Rationale for omitting post-doctoral training

A postdoctoral fellowship is a valuable period to learn how to secure funding, diversify one's training, begin managing a laboratory environment, and establish oneself as an independent investigator. My uniquely independent graduate career, however, has engendered many of the leadership and planning skills developed during a postdoctoral fellowship, provided the basis for a robust research program, and formed a scientific network of support to develop new skills as necessary. I am ready to be an independent investigator and am committed to a career in basic research studying fundamental questions in cell signaling regulation using new approaches to examine basic biology and computational techniques.

The past five years as a graduate student training under Doug Lauffenburger and Frank Gertler at MIT have given me a unique breadth of both engineering and biology knowledge, such that my experience will not restrict me to work in a similar area to them, and I am prepared to develop a distinct research program. My thesis work started with a project evaluating the cell migration models used in an *in vitro* context, teaching me the core methods of cell migration research, including live-cell tracking, image analysis, and quantitative microscopy methods. Interesting results from this first pursuit led me to develop a deep understanding of biochemical methods to interrogate cell signaling, and expand my knowledge of both RTK signaling and data-driven modeling methods to understand how activation of TAM receptors *in trans* contributes to migratory responses. This work also explored new methods of measuring RTK interaction and localization, and is the basis for some of the proposed methods herein. Now, recognizing we need a better understanding of exactly how TAM receptors become activated, I have been using methods from chemical engineering to assemble a quantitative kinetic model of TAM receptor response to ligand. I have a set of both computational and experimental biological and engineering knowledge truly unique in combination, which will allow me to carve a scientific and career niche. Indeed, in a way, I have completed a postdoctoral fellowship; I might have completed my thesis based on my initial migration work within the Gertler laboratory, then moved to the Lauffenburger laboratory to apply my cell migration expertise and engineering methods to study AXL signaling as my postdoctoral fellowship.

Others also already see me as an independent investigator. Having successfully worked in an area new to both my advisors and myself, my graduate studies challenged me to identify the pressing questions within a field, design the necessary project to answer those questions, and acquire the skills to carry out the project with considerable autonomy. I have been the motivating force during these projects, have proactively taught myself the necessary biology and computational methods, and designed every day-to-day experimental detail. I have independently sought out and worked with collaborators, and managed distribution of larger projects into those of feasible scope for undergraduate researchers or collaborators working under my supervision. As an expert in TAM receptor signaling, potential industry partners such as Pfizer, Teva and Merrimack Pharmaceuticals have approached me to talk about our research findings and the consequences on relevant drug development. I discuss research, plan experiments, and communicate with collaborators and associates such as Jennifer Cochran (Stanford), Kevin Janes (University of Virginia), Rebecca Carrier (Northeastern University), and Shelly Peyton (University of Massachusetts, Amherst) with essential autonomy.

I not only completed my thesis project but funded nearly all of it. I have secured graduate fellowships through many generous organizations, and have successfully acquired funding requiring full research proposals through the Department of Defense's Breast Cancer Research Program and the Frontier Research Program at the Koch Institute. The former proposed to look at 3D breast carcinoma cell migration more quantitatively and resulted in my first thesis publication examining cell migration models[1]. In the second I proposed to look at AXL signaling as a driver of cell migration, work included in my later publications[2,3]. As the PI for each of these awards, I carried out all aspects of the proposed projects, filed the annual reports, and communicated with the funding organizations.

I have considerable experience managing a laboratory environment. I mentored six undergraduate students who contributed to my thesis work, and during the later years of my thesis work also mentored graduate students. I manage much of the advanced equipment within the laboratory, and this responsibility means I routinely train students, oversee use, and work with vendors, which has challenged my time management and delegation skills. My laboratory responsibilities mean I help purchase new equipment and advise users as to the best approach for experiments.

A traditional postdoctoral position would not be detrimental to my career, but would insufficiently challenge me to develop as a scientist. Completing another project with the same responsibilities would

preclude both development of a research program of my own and demonstration of my ability to lead such a group. Thus, for the reasons above, I am eager to utilize this award to establish a unique research program.

Personal/career development plan

Strengths and weaknesses, and how they will be developed/addressed during this training period. My tenure during this award will be extremely valuable to develop the distinct requisite strengths to lead an independent research group. I am highly proficient at identifying important and interesting problems, then assembling the knowledge needed to develop a set of relevant testable hypotheses. From there, a particular strength of mine is applying the right methods to most directly and definitively challenge each hypothesis. This is a skill I plan to further develop during the award, with the help of and also benefiting personnel working with me who also require these skills as scientists. I am adamant about developing focused, well-defined projects, which will be a strength of mine when managing multiple personnel.

While I am confident that I am ready to take on an independent project, it is still true that, coming straight out of my thesis work, I necessarily have fewer years of experience doing science. As a result there are likely edge-cases and uncommon events that will arise that senior researchers have seen before but I have not. A particular value of this award is when these new challenges are presented, I will have highly experienced mentorship support within the Koch Institute to provide advice.

How will receipt of this award accelerate entry into an independent research career? This award, in contrast to a postdoctoral fellowship or directly entering a tenure track position, will allow me to concentrate on managing a small group extremely well, performing exemplary research, and preparing to apply for tenure track positions, without the distraction of obligations created by being part of a larger group or having teaching responsibilities. It will allow me to concentrate on development of the skills I need to lead an independent research group, beyond those developed during a postdoctoral fellowship. At the same time, the work performed during this time will demonstrate my proficiency as an independent research scientist most distinctively. While I have managed a number of undergraduates over the past five years, my responsibilities will be different during this award period. I will need to create well-defined research areas that can be carved out in the future for postdoctoral fellows or graduate students, and learn how to delegate both technical and creative responsibilities. With this focused base of expertise and a demonstration of my abilities, this award will uniquely prepare me to apply for a more permanent independent research position and additional sources of support.

Career path if Early Independence Award is not provided. Should I not receive this award I plan to pursue established independent fellowship positions such as those at the Whitehead Institute, Rowland Institute, and University of California, San Fransisco. As an alternative to those positions I have been offered the opportunity to undertake a short postdoctoral position while applying directly to tenure-track positions.

Evidence of training ability and leadership

Over the past five years I have had continual experience training, teaching, and mentoring undergraduates, visiting scientists, and new graduate students and postdoctoral fellows. I mentored six undergraduate students within my own research project, training them both in good science practices generally and the techniques used in my research specifically (Table 1). Individual students had very different starting experience: [redacted], [redacted] and [redacted] had done considerable research before and wanted to broaden their expertise; [redacted], [redacted] and [redacted] had never been in a laboratory before but were enthusiastic learners. I am a hands-on mentor, always in the lab with my students, but careful to not micromanage. Higher-level design and interpretation of experiments is perhaps one of the most important skills in science, and I am careful my students do not become an extra set of hands. Each student made immense progress in their understanding of the science and their ability to contribute to the project. [redacted] and [redacted] made essential contributions to the live-cell tracking assay in one of my manuscripts and became coauthors[1]. [redacted], after some necessary troubleshooting, cloned mutants of the AXL receptor, introduced them into cells, and then assayed the receptor properties, and will be a coauthor on a forthcoming manuscript. I have enjoyed seeing my students progress in their careers—[redacted] is now a Ph.D. candidate in the Biological Engineering department at MIT, [redacted] is applying to medical schools, [redacted] is a scientific consultant and planning to return to graduate school, and [redacted] helps with the scientific discovery process at a law firm. Outside of my own project, as the engineer of my biology advisor's lab and the biologist of my engineering advisor's lab, fellow graduate students and postdoctoral fellows routinely consult me for help with assay development, data analysis, and questions of biology. This occasionally has expanded into full-fledged collaborations, such as with a fellow graduate student Miles Miller into what became a publication[2]. Outside of MIT, I often help scientists learn some of the cell migration assays we have developed, such as Shelly Peyton's laboratory at University of Massachusetts, Amherst, and Rebecca Carrier's laboratory at Northeastern University.

Period	Mentee
2010	[redacted], UPitt BE
2010-11	[redacted], MIT Biology
2010-12	[redacted], MIT Biology
2011-13	[redacted], MIT BE
2011-12	[redacted], UPR BE
2013	[redacted] MIT BE

Table 1: Undergraduate mentoring experience. (BE: Bioengineering)

Host institution interactions

Arrangements with host institution The Director of the Koch Institute (KI), Tyler Jacks, enthusiastically supports the possibility of me launching an independent research program. Upon receipt of this award I will receive the necessary laboratory space for members of my lab and myself. I will have access to all of the core facilities within the KI, including mass spectrometry, sequencing, biopolymer synthesis, and microscopy facilities, and will have administrative support identical to that of a junior faculty.

While I will have complete autonomy over my own projects, personnel, and funding, I will receive mentoring from other faculty members at MIT like a junior faculty member. These mentors will provide career development, management, and scientific advice, through a formal process of feedback and evaluations as well as when requested, and will be available to review funding applications. While I of course intend to continue scientific collaboration with my current advisors Douglas Lauffenburger and Frank Gertler, my future mentors will be other members of the KI faculty to help me establish independence. The research I propose is also distinct from my current mentors, and I do not anticipate difficulties establishing career and scientific independence.

Integration within the KI and MIT Upon receipt of this award I will be integrated into the KI community like a new junior faculty member and will attend KI faculty meetings and annual retreats. The KI has a unique integration of biology and engineering-focused labs, and an essential part of this interaction is continual discussion between labs with complementary interests. My lab will have full participation in floor meetings, KI seminars, and annual retreats. I will continue collaborations I currently hold within the KI but also seek out additional mentorship through others with similar interests.

Research Challenge

Receptor tyrosine kinases (RTKs) play a central role regulating cell response during development and homeostasis[4], and dysregulation contributes to diseases such as cancer[5]. RTK-targeted therapies have been applied successfully in cancer treatment though with limited effectiveness as activity of non-targeted RTKs can enable cells to become resistant[6]. While redundant signaling is now appreciated as a common mechanism of acquired and innate resistance, exactly what signaling is essential to resistance, and whether it is conserved or varies across cancer contexts, has not been addressed[7–12]. RTKs lead to a common set of downstream signals, but in vastly different quantitative combinations, and differ in their ability to confer resistance in a context-dependent manner[7]. A fundamental, rigorous understanding of resistance is necessary if we are to develop better therapies to overcome this redundancy. TAM receptors (Tyro3, AXL, MerTK) are a family of RTKs that have attracted interest for their widespread roles in tumor resistance and metastasis[8,13–16]. However, while the ligands for these receptors have been identified, we lack even a basic understanding of the contexts that lead to activation of these receptors[17–19].

RTKs work by auto- and trans-phosphorylation, recruitment of adapter proteins, and then phosphorylation those adapters and other associated proteins. Systems biology has generally concentrated on easily measurable factors such as phosphorylation, but comparisons of signaling between receptors with this information is not possible as phosphosites do not equate (e.g. vary in their stoichiometry, affinity for particular adapters, competition with other sites in binding, localization and thus local concentration of adapters, and effects of avidity for multivalent interactions). The amount of receptor-bound adapter molecules is one quantity that should be directly comparable however. Protein interactions are generally studied by affinity purification or distance-based measurement. These methods provide either a snapshot of many interactions with one protein or, alternatively, a qualitative comparison of a single interaction pair over a few conditions[20,21]. New methods that provide quantitative information about sets of proteins interacting over many conditions are needed to construct multivariate models of intracellular signaling–a complex environment with myriad simultaneous, dynamic interactions[22,23]. As RTKs act most proximally through interaction with their direct binding partners, this is an area where systems-level, quantitative comparisons of protein interactions will provide a vital addition to biological investigation.

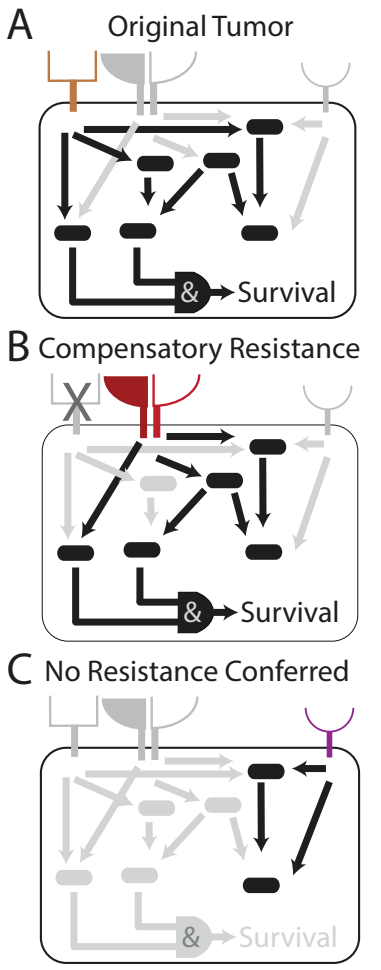

Figure 1: (A) In RTK-driven tumors, signals are transduced from the receptor to various kinases. (B) Upon blocking the original cancer driver, resistance can be conferred by an untargeted receptor. (C) Some receptors, however, do not provide essential resistance signals. This modeling assumes that a common signaling state defines resistance or sensitivity.

Thus, I plan to develop techniques to measure RTK-adapter interaction quantitatively and across the multiple potential interactions within a cell simultaneously with the intention of completely capturing signaling from these receptors. I will use these techniques combined with quantitative modeling to examine interactions during receptor activation to understand how different RTKs can provide redundant signaling leading to RTK-targeted cancer therapy resistance. These resistance and interaction models will then be applied to more specifically understand resistance conferred by the TAM family of receptor tyrosine kinases. Through mechanistic reaction-diffusion models for ligand-dependent and -independent signaling, linked to adapter interaction, downstream signaling, and resistance, I plan to develop an integrative understanding of this dysregulated signaling that can be used to overcome RTK-targeted therapy resistance.

Approach

Specific Aim 1: Measure context-dependent RTK-adapter interactions upon compensatory resistance in carcinoma cell lines

Rationale Cancers frequently acquire resistance to targeted therapeutics against a specific RTK by increasing the activity of one or more alternative RTKs[6]. For example, inhibition of epidermal growth factor receptor in non-small cell lung cancer leads to resistance by compensatory activation of AXL[13]. It is not currently known which signaling adapter proteins recruited by these compensatory RTKs, or subset of subsequent signaling events, mediate resistance. I hypothesize that these compensatory RTKs recruit a common set of adapter proteins, and that a quantitative measure of signaling output from these adapters can be used to predict drug sensitivity. To address this hypothesis I will first develop a quantitative assay to measure RTK-adapter interactions in a high-throughput format. Careful analysis in this phase of the study will develop a predictive model of resistance on a more integrative scale than is found elsewhere.

Preliminary Data, Plan, and Methods

1. Measure context-dependent RTK-adapter interactions. A well-characterized set of cell lines with documented sensitivity to RTK-targeted inhibitors, along with resistance conferred by stimulation with growth factor, exists[7]. To produce lysates for analysis, cells will be treated with the drug of sensitivity, with or without a set amount of growth factors for other receptors concurrently, then crosslinked and lysed. Adapter interaction will be measured by a multiplexed bead immunocapture-based assay in which each RTK is immunoprecipitated and then probed with antibodies targeting each interacting adapter (Fig. 2). Table 2 shows a tentative list of adapters to be measured. While there are many adapters, only a subset will be expressed in each cell line, and our focus will be on those with the most evidence of roles in resistance.

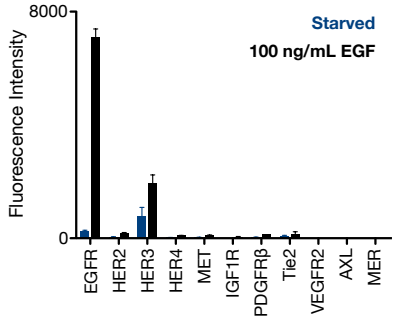

Figure 2: Preliminary test of Grb2-RTK quantification in MDA-MB-231 before and after stimulation with 100 ng/mL EGF for 5 min.

One concern with crosslinking interaction measurement is that interactions may arise after lysis due to affinity between an RTK and particular adapter that did not occur within the environment of the cell (for example, adapters present in different subcellular compartments prior to lysis). To address this, I plan to optimize my use of buffers during lysis and initial bead incubation to reduce interactions after lysis. After measurement of each adapter-RTK pair association in a set of cell lines, direct comparison and statistical testing between conditions is entirely possible to identify adapters that have switched binding between receptors. However, deconvolution of these data across cell lines can aid in interpretation and generalization of the results. If one assumes a constant, intrinsic binding affinity for each activated receptor-adapter pair independent of cell line, thus assuming that the only variation between cell lines is in the abundance of activated receptor and adapter (measured experimentally), a generalized linear model should then be able to fit the entirety of interaction quantitation across cell lines by optimization of the parameters for efficiency of crosslinking and detection. This analysis would then provide inferred parameters for a relative RTK-adapter affinity, corrected for differences in abundance, as was demonstrated in our previous work examining RTK interaction[3].

Modeling RTK-adapter interaction across cell lines may fail if the principle assumption, that abundance of the two active proteins is the only relevant difference between cell lines, is false. Competing interaction partners influence the binding of one another for example[24]. However, this might suggest interesting biology to be examined in greater depth experimentally. Such a problem would not hinder the subsequent phases of this study.

2. Measure context-dependent downstream resistance signaling. In order to integrate measurements of adapter binding with downstream response, I will measure phosphorylation of a subset of central downstream signaling nodes such as Erk, Akt, Jnk and P38 across growth factor conditions conferring varying degrees of

resistance, in the presence or absence of drug, as well as cell viability at appropriate times after treatment. It is then possible with these measurements to test whether the particular repertoire of adapters bound to the RTKs of interest can predict activity of signaling intermediates, whether the signaling intermediates can predict viability response, and whether adapter measurements can improve prediction capacity thus suggesting important information otherwise missed by the downstream signaling nodes measured.

3. Model RTK adapter to downstream signaling relationships. With measurements of downstream signaling driven by measured RTK-adapter regulation, I will build multilinear regression models of the relationship to identify the relevant interactions driving each downstream signal. This information will be important for identifying the essential interactions that mediate resistance via resulting signals upon RTK 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[12,26]. In each case, signaling from both receptors was important to tumor progression, but for unknown reasons on a mechanistic level. I hypothesize that this is because certain receptors lack the full complement of interactions to provide resistance and that these signals must then come from other receptors[27]. This data set will be uniquely powerful for identifying this complex regulation as concomitant signaling from each receptor is only deconvoluted and comparable with interaction measurement.

Adapter	Evidence for role in resistance
CrkL	Overexpression commonly linked to resistance[8].
Grb2	Central activator of Ras signaling[25].
Shc	Central activator of Ras signaling.
PI3K p80	Central PI3k activator.
Gab1	Central PI3k activator.
IRS-1	PI3k and Ras activator.
SHP2	Phosphatase important to robust Erk activation.
PLGγ	Activates PKC signaling via DAG.
SRC	Pleitropic downstream signaling.
Abl	Pleitropic downstream signaling.

Table 2: Initial list of adapters identified for interaction measurement.

One study looked at whether linear combinations of docking affinities can predict the phosphorylation of relatively RTK-proximal signaling nodes[28]. 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. This 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 outcome[29].

As there are many more proteins that interact with RTKs than can conceivably be measured, the possibility exists for important interactions to be missed. This will likely appear as a failure to predict particular growth factor conditions during the modeling phase. If this is the case, immunoprecipitation of the receptor, followed by mass spectrometry to identify interacting partners, could be used in the condition to identify the missing interaction partner to be analyzed. This modeling effort will also combine interaction and downstream signaling data from multiple cell lines. This carries the implicit assumption that the signaling effect produced by a particular RTK-adapter interaction is similar across cell lines, and not affected by expression changes of intermediate species for example. If this is an inappropriate assumption, it will manifest as a failure to predict measurements from certain cell lines. Distinct models for each cell line may need to be assembled in such a case.

4. Develop predictive models of resistance signaling. In principle, some set of cellular signaling states results in survival and thus resistance, and other states result in cell death and thus sensitivity. I hypothesize there is a common resistance/sensitivity set regardless of the relevant driving RTK or other cell line properties[30]. Thus, I will use decision tree modeling as a computational framework to derive a signature of resistance, with downstream signaling/adapter interaction as inputs and cell resistance as output (Fig.

3)[31–33]. The general consensus currently is that Akt and Erk activation are most central determinants of this process[6], however complexities in resistance phenotypes of different cell lines suggest this picture is incomplete[34]. This modeling would directly test the relationship between activation of these kinases and resistance. I certainly anticipate that Erk and Akt phosphorylation will be important inputs to the model, but that additional predictive capacity will be gained by the other inputs due to additional or alternative essential signals. If overall cellular state, and thus sensitivity or resistance, is completely captured by downstream signaling measurements, the resulting model will not rely on interaction measurements directly. Adapters are included in this modeling as current standard downstream measurements provide a myopic view of overall cellular signaling[35]. If adapter interaction is required to predict resistance, any interaction measured certainly has downstream signaling consequence. Interactions predicted and validated to be important to development of resistance will be investigated for their downstream signaling consequence by literature investigation and further detailed biochemical analysis. All hypotheses formed in the modeling phase will be tested by knockdown of the relevant adapter proteins and/or mutagenesis of the relevant interaction site to validate mechanistic significance. This will likely reveal therapeutic combinations that can delay or prevent the onset of resistance, and will be tested *in vitro* and potentially *in vivo* with a collaborator for their efficacy. Genetic manipulation may be used if no drugs target the proteins identified.

5. Combine the decision tree and RTK-adapter models to enumerate all possible routes of resistance. With a quantitative relationship between signaling state and resistance phenotype, and a relationship between RTK-adapter interaction and downstream signaling, it should be possible to identify all combinations of RTK-adapter interactions or RTK activation patterns that give rise to resistance.

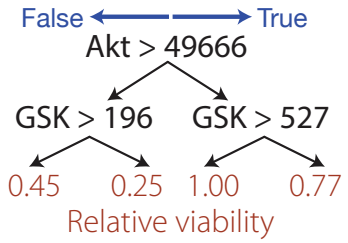

Figure 3: Ideal two-input decision tree model for SKBR3 and BT474 cells treated with lapatinib with or without EGF, TGFα, PDGF, HRGβ1, HGF, IGF, or Gas6. Consistent with previous literature, Akt reactivation generally indicates resistance for HER2-driven cells. Inclusion of more diverse cell lines will more stringently challenge the model. Akt: Akt pS473. GSK: GSK-3α/β pS21/9.

This could be examined in two fashions. First, by computationally modeling activation of each receptor, then predicting compensatory resistance, we will identify whether all RTK families examined can compensate for one another, or if particular combinations of receptors cannot provide the requisite signals. This provides very critical information for the design of combination therapies. If all RTKs can compensate and provide resistance for one another, the extent of this redundancy suggests overcoming resistance will be challenging at the level of RTK inhibition. If this process of resistance is more specific to particular combinations of RTKs, there is a limited set of other receptors to target with new therapies.

Second, we will test for sensitivities in the interaction components of the model. Computational modeling allows us to manipulate many components at once, such as perform all combinations of protein knockdowns, in a manner that is not feasible for functional genetic studies. As an example of important conclusions that might arise from this, many other RTK families may be able to provide resistance to MET-targeted therapies, but other receptors may all rely on interaction with Grb2 to provide resistance signaling. The modeling could then identify that combination targeting of MET and Grb2 would overcome resistance regardless of the compliment of RTKs activated. While RTK-binding proteins that do not themselves have kinase activity present a difficult set of therapeutic targets, this information would be valuable to focus the design of interaction inhibitor and gene silencing therapies.

In total, this effort will be extremely important for the design of combination treatments across RTK-driven cancers. For example, a quantitative understanding of the signaling states that provide resistance will provide a state that can be examined when measuring tumor response to a treatment, or could allow sorting heterogeneous cell populations to predictively examine resistant subpopulations rather than examining resistant subclones after adaptation has occurred. Understanding the relationship between RTK-level regulation and downstream signaling will provide an upper limit to the combination of resistance mechanisms we expect to observe. For example, if Met activation alone is sufficient to activate one essential signal, such

as Erk, but not another such as Akt, Met activation is unlikely to arise in Akt-targeted therapies. Finally, this multivariate understanding of this process builds upon the many single-component studies attempted thus far, to provide an integrative picture of the process of resistance. A principals-based understanding of resistance will provide a framework for understanding new discoveries—e.g. if A provides resistance to B, does it do so by manipulation of the same core set of signals, or is it an example of truly novel signaling regulation?

Specific Aim 2: Develop a quantitative understanding of TAM receptor activation and validate resistance modeling using the important case of TAM receptor activation

Rationale While pan-TAM (Tyro3, AXL, MerTK) inhibitors have entered clinical trials for carcinoma treatment, very little understanding of the relevant activation mechanisms for these receptors exist[17–19]. AXL has emerged as a promising target to combat metastasis and resistance to other targeted therapies in breast carcinoma, lung adenocarcinoma, and glioblastoma[8,13,14], and MerTK is a promising target in melanoma and acute myeloid leukemia[15,16]. Overly broad targeting is likely to have deleterious effects however, as these receptors have important roles in synapse turnover, innate immune response, spermatogenesis, photoreceptor turnover, and phagocytic clearance[36–39]. A better understanding of TAM receptor activation generally, and the activation contexts which lead to resistance specifically, will lead to better cancer therapies.

Ultimately the goal of this proposal is to improve our understanding of the process of resistance from an anecdotal set of many mechanisms to principles of exactly which signaling is essential for resistance and the regulatory mechanisms that provide this signaling. This aim will apply the improved understanding of resistance from Aim 1, combined with thorough understanding of activation mechanisms for TAM receptors, to develop better TAM receptor-targeted therapies, with focus on breast carcinoma. While the exact mechanisms of resistance elucidated in this aim will be interesting on their own, many mechanisms of resistance have been identified. The ultimate goal of this effort is to demonstrate a quantitative framework for resistance analysis, so that treatments might one day be tailored based on the exact mechanism of resistance.

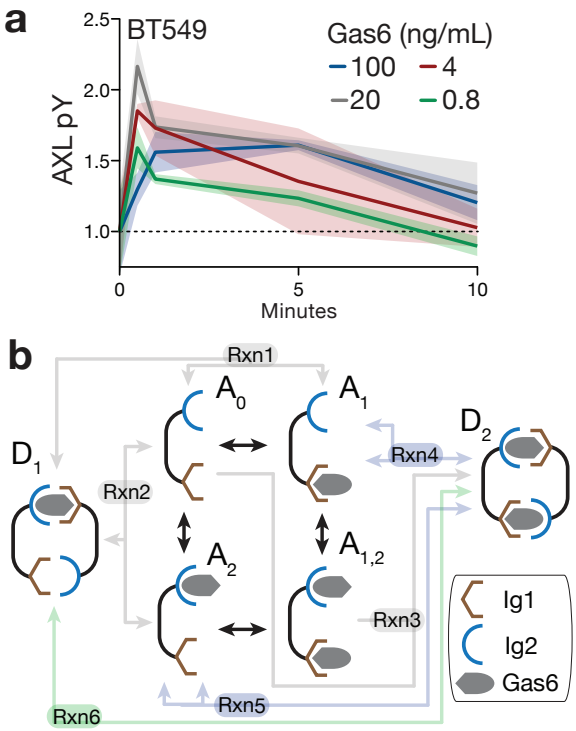

Figure 4: TAM receptor mechanistic modeling applied to AXL. a) BT-549 cells were starved and stimulated with increasing doses of Gas6. AXL pan-phosphotyrosine was subsequently measured over 10 min. b) Data from cell lines with varying expression of AXL were used to train an ordinary differential equation model incorporating TAM receptor-ligand interaction.

Preliminary Data, Plan, and Methods

1. Assemble models for Tyro3 and MerTK ligand-mediated activation. We recently assembled a quantitative kinetic model of Gas6/AXL binding, dimerization and activation. Using this model and diverse experimental validations, we have shown that the receptor most robustly senses ligand concentration discontinuities as arise after immobilization of the ligand on phosphatidylserine-presenting surfaces, in contradistinction to simply ligand concentration. To develop a better understanding of Tyro3 and MerTK function, I will treat cell lines expressing MerTK or Tyro3 with Gas6 or Protein S and quantitatively measure receptor phosphorylation response (Fig. 4a). These data will be used to train models of activation for these other receptors, using the same reaction-diffusion modeling framework, and will allow evaluation of ligand-mediated activation for all three receptors (Fig. 4b).

This information for other TAM family members will be important even if just one is expressed by tumor cells, as these receptors are expressed in many cell types known to infiltrate tumors and throughout the body[40,41]. As these receptors are also used by immune cell types during clearance of apoptotic cell debris, manipulation of this system may hold promise for immune targeting of tumors[40]. Outside of cancer, this information will be profoundly useful for basic study of these receptors, as it will provide a detailed,

mechanistic understanding of the conditions under which these receptors are activated. Our modeling for AXL has indicated that ligand-mediated response is regulated by more than just ligand concentration, necessitating a more rigorous approach such as this. Importantly, since normal function of the TAM receptors relies on localized signaling, and tumor-related signal dysregulation on overall receptor activation, a more careful understanding such as this may allow direct manipulation of tumor-associated signaling while preserving some normal function.

2. Evaluate ligand-independent signaling in Tyro3 and MerTK. Surprisingly our AXL signaling model required ligand-independent phosphorylation for proper model training, which acted as a threshold for response to ligand and was essential for normal function of this receptor as a specific signal for phagocytic uptake of cellular debris. This prediction of some amount of AXL signaling being independent of ligand was confirmed with mutants unable to bind ligand, which retained considerable phosphorylation. On the level of basic understanding, this regulation is interesting as a distinct paradigm in RTK function. Clinically, ligand-independent signaling cannot be targeted by manipulation of ligand interaction. To extend this, we will assemble mutants with ablated ligand binding capacity for Tyro3 and MerTK, and assess the relative contribution of ligand-independent activation effects. This will serve as validation, as ligand-independent activation will also be predicted from the mechanistic model trained using the previous stimulation measurements.

3. Compare ligand-dependent versus ligand-independent phosphorylation state. While we previously identified that AXL is phosphorylated to some extent independent of ligand, ligand-mediated and ligand-independent phosphorylation may occur on a distinct complement of sites. This would mean that overall receptor phosphorylation may not completely capture receptor activation state. To test this, I will use both A172 glioblastoma cells which express AXL and very little Gas6 (< 2 pM, when the high affinity K_D is $\sim$1 nM[18], and our modeling infers that phosphorylation is predominantly not from the ligand-mediated complex), and MDA-MB-453 cells (which do not express AXL or Gas6) with a mutant of AXL expressed that cannot bind Gas6, both to complementarily model ligand-independent signaling. For ligand-dependent signaling, we will treat A172 cells with Gas6 immobilized on phosphatidylserine-containing vesicles to elicit a maximal ligand-mediated phosphorylation response.

Cells for each condition will be SILAC labeled, lysed, and then AXL immunoprecipitated from lysates. The combined samples will then be submitted for mass spectrometry to quantitatively compare each phosphosite across conditions.

4. Measure ligand-dependent versus ligand-independent adapter interaction and downstream signaling. If the complement of phosphorylated residues is distinct for each AXL activated state, the adapters bound are likely to be distinct as well. Using SKBR3 cells which are sensitive to HER2 inhibition by treatment with lapatinib (and do not express TAM receptors), matched with the same cells made resistant to inhibition by expression of AXL, I will examine the requirements for AXL-mediated resistance[7,42]. Specifically, I will express either wild-type AXL receptor or a version of the receptor with a mutation to prevent ligand binding to the Ig1 domain, which completely blocks ligand-mediated signaling[18]. I will then measure adapter interaction and downstream signaling in the presence or absence of lapatinib with or without Gas6 treatment. Previous quantitation of phosphosites on the receptor in each condition will be used to help inform which proteins should be measured for RTK interaction, using tools such as ScanSite[43].

If the other TAM receptors display similar ligand-independent signaling during the earlier modeling effort, they will be tested in a similar fashion here. If they do not, only ligand-mediated signaling will be evaluated. Little is known about the functional differences between AXL, Tyro3, and MerTK, and so comparisons of the downstream signaling and adapter interactions themselves will be of considerable interest, especially since all three receptors share only two ligands[41].

5. Evaluate the capacity of ligand-independent and ligand-dependent signaling to promote resistance with each family member. This will be accomplished two ways. In the first, we will use the signaling and adapter interaction measurements to predict using our decision tree model from Aim 1 whether the signaling consequences upon expression and activation of each TAM are sufficient to confer resistance. Next, we will

confirm these predictions by directly testing resistance in corresponding conditions. Importantly, the modeling effort will directly identify methods of overcoming resistance. For example, if Akt reactivation is a critically changed input to the model, this would identify that Akt inhibition, or blocking the relevant RTK interactions which give rise to Akt reactivation, should prevent resistance.

6. Identify methods of tumor-specific TAM targeting.
The TAM receptors are potentially unique in that many of the potentially deleterious effects of TAM inhibition indicated by the knockout mice relate to phagocytic uptake of phosphatidylserine-presenting particles (to which TAM ligand binds), and thus localized signaling which we can model by taking into account diffusion of receptor (Fig. 5)[44,45]. In contrast, resistance likely is a consequence of the overall magnitude of RTK-driven signaling, rather than its detailed localization. I will use this reaction-diffusion model, and an understanding of the activation contexts that give rise to TAM-mediated tumor resistance, to computationally compare different methods of targeting these receptors (for example, by competitively or noncompetetively binding to different domains of the receptors with different affinities, down regulating receptor abundance, etc). This will likely identify methods to selectively target resistance-promoting signaling, while preserving some of the localized signals that promote phagocytosis and its associated signaling.

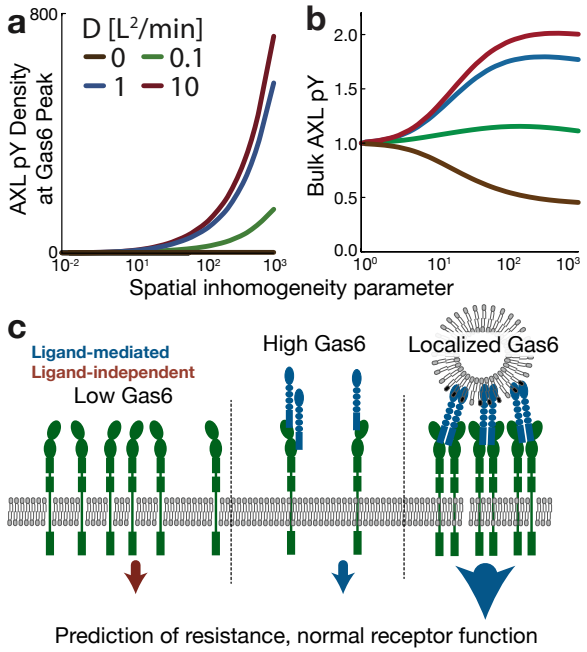

Figure 5: Spatial model of TAM receptor signaling to identify tumor-specific targeting. This model has so far been used to show that robust phosphorylation response comes principally from localized stimulation. Using finite differencing with a trained kinetic model, we can evaluate local (a) and overall (b) receptor activation under bulk or localized stimulation conditions. c) This allows for evaluation of signaling under different stimulation conditions that occur within the *in vivo* environment, and evaluation of therapeutic manipulations to this system.

I will then test these predictions *in vitro* for their ability to selectively target tumor-resistance promoting signaling. For example, competitive binding of the Ig2 domain might be identified as tumor-specific, which could be evaluated using Gas6 with only the Ig2-binding portion of the ligand. I will model normal phagocytic function of these receptors using macrophages or another appropriate cell type with Gas6- or Protein S-coated particles, evaluating uptake of the particles in the presence of drug. Promising candidates will be further developed with collaborators possessing appropriate expertise (for example, to engineer therapeutic peptides of specific affinity, *in vivo* preclinical evaluation, etc).

In total, this Aim will considerably advance our understanding of TAM receptor-mediated activation and resistance. Improved models of receptor activation will greatly aid future studies, as a critical limitation in studying these receptors at the moment is limited understanding of what actually leads to receptor activation. Simultaneously, it acts as a stringent test of the modeling effort proposed in Aim 1, by examining resistance conferred by a new receptor family.

Innovation

My work lies at the intersection of diverse computational approaches including machine learning and mechanistic modeling, combined with translating new methods of assessing biology to systems approaches. Critically, this combination is synergistic; models in systems biology will always be only as good as the information used to assemble them, and as our understanding of biology begins to assemble from information about single proteins, we need quantitative models to understand and even communicate these complex processes.

A core novelty of this proposal is expanding the methods used to understand sets of protein interactions in a quantitative and integrated fashion. The approach described here has not, to my knowledge, been used elsewhere, and certainly has not been integrated with quantitative modeling. However, preliminary results with the method suggest little difficulty in implementation for the proposed measurement of RTK interactions. This method will be vital to the biomedical research community and can be adapted to other areas such as study of GTPases where amenable high-throughput methods of protein activity measurement have been difficult.

While building upon well-developed methods in machine learning and systems biology, the conceptual approach to understanding resistance herein is quite novel. Considerable literature exists on resistance mechanisms to RTK-targeted drugs in cancer[7], but the underlying principles of exactly what signaling is required for conferring resistance hasn't been approached in a rigorous manner. Many studies have approached this using synthetic lethality screens and drug combination approaches[8], but while this has found many examples of resistance mechanisms and corresponding effective approaches to overcoming resistance, the information is largely anecdotal in nature. Single component approaches cannot effectively address the principles of resistance due to the dense, nonlinear connectivity of cellular signaling and feedback/compensation. From many accounts of resistance mechanisms, a general consensus has developed that Erk/Akt reactivation are the essential elements of resistance development, but this idea has not been directly tested. Logic and decision tree methods are the exact frameworks for testing such a hypothesis, and the outcome of this modeling will be vital to our understanding of cancer resistance mechanisms and for integration of our understanding of cancer regardless of its outcome.

Finally, the new insights into TAM receptor signaling will considerably accelerate design of drugs targeting the receptors without deleterious side effects. Clinical trials are already underway using pan-TAM receptor inhibitors, which have shown striking effects in pre-clinical models. However, global TAM inhibition in genetic studies has shown effects such as infertility, retinal degeneration, autoimmune disease, central nervous system defects, and increased carcinogenesis[36–39,46,47]. These broad inhibitors will likely be followed by better-targeted molecules to dysregulation in cancer, but study of signaling from the receptors has been limited by limitations in our understanding of the relevant activation mechanisms. The rapid development systems methods being used to assemble a quantitative understanding of these receptors is distinct from the slower pace of model development for other receptor families, and will be valuable for study of other signaling systems.

Relationship to previous work

This work is similar to my previous work in its focus on TAM receptor tyrosine kinase signaling and targeted cancer treatment. During my thesis studies I studied how activation of AXL by EGFR *in trans* promotes a migration response, and *in vitro* models of carcinoma metastasis[1,3]. I will continue development of the AXL signaling model I began late in my thesis, generalizing it for the other TAM family members and applying it for development of TAM-targeted therapies. While I will continue in this area, the work proposed is well beyond the scope of a graduate thesis. Neither of my graduate mentors had worked on the TAM receptor family before I joined their laboratories—my own interest led me to develop the project with their uniquely flexible support—and so I anticipate an easy transition to working independently from my mentors in this area.

While the connection between resistance and metastasis mechanisms has been a long-standing interest of mine, the more general focus on modeling resistance is a new area of research for me. I anticipate this approach will bring a fundamentally improved understanding to how we examine this form of RTK bypass resistance. The computational techniques are ones used in my advisers' laboratories, but not applied to this

area. Additionally, I developed the method and idea of measuring RTK-adapter interaction independently, so I do not anticipate difficulty in independently working in this area.

During my graduate career I have relied on a diverse set of experts across both biology and engineering for advice. I anticipate this will continue with my graduate mentors as a helpful but small part of the network of scientists I have developed in graduate school.

Timeline

Year	Specific Aim 1	Specific Aim 2	Career
2014-15	Measure context-dependent RTK-adapter interactions.	Assemble models for Tyro3 and MerTK ligand-mediated activation. Evaluate ligand-independent signaling in Tyro3 and MerTK.	Establish laboratory and begin training technician. Interview potential postdoctoral fellows.
2015-16	Continue RTK-adapter interaction measurement. Measure context-dependent downstream resistance signaling.	Finish modeling analysis of signaling response. Compare ligand-dependent versus ligand-independent phosphorylation state.	Begin training postdoc, present preliminary work at scientific conferences.
2016-17	Model RTK adapter to downstream signaling relationships. Validate important predictions.	Measure ligand-dependent versus ligand-independent adapter interaction and downstream signaling. Publish kinetic model of TAM signaling.	Seek additional funding if warranted. Continue presenting work at scientific conferences.
2017-18	Develop predictive models of resistance signaling. Combine the acquired logic and RTK-adapter models to enumerate all possible routes of resistance.	Evaluate the capacity of ligand-independent and ligand-dependent signaling to promote resistance in each family member.	Explore tenure-track positions.
2018-19	Validate model predictions with combination targeting. Publish integrated resistance model.	Evaluate methods of tumor-specific TAM targeting.	Identify tenure-track faculty position. Transition lab to new environment.

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