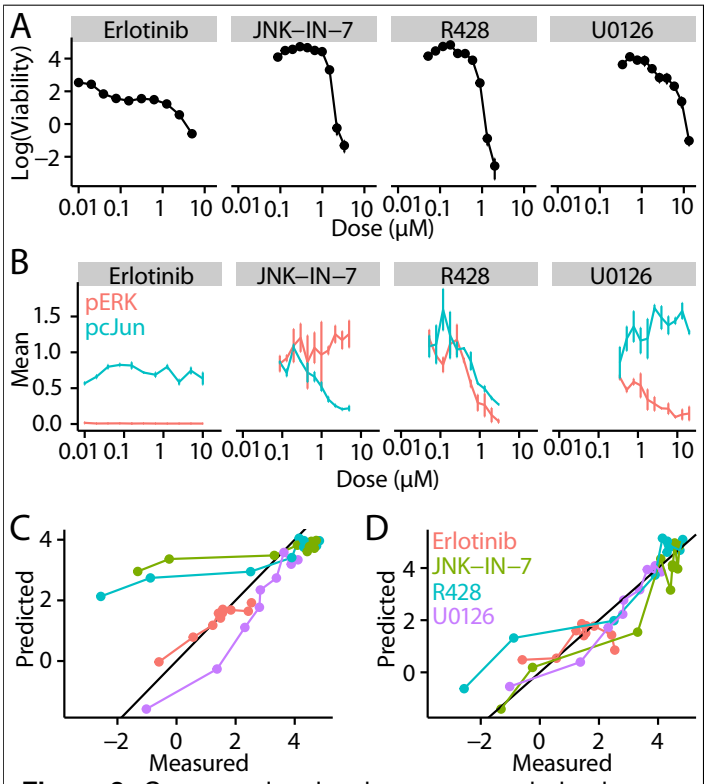

Figure 3: Conserved molecular programs during bypass resistance can capture the effects of RTK co-activation. A) PC9 viability measured by Incucyte cell counts at 72 hrs. B) pERK & pcJun measurement across identical conditions. Both of these measurements were chosen based on the models in Fig. 2. Notably, an EGFR inhibitor (erlotinib) potently inhibits pERK while an AXL inhibitor R428 inhibits pERK & pcJun. C) Regression model prediction of viability using just pERK versus actual values. D) Multiple linear regression model prediction of viability with both pERK & pcJun. Coordinate activation of EGFR/AXL is necessary for PC9 viability via pERK & pcJun.

Addressing the role of RTK co-activation Different receptors seem to show context-dependent abilities to supply resistance, dependence upon transactivation of other receptors, and coactivation patterns. For example, mutant EGFR has been found to transactivate Met, and Met has been observed to activate HER3[12,40]. In each case, signaling from both receptors was important to tumor progression, but for unknown reasons on a mechanistic level. At the same time, RTK co-activation can endow cells with new phenotypes, such as EGFR-mediated invasive ability in the case of AXL expression[34,39]. We hypothesize this is because certain receptors lack the full complement of interactions to provide resistance and that these signals must then come from other receptors. Understanding the receptor-proximal similarity and differences of RTKs on a systems-level will contribute to our understanding for the role of RTK co-activation in resistance (Fig. 3).

Innovation

Bypass resistance to targeted therapies represents a considerable challenge due to the many tumor-intrinsic and -extrinsic factors through which it can arise. Resistance exists on the level of downstream signaling network reactivation, but must be traced back to the upstream changes driving resistance for therapeutic benefit. To integrate these distinct signaling scales, **we have combined multiple cutting-edge analytical technologies to develop a highly innovative, integrated strategy to quantitatively map RTK-proximal adapter interactions to essential signaling network changes driving bypass resistance (Fig. 1).**

In this approach, we will first use a diverse set of bypass resistance mechanisms. By globally interrogating the phosphorylation network by mass spectrometry and then applying machine learning for interpretation, we will identify the critical signaling network changes leading to resistance. We will then map these signaling network changes to RTK-adapter interactions by quantitatively measuring adapter interaction in each bypass resistance condition using tandem affinity purification (TAP)-MS, combined with machine learning approaches to identify the causal interactions driving the implicated downstream signaling events. Lastly, we will apply the results of these earlier efforts, directly measuring RTK-adapter interactions in individual PDX tumors to predict resistance mechanism. **Taken together, this innovative approach uniquely enables one to map RTK-proximal interactions to their resistance promoting capacity, and then apply this information to design treatments in a precise manner.** The experimental techniques–including quantitative MS, PLA, and multiplexed biochemical measurement–along with machine learning approaches, enables this in their combination and is a critical component of achieving this goal.

Two common challenges when interpreting statistical modeling results are (1) sensitivity of the results to parameter choice, such as the number of clusters and (2) model non-identifiability given the data. Both of these can be especially problematic for high-dimensional data such as obtained here, with an extremely large number of measurements over a limited set of conditions. Techniques such as partial least squares regression (PLSR) are generally predictive with such data, but are not prescriptive (i.e. with new measurements of signaling they can predict new conditions, but do not identify the causal factors). **In order to analyze this data in a manner that addresses these limitations, we plan to take an innovative approach for data analysis, using a Bayesian framework with Monte Carlo sampling of our analysis pipeline model posterior distribution.** This approach will allow us to make rigorous predictions despite parameter uncertainty and update our predictions dynamically with new observations from our validation experiments.

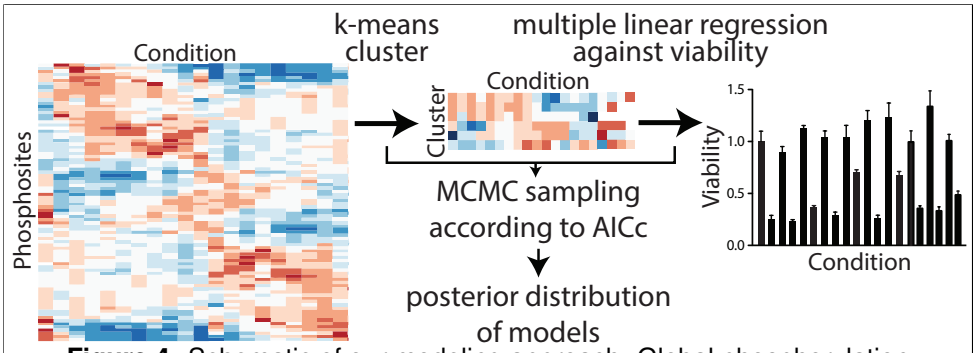

Figure 4: Schematic of our modeling approach. Global phosphorylation measurements will be clustered by k-means and then regressed against viability outcome of the same conditions by MLR. This modeling will provide specific predictions as to the role of phosphorylation clusters in promoting the bypass-mediated resistance of cells. We will use Markov Chain Monte Carlo (MCMC) sampling of the model posterior distribution across cluster sizes to rigorously account for parameter uncertainty and non-identifiability. Thus, we will be left with a family of models, which will provide alternative hypotheses and can be updated with new observations from our validation experiments.

Beyond implicating specific RTK-adapter interactions in bypass resistance, we will take an innovative approach utilizing multiplexed PLA measurements across RTKs to predict resistance mechanism. We will perform these predictions in individual PDX tumors, and then directly test our predictions by designing combination treatments based on our measurements. Each interaction will be selected based upon the earlier work identifying each as mechanistically critical to resistance. The dynamic range of each assay will be optimized to quantitatively identify the resistance being conferred within each tumor by each RTK. These measurements will additionally provide us single cell, spatially resolved interaction measurements. By utilizing this information, we will be able to assess the contribution of spatial and single cell intratumoral heterogeneity and intricately tie it to specific resistance mechanisms. **Critically, this approach is both novel and vital to ensuring the translational value of these findings and maximizing the clinical benefit of targeted therapies in lung cancer.**

Approach

Aim 1: Map the global signaling state changes during switched RTK activation to identify the essential features of bypass resistance

Rationale Cancers frequently acquire resistance to targeted therapies by increasing the activity of one or more alternative RTKs. For example, resistance to inhibition of the epidermal growth factor receptor (EGFR) in non-small cell lung cancer (NSCLC) can arise by compensatory activation of AXL[15]. While studies have identified many RTKs capable of providing resistance to targeted agents, the number of receptors capable of doing so present a challenge for identifying ideal therapeutic approaches[10,41]. This challenge of predictively identifying efficacious therapeutic combinations is compounded by RTK regulation through genetic, expression, and microenvironmental changes[10,42]. We hypothesize that a conserved downstream activation signature governs the development of resistance. Identifying this signature may inform future therapeutic development and diagnostic strategies for interpreting tumor response.

While many mutational and expression changes have been identified that lead to therapeutic resistance, a more unified understanding of resistance from the perspective of the overall signaling network has not emerged[25,43]. Erk reactivation is most commonly quantified during resistance to erlotinib[15], but other pathways are also important to viability and proliferation in the presence of the inhibitor[25]. In principle, some set of cellular signaling states must result in survival and thus resistance, while others result in cell death and thus sensitivity. We hypothesize there is a conserved resistance/sensitivity profile regardless of the relevant driving RTK. If this is the case, examining multiple resistance mechanisms coordinately should reveal an overall signaling network pattern of resistance, informing future therapeutic development.

1.1. Functionally evaluate the ability of each RTK and AXL mutant to promote resistance in each cell line model In order to quantitatively assess the capacity of individual RTKs to mediate EGFR-targeted therapy bypass resistance, and to enable downstream analyses in subsequent Aims, we will stably express a subset of RTKs (IGF1R, FGFR1, ALK, AXL, PDGFRb, cMET), each implicated in mediating bypass resistance, in HCC827 and PC9 mutant EGFR lung carcinoma cells. Ectopic expression will serve to reduce the influence of varied RTK expression on our results, as each receptor studied will be expressed to similar levels and activated by its cognate ligand. Each receptor will carry a TAP tag comprised of both His and Myc tags for analysis in subsequent Aims (Tbl. 1).

#	Construct	Erl	GF
1	GFP-TAP	N	-
2	GFP-TAP	Y	-
3	AXL-TAP	Y	Gas6/PS
4	AXL(KD)-TAP†	Y	Gas6/PS
5	AXL(mut1)-TAP	Y	Gas6/PS
6	AXL(mut2)-TAP	Y	Gas6/PS
7	AXL(mut3)-TAP	Y	Gas6/PS
8	AXL(mut4)-TAP	Y	Gas6/PS
9	AXL(mut5)-TAP	Y	Gas6/PS
10	AXL(mut6)-TAP	Y	Gas6/PS
1	Parental	N	-
2	Parental	Y	-
3	GFP-TAP	Y	-
4	IGF1R-TAP	Y	IGF1
5	cMET-TAP	Y	HGF
6	FGFR1-TAP	Y	FGF1
7	ALK-TAP	Y	-
8	AXL-TAP	Y	Gas6/PS
9	PDGFRb-TAP	Y	PDGF
10	EGFR(T790M)-TAP	Y	EGF

Table 1: Conditions to be measured for global signaling network state. As measuring all 17 AXL mutants would be challenging, we will prioritize a subset of mutant constructs for analysis. We will select mutants based on whether they exhibit differences in our resistance assays as compared to the wild-type receptor. Selecting in this way will maximize our chances of capturing the most salient signaling differences. †Kinase dead mutation K562R. Erl: Erlotinib

While the above constructs will be extremely valuable in determining how bypass resistance occurs via many different receptors, more closely matched mechanisms of promoting bypass resistance will more sensitively identify the key molecular events. For this, we will focus on the RTK AXL, activation of which drives resistance to EGFR-targeted inhibitors, invasion, and metastasis[15]. We have developed a panel of vectors for ectopic AXL expression in which each intracellular tyrosine is mutated to a phenylalanine. As a consequence of these matched constructs, we can attribute variation in bypass resistance capacity to individual phosphorylation events. We will, using retroviral infection, express each of these in HCC827 cells, which have minimal endogenous expression of AXL but can be made resistant to erlotinib through expression and activation of the receptor[15]. We will verify by mutation-specific qRT-PCR that the vast majority of expressed AXL comes from our expression vector, and will use dCas9-mediated knockdown of endogenous AXL if needed[44]. Each receptor mutant cell line will be treated with a mixture of 1 nM Gas6 and PS-presenting vesicles as work from the Meyer lab and others has shown these are critical to activation of AXL[4,36,37].

We will quantify the extent to which each panel of cells are resistant to erlotinib in response to distinct bypass receptors and AXL mutants. To do so, the proliferation of cells with each receptor expressed will be monitored

hourly for seven days by live-cell microscopy within a tissue culture incubator (Fig. 5). Some investigations into AXL-mediated resistance have suggested the receptor is more capable of mediating cell survival rather than proliferation[45]. Similarly, IGF1R activity has been identified in PC9 cells as essential for cell survival but not sufficient to induce resistance[18]. Our approach will allow us to deconvolve whether GF signaling is merely extending cell survival or is indeed allowing for proliferation in the presence of targeted therapy. Quantitation of cell numbers over time will ensure our later analyses are independent of the time point selected for the assay.

1.2. Measure the global signaling network state of cells with RTK-induced bypass resistance For initial analysis, we will treat cells with erlotinib with or without expression and activation of a bypass receptor (Tbl. 1). To define the signaling network response we will quantify protein phosphorylation, using enrichment strategies for different subsets of the phosphoproteome and performing the analysis by multiplexed LC-MS/MS. We will provide an overview of the approach here and note that detailed protocols for each aspect are available, either as separate publications[46–49], supplemental methods to our publications[50–52], or through the White lab website. After 4 hrs, we will lyse cells in 8 M urea. We have selected 4 hours as it is before we observe cell death, but optimally predicted outcome as compared to earlier measurements in our preliminary studies[3]. Lysates will be reduced with dithiothreitol (DTT), alkylated with iodoacetamide (IAA), and then diluted and digested to peptides using trypsin at a 50:1 substrate to trypsin ratio overnight at room temperature. Following digestion, samples will be acidified, desalted, lyophilized and stored at -80°C. For multiplex quantification, lyophilized peptides will be labeled with Tandem Mass Tag (TMT) 10plex mass tag labeling kits (Thermo), with one label per stimulation condition. After labeling, samples will be mixed and lyophilized.

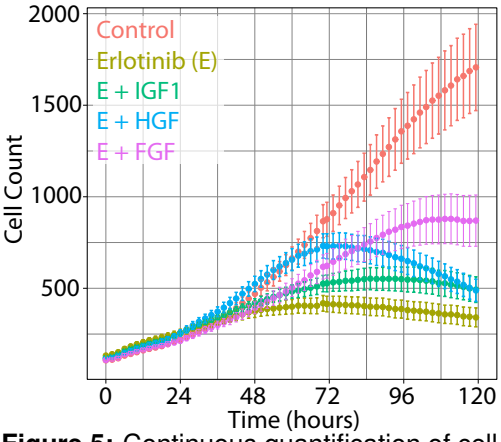

Figure 5: Continuous quantification of cell proliferation response. PC9 cells were treated with erlotinib and different GFs, then monitored for 5 days. GF consumption over long-term culture is a concern, and so we will replenish the media every 48 hrs.

To quantify a wide range of biologically relevant phosphorylation sites, protein phosphorylation analysis will be performed in two steps for each set of labeled samples. (1) Initially, tyrosine phosphorylated (pTyr) peptides will be enriched through immunoprecipitation (IP) with a set of pan-specific anti-pTyr antibodies[53]. Following elution from the IP, samples will be subjected to a second stage of enrichment on an Fe^{3+}-immobilized metal affinity chromatography (IMAC) column to remove non-specifically retained non-phosphorylated peptides. Phosphopeptides will be eluted and analyzed by ultra-low-flow nano-LC-MS/MS on custom microcapillary columns with integrated electrospray emitter tips. Additionally, to quantify remaining phosphorylation site changes throughout the network, the TMT-labeled samples will be separated into 20 concatenated fractions by basic reverse-phase HPLC. Each fraction will be subjected to phosphorylation enrichment by IMAC, followed by quantitative ultra-low nanoflow LC-MS/MS on an Q Exactive Plus orbitrap mass spectrometer. Based on several years of results from application of variants of this protocol to a variety of biological samples, we expect that this approach will yield the identification and quantification of $\sim$500 pTyr sites, and $\sim$10,000 additional pSer/pThr sites across thousands of proteins. This level of network coverage will allow for the in-depth characterization of response to each bypass resistance-inducing receptor, across a wide range of cellular pathways. This general protocol will be repeated for two panels of conditions (Tbl. 1), thereby producing a massive compendium of quantitative signaling data that will provide novel insight into the mechanism of action for cellular response.

1.3. Identify the conserved elements promoting resistance by multivariate statistical modeling With measurement of the phosphorylation (§1.2) and phenotypic response (§1.1) we will build MLR models to determine the relationship between signaling (our global phosphoproteomic measurements) and phenotype (viability in the presence of erlotinib). Two common challenges when interpreting statistical modeling results are (1) sensitivity of the results to parameter choice, such as the number of clusters and (2) model non-identifiability given the data. Both of these can be especially problematic for high-dimensional data such as obtained here, with an extremely large number of measurements over a limited set of conditions. Techniques such as PLSR are generally predictive with such data, but are not prescriptive (i.e. with new measurements of signaling they can predict new conditions, but do not identify the causal factors). The same limitation exists for L1-regularized regression, which can help form predictive models but at the cost of model interpretation upon deconstruction.

In order to analyze this data in a manner that addresses these limitations, we plan to take a new approach

(Fig. 4). We will first utilize k-means clustering to identify sets of similarly regulated phosphosites across our conditions. Phosphorylation site co-variation is able to reconstitute cell signaling networks and identify causal interactions[54–56]. Thus, we expect these clusters to represent biologically meaningful signaling nodes. The averages of these groups will then be used in MLR models, optionally with interaction parameters between clusters (Fig. 2). These nonlinear relationships will be important to understanding the signaling-viability relationship as we expect that pathway dependencies may not be linearly separable (e.g. cells may rely on Akt AND Erk being activated for survival). We will then utilize a Bayesian approach, using MCMC to sample the model posterior distribution across parameter weights and number of clusters[57,58]. (Note that clustering will be performed once for each number of clusters within k-means, but with a posterior likelihood we will be able to combine our sampling for different numbers of clusters.) Model likelihood will be estimated using the second-order Akaike information criterion[59]. We will ensure mixing of our MCMC model through trace inspection, autocorrelation, and the Geweke convergence diagnostic[60]. Through this approach, we will be left with a family of models that perform equivalently well in explaining our measurements, and will have removed our dependence on cluster number by integrating across all number of clusters (Fig. 6).

Once we have assembled a family of models, one challenge will be interpretation of these. We will examine the joint posterior probability distributions for different parameters and the parameter distribution within principle component space for significantly non-zero parameter weights and higher-order relationships. Another challenge, if different cluster numbers provide comparably likely models, is that the clusters will contain different combinations of phosphorylation sites. We expect that in most cases we will be able to match clusters through their profiles and fraction of shared phosphorylation sites. However, if models with differing numbers of clusters provide differing hypotheses, we plan to test both. Validation experiments will be used to construct priors within our modeling effort, in order to further refine our predictions.

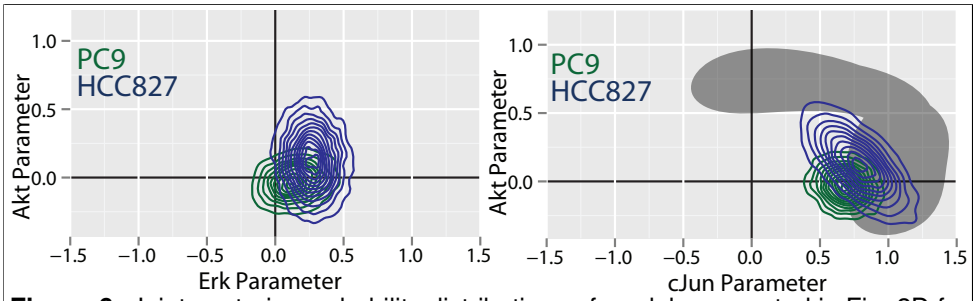

Figure 6: Joint posterior probability distributions of models presented in Fig. 2D for Akt/Erk and Akt/cJun. In this case, there are relatively few phosphorylation measurements and so the model is easily well-specified. Significance in Fig. 2D is for significantly non-zero parameters, which is reflected here in the distribution lying off-axis. However, bivariate dependencies in the model specification exist (e.g. the pAkt vs. pcJun values). This indicates that if, in validation experiments, the pAkt parameter were to be positive, the model would predict a smaller pcJun parameter. Shaded in grey is a likely possibility for higher-dimensional data, where significance for a single parameter may break down. Such a distribution would indicate the model can roughly either weight pAkt OR pcJun. Analysis only looking at the optimal model would miss these competing hypotheses, or would find that the model relies on neither parameter (since neither would be significantly non-zero when examining single-variable marginal distributions). In this way sampling a family of models can help make a set of testable hypotheses in the face of incomplete model specification.

An additional challenge during interpretation and validation of our model will be that we are measuring and modeling phosphosite response, but will likely validate our model through manipulation of kinase activity. To overcome this limitation, we will map the predicted kinase(s) for each phosphosite to the extent possible, and can look for overrepresentation within particular clusters[61]. This mapping step will serve to identify the likely kinases driving the effects observed via a set of phosphosites, using the consequential phosphosites as a readout of kinase activity.

1.4. Validate model predictions of the relationship between signaling network state and resistance In order to validate novel findings from our modeling approach, we will investigate the effects of novel targeted inhibitor combinations and/or knockdown particular proteins and evaluate their effect on viability. Depending upon the results of this validation, we may choose to examine the detailed mechanisms of these novel targets.

For example, in preliminary work examining a targeted subset of phosphosites[3], we have identified JNK activity as measured by pcJun as an important predictor of viability due to bypass resistance (Fig. 2). Using inhibitor combinations across panels of cell lines, we have validated that JNK activity does indeed contribute to lung cancer cell viability (Fig. 7), and is essential in combination with Erk for bypass resistance. This demonstrates that, with a broader set of measurements, this theoretical framework for probing bypass resistance can uncover therapeutically valuable molecular sensitivities.

This validation step will also be essential for distinguishing among multiple competing models. For example, one subset of models may show dependence upon a cluster of known Erk substrates, while another subset of models may replace this dependence with a cluster of known Akt substrates. By examining the effect of inhibitors for each of these kinases, we will be able to distinguish between subsets of models. Importantly, the standard approach of identifying a single model to validate would miss one or both of these competing hypotheses.

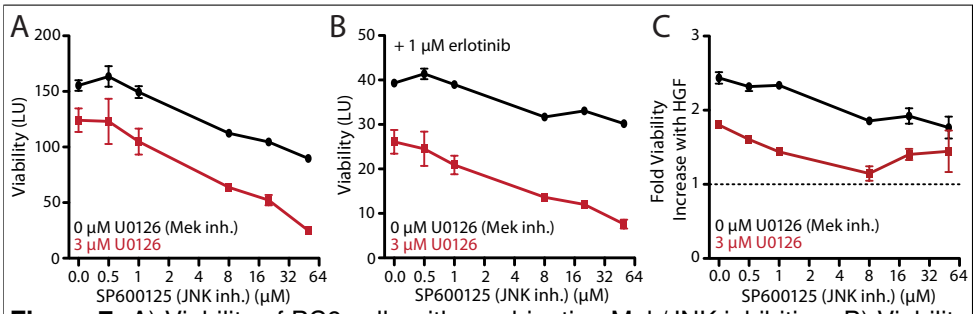

Figure 7: A) Viability of PC9 cells with combination Mek/JNK inhibition. B) Viability of PC9 cells with combination Mek/JNK inhibition in the presence of 1 μM erlotinib. The y-axis is the same as that in (A). C) Measurement of the viability increase conferred by HGF stimulation in the presence of 1 μM erlotinib combined with Mek and JNK inhibition. Two unrelated JNK inhibitors and a panel of three other EGFR-mutant cell lines elicited similar results.

Challenges & Alternative Approaches All of the computational and experimental approaches proposed here are commonplace in the Meyer and White labs, and so we do not anticipate challenges in execution of this Aim.

One concern with this approach is that exogenous AXL expression may lead to non-physiological levels of signaling. We will quantitatively measure the expression levels of AXL after transduction and, if needed, sort for relatively low expressing populations of cells by FACS. AXL is observed to be very highly expressed in many cancer cell lines however, and we expect our expression will be similar to endogenous levels observed[4].

A central assumption of our modeling across different wild-type RTKs is that common changes direct resistance regardless of the driving RTK. If this is not true, this will present a challenge for interpreting the signaling changes that occur in each condition. However, the various AXL mutant receptors will help in this case, since they will be more closely matched in their signaling consequence. Studying AXL-mediated resistance would then also demonstrate an approach that could be adopted to study resistance via other RTKs.

Aim 2: Quantify the corresponding RTK interaction profiles to identify the requisite receptor-level interactions promoting resistance

Rationale While understanding the signaling network changes that lead to bypass resistance is critical to our understanding of how cells overcome targeted inhibition, blocking activation of signaling pathways common to many cell types may not be feasible due to prohibitive toxicity. Inhibition at the level of RTK signaling has shown clinical efficacy both as single agents and combinations with serious but manageable toxicity. However, targeting bypass mechanisms requires that one is able to identify the salient RTK for individual malignancies.

Directly measuring RTK-adapter interaction and identifying the essential interactions that mediate resistance upon RTK activation will be uniquely powerful for identifying which bypass receptor is driving resistance. Different receptors seem to show context-dependent abilities to supply resistance, dependence upon transactivation of other receptors, and coactivation patterns. For example, mutant EGFR has been found to transactivate Met, and Met has been observed to activate HER3[12,40]. Adapter interaction measurement will be uniquely powerful for identifying this complex regulation as concomitant signaling from two receptors can only be deconvolved and compared with RTK-proximal measurements. In each case, signaling from both receptors was important to tumor progression, but for unknown reasons on a mechanistic level. We hypothesize this is because certain receptors lack the full complement of interactions to provide resistance and that these signals must then come from other receptors.

To determine the influence of particular RTK-adapter interactions, it is necessary to examine them within the proper cellular environment. The signaling consequence of RTK activation is the product of multiple factors including receptor abundance, docking affinity, adapter abundance, and competition. One study has looked at whether linear combinations of docking affinities can predict the phosphorylation of relatively RTK-proximal signaling nodes[62]. However, docking affinities address which proteins *can* interact directly outside the environment of the cell, rather than which *do so* in the particular physiological context. As an example of this shortcoming, adapter phosphorylation was often only predicted with combinations of affinities for other proteins included as well, and downstream signals could not be predicted. Lack of prediction may be a result of competition between adapters, or affinities simply serving as RTK identifiers to the modeling effort, rather than mechanistically significant measurements. This previous modeling also cannot take into account differences in stimulation state of a single

receptor, which is well known to often influence phenotypic outcomes[63].

The information obtained here will directly inform the development of predictive assays based on adapter interaction in Aim 3.

2.1. Quantify the complement of receptor-interacting proteins during bypass-mediated resistance In order to obtain a comparison of the receptor-proximal interacting components, we will examine samples after IP of each activated RTK from cell lysates. In conditions matching those in §1.2, we will lyse cells in NP-40 lysis buffer, and IP each TAP-tagged receptor using a monoclonal antibody binding 6xHis. After this IP and washing, we will compete off the interacting complexes with an excess of 6xHis peptide, and bind the elutate to a monoclonal antibody binding the Myc tag. Interacting complexes will again be eluted after washing using an excess of Myc peptide. Elutions will be reduced, alkylated, and then be diluted and digested to peptides using trypsin overnight at room temperature. Following digestion, samples will be acidified, desalted, lyophilized, and labeled with TMT 10plex mass tag labeling kits (Thermo), with one label per stimulation condition. After labeling, samples will be mixed and lyophilized. Peptides will be analyzed by quantitative ultra-low nanoflow LC-MS/MS on an Q Exactive Plus orbitrap mass spectrometer. Control samples either with TAP-tagged GFP or with the expressed receptor inhibited will serve to identify specifically interacting components (Tbl. 2). We will filter for interacting peptides that are at least two-fold more abundant than when the purified receptor is inhibited or GFP as a control is purified. Through polyacrylamide gel separation and blotting, we will quantify His tag abundance in a small fraction of each purified and digested sample as normalization for the amount of receptor. Based on prior experience in the Haura lab, we expect that this approach will identify hundreds of interacting proteins[1].

#	Construct	Erl	GF/Inhibitor
1	GFP-TAP	N	-
3	AXL-TAP	Y	Gas6/PS
3	AXL-TAP	Y	R428
4	AXL(KD)-TAP†	Y	Gas6/PS
5	AXL(mut1)-TAP	Y	Gas6/PS
6	AXL(mut2)-TAP	Y	Gas6/PS
7	AXL(mut3)-TAP	Y	Gas6/PS
8	AXL(mut4)-TAP	Y	Gas6/PS
9	AXL(mut5)-TAP	Y	Gas6/PS
10	AXL(mut6)-TAP	Y	Gas6/PS
1	IGF1R-TAP	Y	IGF1
2	IGF1R-TAP	Y	Lisitinib
3	cMET-TAP	Y	HGF
4	cMET-TAP	Y	Crizotinib
5	FGFR1-TAP	Y	FGF1
6	FGFR1-TAP	Y	PD173074
7	ALK-TAP	Y	-
8	ALK-TAP	Y	TAE684
9	PDGFRb-TAP	Y	PDGF
10	PDGFRb-TAP	Y	Sunitinib

Table 2: Conditions to be measured for their RTK-adapter interaction. As measuring all 17 AXL mutants would be challenging, we will prioritize a subset of mutant constructs for analysis. The selected AXL mutants will be matched to those in Tbl. 1. †Kinase dead mutation K562R. Erl: Erlotinib

2.2. Correlate adapter interactions and downstream pathway activation to map the downstream consequences of receptor-proximal signaling With measurements of downstream signaling (§1.2) driven by measured RTK-adapter regulation (§2.1), we will build regression models of the relationship to identify the relevant interactions driving each downstream signal. As we are most interested in the variation in interacting adapter proteins and how this drives variation in the signaling consequences, we will employ PLSR (Fig. 8). Our choice of modeling approach is also based on our expectation that the variation in adapter interaction and signaling outcome will strongly co-vary between each AXL mutant and bypass receptor. Additionally, our relationship is "many-to-many", in which multiple adapters may influence a downstream signaling node, and multiple signaling nodes may be influenced by a single adapter. PLSR models will be derived with our adapter interaction measurements as input and each signaling k-means cluster average as output considered together. Model robustness and parameter significance will be determined through cross-validation and bootstrap sampling, respectively.

If we are interested in the drivers of single downstream signaling changes, we will employ the approach outlined in §1.3. Being more prescriptive, it may offer more direct predictions of adapter interactions to perturb.

2.3. Validate the role of particular adapter interactions in driving therapeutic resistance Once we have identified the particular RTK adapters driving resistance signaling, we will validate our predictions experimentally. Expression of individual adapter proteins implicated in bypass resistance-associated effects will be disrupted. We will pursue CRISPR-mediated knockout and dCas9-mediated knockdown in parallel, as complete knockout may prove sufficiently toxic on its own to prevent derivation of the required cells[44]. First, we will confirm the expected changes to downstream signaling effects. Where possible with available reagents, we will confirm the signaling consequences by multiplexed ELISA measurements, across a wide panel of phosphosites, to not only confirm an effect on the predicted target, but also specificity among many pathways. Next, we will test whether knockdown of implicated adapter proteins has the predicted effect on bypass resistance of cells, using the assays discussed in §1.1. Finally, predictions will be confirmed across a wider panel of EGFR mutant and wild-type lung carcinoma cell lines.

With this work, we anticipate that we will identify particular RTK-interacting proteins that are essential for resistance driven by that receptor. As a hypothetical example, CrkL may be essential for AXL-mediated resistance to EGFR inhibitors in lung carcinoma cell lines. This would indicate that measuring AXL-CrkL interaction would be especially informative for determining which cell populations are susceptible to AXL inhibition. If we are able to identify such relationships, we will examine other, broader sources of genomic data for further evidence of this relationship. For example, in earlier work we examined whether measuring multiple RTKs might be more predictive of erlotinib response in breast carcinoma cell lines than just EGFR. This work identified that AXL and EGFR expression was more predictive than EGFR expression alone or EGFR with other RTKs[34]. If interaction with particular adapter proteins is essential for bypass resistance through particular RTKs, we will examine whether coordinate expression of the adapter and receptor is more predictive of response than either alone[64].

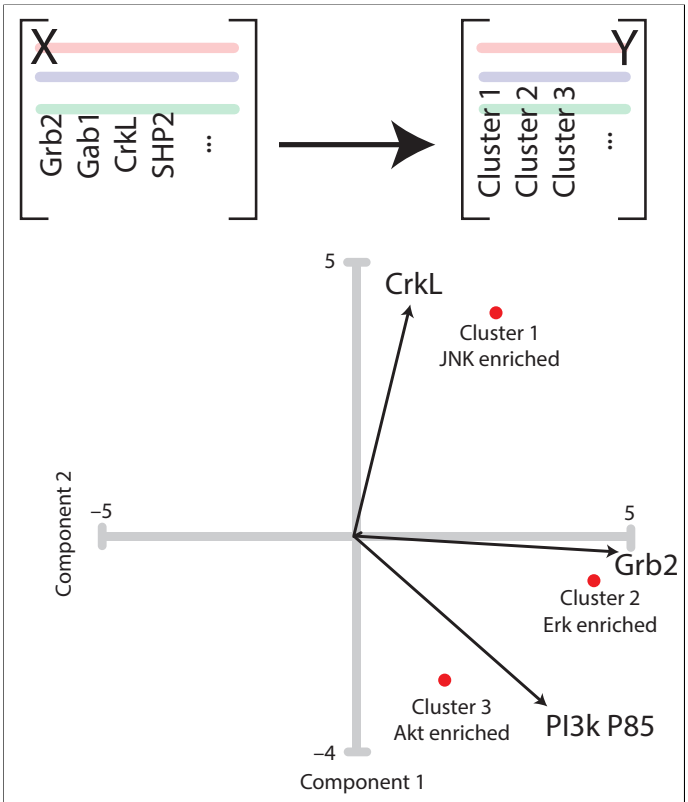

Figure 8: Cartoon of anticipated PLSR modeling results. Colors indicate AXL mutants and/or bypass RTKs. Plot (bottom) shows hypothetical X loadings with arrows and Y loadings with red points. Each cluster is formed from the average of the phosphosites within that cluster. Clusters will be examined for enrichment of likely kinase substrates (see §1.3).

Challenges & Alternative Approaches One concern with sustained knockdown of adapter proteins is widespread cell death, preventing any investigation into the signaling consequences. As an alternative, we will apply transient siRNA transfection, which may provide a period after knockdown but before cell death to analyze the signaling consequences. While aware of limitations of the technology, we will consider shRNA-mediated knockdown of adapters should we have trouble applying CRISPR. If the site of RTK interaction can be identified, it may also be possible to express a mutant version of the RTK lacking interaction with the adapter to study the signaling consequences.

Aim 3: Evaluate multiplexed protein interaction measurement as an effective method to predict resistance mechanism

Rationale Many expression or microenvironmental changes exist that can make tumor cells resistant to a targeted therapy. As a result, we need ways of assessing bypass resistance that are mechanistically based and work across many bypass pathways simultaneously. In this Aim, we will apply our insights from earlier results to evaluate RTK-adapter measurement by PLA as a means of predicting resistance mechanisms. We will utilize well-established *in vitro* models as well as PDX models of therapeutic resistance to determine the predictive capacity of these measurements and accordingly their utility.

3.1. Validate proximity ligation-mediated detection of selected interactions In order to quantify the abundance of particular RTK-adapter interactions, we will employ PLA[65,66] (Fig. 9). In this assay, two monoclonal antibodies are used against interacting targets in fixed samples. Each antibody is conjugated to an oligonucleotide (oligo) probe. After allowing each antibody to bind its target, samples are incubated with two oligos complementary to both probes. If the antibodies are sufficiently close, the complementary sequences align each oligo such that the two non-conjugated fragments are ligated into a single loop. Polymerase is then added to extend one of the conjugated oligo sequences using the circular DNA as template. Finally, fluorescent probe is added to quantify the spots of DNA produced during the rolling circle amplification. Through these steps, the interaction between two proteins can be specifically quantified.

Before using PLA in order to detect RTK-adapter interactions, we will first need to validate that each antibody we use is specific for the receptors and adapters of interest[27]. The adapters we examine will depend upon our analysis in Aim 2. First, we will verify specific labeling by immunofluorescence (IF) in cells with and without knockdown and ectopic expression of each adapter and receptor. Next, we will stimulate cells with the cognate GF for the RTK of the pair, or inhibit the receptor with a specific inhibitor (Tbl. 3), and verify that we observe low

background levels of PLA in the absence of receptor activity, as well as an activation-dependent increase in PLA signal. The background for PLA assays is likely proportional to the abundance of each target, and so this second test will verify that our assay specifically quantifies active complex. We will only proceed with antibody pairs that satisfy all criteria.

Next, we will optimize each assay for the appropriate quantitative range to evaluate the relationship between PLA signal and cell viability. Each cell line with a tagged resistance-promoting receptor will be treated with increasing amounts of inhibitor for the receptor, and viability will be assessed. For each inhibitor concentration, PLA signal will be assessed while varying the dilution of each antibody independently. The optimal dilution chosen will both maximize the range over which inhibitor dilutions can be resolved by the PLA assay, and maximize the variation in cell line viability explained.

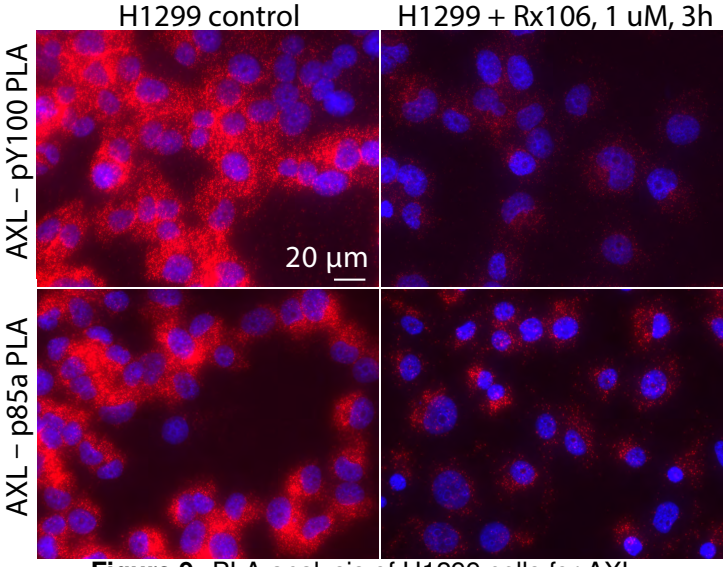

Figure 9: PLA analysis of H1299 cells for AXL phosphorylation (using AXL and pTyr antibodies, top) and AXL-p85a interaction (bottom), in the presence of DMSO or a specific AXL inhibitor. The Haura lab has extensive experience developing and applying these assays in clinical samples[27].

3.2. Evaluate the ability of RTK-adapter interaction measurement to identify rare resistant subpopulations While resistance occurs in part through intrinsic molecular network regulatory changes throughout a tumor, it also occurs through an evolutionary selection process between single tumor cells in the context of tumoral heterogeneity[12,33]. This process dictates that small subpopulations within a tumor can result in considerably different survival outcomes for patients. As a result, measuring average tumor properties may have limited predictive capacity for resistance after selection driven by a targeted therapy. The single-cell results produced by PLA thus might provide valuable information about subclonal populations before tumors are overall resistant.

To test the potential for single-cell PLA analysis to identify resistance subclones, we will employ a dilution strategy with cell lines of known resistance mechanism. First, we will verify that PLA measurement is able to faithfully measure subpopulations of cells with ectopic AXL expression and activation[15]. These stable clones will be diluted at known ratios into parental cells. These mixtures will then be fixed and stained, and the ratio of AXL-mediated resistant cells will be determined by a blinded experimenter. In order to ensure that our ability to distinguish bypass-dependent subpopulations is through measurement of the receptor activity, rather than just an increase in AXL abundance, we will perform the equivalent experiment with dilution of AXL wild-type expressing cells into cells expressing a kinase dead form of the receptor. Separately, by staining for the Myc tag by IF, we will be able to assess the fidelity of assessing PLA in heterogeneous populations of cells in a supervised fashion.

Drug	AXL	EGFR	ALK	cMET	FGFR	PDGFR	IGF1R
R428	Y	-	-	-	-	-	-
Erlotinib	-	Y	-	-	-	-	-
TAE684	-	-	Y	-	-	-	-
Crizotinib	Y	-	Y	Y	-	-	-
PD173074	-	-	-	-	Y	-	-
Sunitinib	-	-	-	-	-	Y	-
Linsitinib	-	-	-	-	-	-	Y

Table 3: Inhibitors to be used in xenograft studies. Crizotinib's polypharmacology will be considered when interpreting results.

3.3. Determine the predictive capacity of multiplexed interaction measurement for evaluating acquired therapeutic resistance *in vivo* using patient-derived xenografts To directly assess the value of multiplexed interaction measurement for predictively determining drug combinations over RTK abundance or activation, we will utilize a panel of RTK-mutant lung cancer PDXs (Fig. 10). These tumors are created by direct transfer of primary tumor and stromal tissue from patients, and effectively recapitulate intertumoral heterogeneity and its effect on response[67]. They retain phenotypic traits of the original tumor and can be serially passaged in mice, allowing for matched analysis of molecular features and therapeutic response[68]. We will include 24 PDX models in our analysis, sufficient for power at 0.8 at a significance level of $p < 0.05$ given a moderate effect size (f = 0.34, balanced one-way ANOVA).

First, from each xenograft, we will obtain tissue sections, tissue lysate, and serum samples. Our inclusion of serum is based on recent work which identified that proteolytically shed receptor can be detected in peripheral blood, and responds dynamically to changes in RTK activity[69]. By quantitative ELISA, we will measure the

abundance of a wide RTK panel (EGFR, HER2, HER3, HER4, ALK, FGFR1, PDGFRb, cMET, IGF1R, AXL) in both tissue lysate and in serum in order to determine the most abundant receptors in each. We have employed this Luminex-based quantitation strategy extensively in clinical samples with highly quantitative and sensitive results[69,70]. By using a pTyr detection antibody along with the same capture antibodies for the RTK ELISA, we will quantify activated receptor within the tissue lysate. Tissue sections from each sample will be quantified by PLA using the optimized strategies from above.

The two RTKs with the most abundant signal will identify the inhibitor combinations (Tbl. 3) to be used according to each strategy. Thus, for each xenograft, up to four combination treatments of inhibitor pairs will be applied (strategies may identify the same inhibitor pair). Animals will be randomized into treatment groups when tumors have reached 100 mm^3, and treated with oral dosing daily until day 21. Tumor volume will be measured twice weekly and calculated according to $\frac{1}{2}(w \cdot h \cdot l)$. Animals will be euthanized on day 28, or when tumors have reached 2000 mm^3.

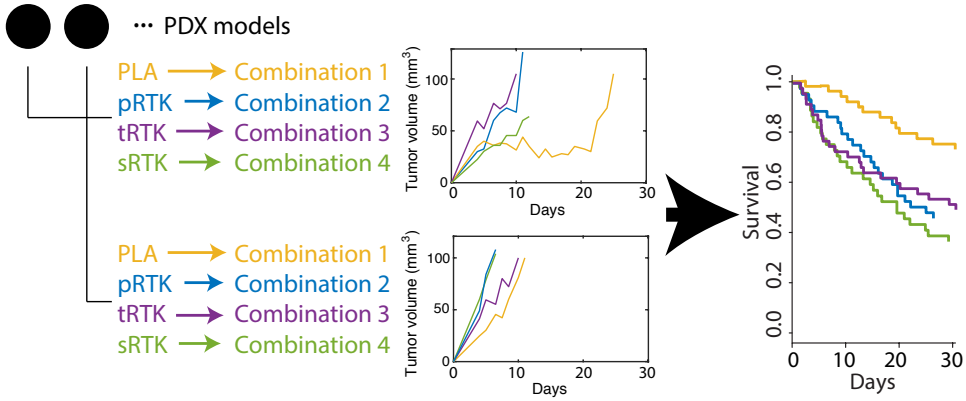

Figure 10: Individualized trial strategy to evaluate the efficacy of each precision therapy approach. Combination therapies will be selected based upon four different strategies. The response of each combination across each PDX model will be evaluated. By this strategy, each diagnostic approach will be evaluated in matched samples.

Predictive regimens (PLA-based, pRTK-based, etc) will be compared by examining response across PDX tumors and their matched combinations. Progression-free survival will be calculated as the time until tumor doubling after the onset of inhibitor treatment. Significance for a difference in progression-free survival will be calculated by a log-rank two-sample test, and response by balanced one-way ANOVA.

After sacrifice, each measurement of RTK abundance, activation, and adapter interaction will be performed on the outgrowth tumors. We will use the pre-treatment single-cell PLA measurements to determine whether subclonal populations with activated receptors not targeted by inhibitor predicts the driving receptor upon resistant outgrowth. Through the tissue bank at Moffitt, we will be able to compare our measurements to those of patients who have been treated with EGFR inhibitors, to examine whether we can identify similar subclonal outgrowth in clinical samples.

Challenges and Alternative Approaches Identifying specific antibodies for each protein target will likely be one of the largest challenges to this effort. To ensure this does not delay our efforts, we will begin this as soon as is feasible. Our strict guidelines for validation will help to prevent setbacks later in this Aim. Notably, four antibody pairs–EGFR-Grb2, MET-Grb2, ALK-Shc1, and AXL-p85–have already been validated by these criteria in the Haura lab[27]. Two additional antibody pairs EGFR-Gab1 and EGFR-Shc have been validated in the White lab[52].

Although we will be examining bypass resistance in a multiplexed manner, and thus look across a set of possible resistance mechanisms, we will be far short of covering all RTKs. Thus, in validation of our assay in cases where the exact resistance mechanism is not defined, the possibility exists for bypass resistance we are not capturing due to our limited coverage of receptors. In this case, one option available to us will be to IP individual RTK-interacting proteins, and quantify the receptors that are pulled down preferentially in the resistant condition. This would potentially allow us to identify underappreciated RTK bypass-mediated resistance mechanisms, and identify how we might improve our assay by wider coverage across receptors.

While selecting the most active two targets by PLA should identify the ideal inhibitor combinations to administer, this selection strategy may not be fully predictive of the resulting response. For example, if more than two RTKs are fully redundant in providing survival signaling, two inhibitors may be insufficient. Our multi-faceted measurements of RTK abundance, phosphorylation, and adapter interaction matched to responses with inhibitor combinations will provide a valuable data set for testing hypotheses for predicting response to inhibitor combinations. For example, PLA signal for interaction with the targets of an inhibitor combination may favorably predict response. However, a lack of PLA signal for interaction with other bypass receptors may be just as informative for predicting that the inhibitor combination will elicit a response. We will make this set of individual measurements and responses available for others and use modeling to interrogate hypotheses such as this.

Glossary

DTT dithiothreitol. 7

EGFR epidermal growth factor receptor. 6, 13

GF growth factor. 3, 7, 11

IAA iodoacetamide. 7
IF immunofluorescence. 11, 12
IMAC immobilized metal affinity chromatography. 7
IP immunoprecipitation. 7, 10, 13

MCMC Markov Chain Monte Carlo. 5, 8
MLR multiple linear regression. 3, 5, 7, 8
MS mass spectrometry. 2, 5

NSCLC non-small cell lung cancer. 6

oligo oligonucleotide. 11

PDX patient-derived xenograft. 2, 5, 11–13
PLA proximity ligation. 2–5, 11–13
PLSR partial least squares regression. 5, 7, 10, 11
PS phosphatidylserine. 2, 6
pTyr tyrosine phosphorylated. 7, 12, 13

RTK receptor tyrosine kinase. 2–6, 9–13

TAM Tyro3, AXL, MerTK. 4
TAP tandem affinity purification. 5, 6, 10
TMT Tandem Mass Tag. 7, 10

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