THE EMERGENCE OF DIVERSE DRUG-RESISTANCE MECHANISMS FROM DRUG TOLERANT CANCER PERSISTER CELLS

APPROVED BY SUPERVISORY COMMITTEE

Steven J. Altschuler, Ph.D. (Co-mentor)

Lani F. Wu, Ph.D. (Co-mentor)

Melanie H. Cobb, Ph.D. (Co-mentor)

Joshua T. Mendell, M.D., Ph.D. (Chair)

Jerry W. Shay, Ph.D.

Jiang I. Wu, Ph.D.

For my family, past, present, and future

THE EMERGENCE OF DIVERSE DRUG-RESISTANCE MECHANISMS FROM DRUG TOLERANT CANCER PERSISTER CELLS

by

MICHAEL EDWARD RAMIREZ

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THE EMERGENCE OF DIVERSE DRUG-RESISTANCE MECHANISMS FROM DRUG TOLERANT CANCER PERSISTER CELLS

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Michael Edward Ramirez

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Supervising Professors: Steven J. Altschuler, Ph.D., Lani F. Wu, Ph.D., Melanie H. Cobb, Ph.D.

Cancer therapy has traditionally focused on eliminating fast-growing populations of cells, yet a growing body of evidence suggests that small subpopulations of cancer cells can evade strong selective drug pressure by entering a slow-growing "persister" state [1]. This drug-tolerant state has been hypothesized to be part of an initial strategy towards eventual acquisition of *bona fide* drug-resistance mechanisms. However, the diversity and clinical relevance of drug-resistance mechanisms that can expand from a persister bottleneck is unknown. Here, we compared persister-derived, erlotinib-resistant colonies that arose from a single,

EGFR-addicted lung cancer cell. We found, using a combination of large-scale drug screening and whole-exome sequencing, that our erlotinib-resistant colonies had acquired diverse resistance mechanisms, including the most commonly observed clinical resistance mechanisms [2]. Thus, the drug-tolerant persister state does not limit—and may even provide a latent reservoir of cells—from which drug-resistance heterogeneity can emerge.

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PRIOR PUBLICATIONS

Ramirez, M.E.*, Rajaram, S.*, Steininger R.J. 3rd*, Osipchuk, D., Roth, M., Evans, L., Hsu, C., Thurley, K., Wei, S., Zhou, A., Posner, B.A., Wu, L.F., Altschuler, S.J. Diverse drug-resistance mechanisms can emerge from drug-tolerant cancer persister cells. Manuscript Submitted.

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Han, J., Sridevi, P., **Ramirez, M.E.**, Wang, J.Y.J. β-Catenin-Dependent Lysosomal Targeting of Internalized Tumor Necrosis Factor-α Suppresses Caspase-8 Activation in Colon Cancer Cells. *Molecular Biology of the Cell, (4):465-73, 2013 February 24.*

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DTP – drug tolerant persister

DTEP – drug tolerant expanded persister

PERC – persister-derived erlotinib resistant colonies

NSCLC – non-small cell lung cancer

EGFR – epidermal growth factor receptor

UV – ultraviolet

DNA – deoxyribonucleic acid

ATP – adenosine tri-phosphate

MEK – mitogen-activated protein kinase kinase

ERK – Extracellular signal-regulated kinase

PI3K - Phosphatidylinositol-4,5-bisphosphate 3-kinase

MTOR – Mammalian target of rapamycin

CML – Chronic myeloid leukemia

PIK3CA – Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha

HER2 – Human epidermal growth factor receptor 2

EMT – epithelial to mesenchymal transition

SCLC – Small cell lung cancer

IGF1R – Insulin-like growth factor 1 receptor

IC50 – Half maximal inhibitory concentration

NRAS – Neuroblastoma RAS viral oncogene homolog

MET – Mesnechymal epithelial transition factor

RPPA – Reverse phase protein array

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

PARP – Poly (ADP-ribose) polymerase

siRNA – small interfering ribonucleic acid

RNAi – riboneucleic acid interference

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

BCA – Bicinchoninic acid assay

DAB - 3, 3'-diaminobenzidine

AUC – Area under the curve

SNV – Single nucleotide variation

CNV – Copy number variation

KDM5A – Lysine (K)-specific demethylase 5A

ROS – Reactive oxygen specie

CHAPTER ONE

Introduction

1.1 Cancer Cell Persisters: an Emerging Paradigm in Drug Resistance

The advent of molecularly targeted therapy represents a significant achievement in medicinal cancer therapy; it is the first time that knowledge of the molecular underpinnings that differentiate cancer cells from normal cells have lead to the development of drugs that target molecular drivers of cancer cell growth [3-5]. Such drugs have the potential to change the landscape of cancer therapy, in principle offering hope for less noxious cancer treatments owing to a more specific targeting of the molecular changes driving cancer cell growth [6-8]. This is in contrast to the more commonly used medicinal cancer treatment, conventional cytotoxic chemotherapy, whose use leads to massive side effects owing to the fact that chemotherapy compounds are designed to target all rapidly proliferating cells in the body [9-11]. Despite this step forward, the success of most targeted therapies to date has been limited by the eventual development of drug resistance [12-14]. Therefore,

understanding of drug resistance is crucial to more effective use of molecular targeted therapies.

Resistance to targeted therapies has been vastly studied, and subsequently, it has been discovered that resistance can be driven by the expansion of rare, pre-existing cells within a treatment-naïve tumor [15-17]. Ongoing efforts seek to limit drug resistance through targeting of such rare, intrinsically drug resistant cells [18-20]. However, an additional model of drug resistance has recently been proposed in which a subpopulation of cancer cells referred to as “drug-tolerant persisters” (or simply persisters) are able tolerate a formidable drug challenge, and subsequently grow in the presence of drug without the requirement of resistance-conferring mutations [1]. Further understanding of drug-tolerant persisters is therefore of upmost importance, as this path to drug resistance would not be targeted by ongoing efforts to eliminate pre-existing resistance mutants.

In this dissertation, I will discuss research conducted on persisters in the particular context of “EGFR addiction” in non-small cell lung cancer (NSCLC) (Fig 1.1), a context where a targeted therapy, erlotinib, is very effective at first, but is limited by drug resistance [21]. In this context, multiple resistance mutations have been characterized [2,22,23], but whether or not the drug-tolerant persister state is compatible with these mutations is unknown. Is there a link between the observation of resistant

cancer cell populations bearing resistance mutations and the persister state? Are the cells that utilize persisters states able to obtain resistance mutations

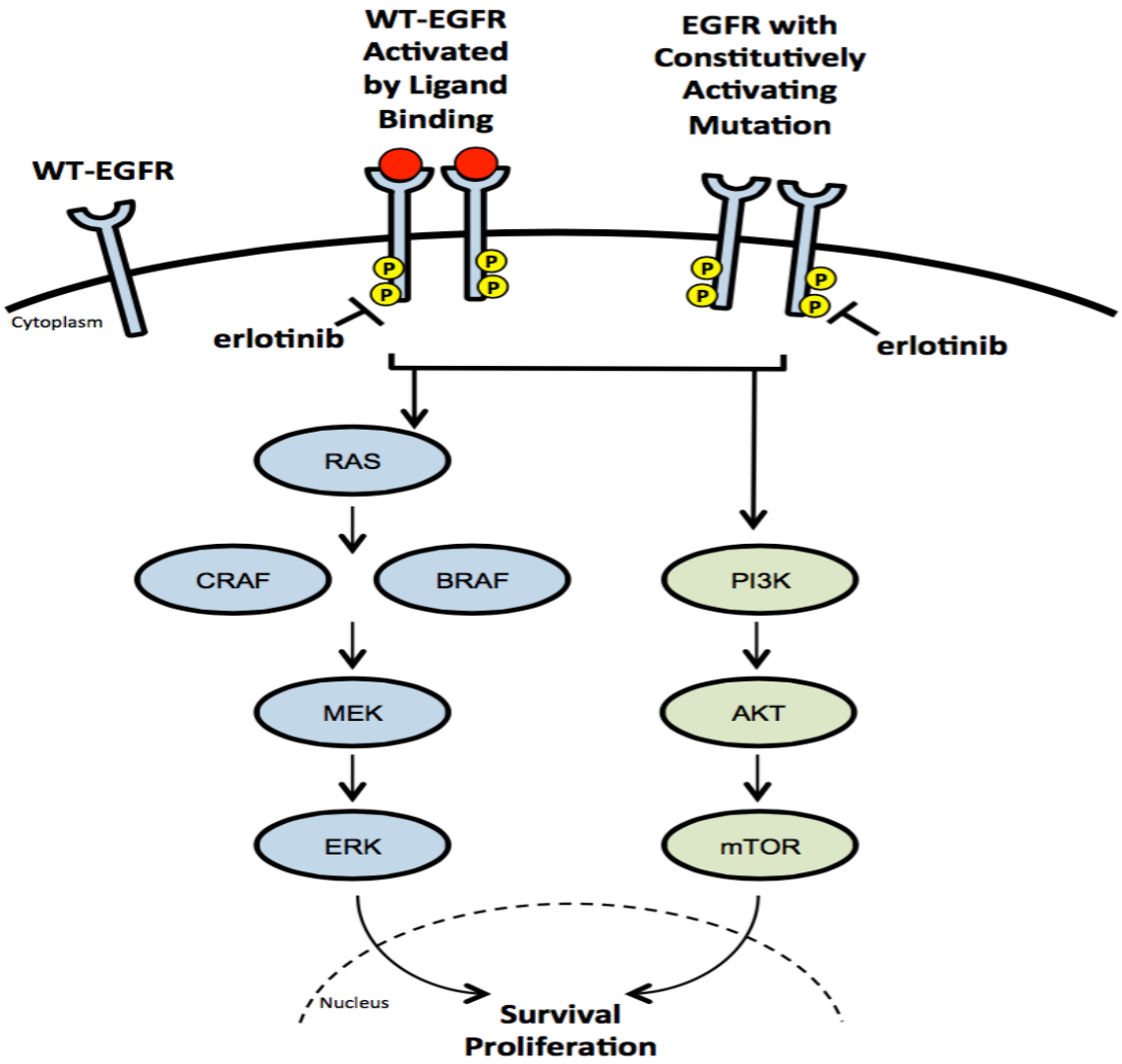


Figure 1.1: Overview of EGFR signaling
Schematic representation of the EGFR signaling pathway. In a normal setting, EGFR binds ligand and dimerizes, resulting in autophosphoylation. In EGFR variants with a constitutively activating mutation, autophosphorylation is achieved without ligand binding. In both settings, downstream signaling to the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways is stimulated, resulting in survival and proliferation.

or does the persister state represent an evolutionary dead end? The hypothesis of this dissertation is that drug-tolerant persister states can be utilized by cancer cells as an intermediate strategy for tolerating a drug challenge before resistance mutations eventually become fixed and utilized for growth in the presence of drug. The data presented here support a model where persister states are compatible with the fixation of resistance mutations. Additionally, we find that between persister colonies, a variety of different resistance mutations can become fixed, raising the possibility that resistance derived from persister clones can lead to the emergence of resistant populations heterogeneous in terms of their resistance mutations.

1.2 Experimental Approach

To test the hypothesis of this dissertation, our laboratory chose to examine persister-derived resistance to the targeted therapy drug erlotinib, in the context of EGFR-addicted non-small cell lung cancer. A crucial advantage of conducting our study in this context is that multiple examples of resistance mechanisms have been previously characterized [2,22,23]. Primarily, these mechanisms involve the utilization of one of several resistance-conferring mutations in various kinase genes. Therefore, in this study, we chose to profile persister-derived populations using whole exome sequencing in order to identify putative resistance mechanisms. In order to broadly corroborate putative resistance mechanisms with functional data, we chose to assay

each persister-derived population for their response to a variety of anti-cancer compounds, the majority of which were developed to target various kinases. Together, exome sequencing and large-scale drug response experiments provide a wealth of information on resistance mechanisms, and have been used in combination in the literature to infer resistance mechanisms of large numbers of resistant populations [24].

1.3 Dissertation Aims

Here, we aim to link persisters with the acquisition of resistance mutations. Currently, the field of resistance to targeted therapy drugs is dominated by the model of resistance that has been confirmed in the clinical setting, that is, the expansion of rare pre-existing cells with resistance mutations. Although the relative role of persister-derived resistance has not been determined in the clinical setting, we hypothesize that persister-derived lineages may fix some of the resistance mutations that have been associated with the expansion of intrinsically resistant, pre-existing cells. We hope that the linking of persisters with such resistance mutations will further implicate their potential role in resistance in the clinic, leading to further investigation into the molecular mechanisms that underlie persisters, and subsequent identification of potential avenues for therapeutic intervention.

In Chapter 2, I will review the crucial literature regarding oncogene addiction, cancer evolution, and drug-tolerant persisters. In Chapter 3, I will discuss the primary results of this study that support a model where persisters are used by cancer cells as an intermediate strategy for surviving a drug challenge in the absence of resistance mutations, before eventual fixation of resistance mutations. In Chapter 4, I will discuss in detail the methods and analyses that were used in this study. Finally, in Chapter 5, I will conclude with a discussion of new questions raised by this study.

CHAPTER TWO

Literature Review

2.1 On Mutations in Cancer and Oncogene Addiction

Over the past few decades, great strides have been made in the molecular understanding of cancer. This understanding has lead to the development of drugs that target specific molecular changes in cancer cells that distinguish them from normal cells, a type of therapy known as “targeted therapy” [25,26]. In this section, I will briefly review the role of mutations in cancer, and how mutations in some cases confer “oncogene addiction” to cancer cells. Finally, I will end with a discussion on how oncogene addiction is the basis for the use of targeted therapy in a subset of non-small cell lung cancer cases.

2.1.1 Cancer Initiation through mutation

Normally, a cell has mechanisms of tightly controlling growth, survival, and entry into cell death. In cancer, cells undergo a process commonly referred to as “transformation”. Transformation is characterized by the acquisition of mutations in certain genes; collectively, the acquisition of mutations in these genes serves to

deregulate the tightly controlled cellular programs of cell growth, survival, and cell death [27-30]. Cancer-causing mutations can be inherited in the germline [31-33], but more commonly, they are acquired [34-36]. They can be acquired by exposure to carcinogens in the environment [37](i.e. tobacco [38], asbestos [39]), exposure to UV radiation [40], viruses [41], and random errors in rapidly proliferating cells [42,43], among other things. However, it is believed that mutation in only a subset of genes can lead to cancer [29]. These genes fall into three categories: oncogenes, tumor suppressor genes, and DNA repair genes [44]. Oncogenes are genes that typically promote growth and prevention of cell death, and mutations often cause loss of negative regulation, leading to aberrant, unregulated signaling [45]. Tumor suppressor genes are genes whose normal function is to limit cell growth, and mutations in these genes causes the normal negative regulation of growth to be abolished, leading to the allowance of growth [46]. DNA repair genes are involved in fixing mistakes when DNA is replicated, and mutation in these genes leads to the retention of further mutations in the genome [47]. Subsequently, further cancer-causing mutations can be acquired. In general, mutation in multiple genes is thought to be necessary for transformation [48,49].

2.1.2 Duality of transformed cells – expanded phenotypic repertoire, and exposed weaknesses

Once transformation has occurred, cancer cells take on new properties. Signaling pathways that are normally tightly regulated are now deregulated, and thus the topology of these signaling pathways have been re-organized [50,51]. In undergoing transformation, cancer cells acquire an expanded phenotypic repertoire. Namely, cells acquire the ability to grow in an unregulated way, without normal contact inhibition, and are not as prone to undergoing cell death in stressful conditions, among other hallmarks [52]. Cancer cells in tumors are constantly undergoing stressful conditions (i.e. hypoxia, reactive oxygen species, etc. [53]), but rather than die in these conditions, cancer cells have a remarkable ability to survive stressful conditions compared to normal, untransformed cells [52]. The propensity for cell death is a crucial part of normal physiology, as it is beneficial to negatively regulate aberrant cell growth so as to maintain proper tissue function [54,55]. Transformed cells, in contrast, have lost this relatively higher propensity for cell death, owing to the re-organization of signaling pathways [52]. However, this reordering of signaling pathways that cancer cells use to resist cell death also exposes new molecular features that differentiate them from normal cells [51]. In this way, transformed cells have gained much, but have also exposed new weaknesses that can potentially be exploited therapeutically.

2.1.3 Oncogene addiction and the development of targeted therapies

In many cases, the rationale for the use of targeted therapies is linked to the concept of oncogene addiction [25]. Oncogene addiction refers to the idea that even in the background of many mutations, cancer cells can become extremely dependent on a single mutated oncogene [56]. In this way, drugs that inhibit the mutated oncogene will induce a strong cytotoxic or cytostatic response in cancer cells that harbor this mutation.

Currently, there are three models for oncogene addiction. The first is known as genetic streamlining [57]. This model postulates that during tumor evolution, cells that are better able to use a dominant addictive pathway through inactivation of non-essential pathways (even ones that are compensatory) are selected for. Upon inhibition of the dominant addictive pathway, cells either lose the ability to grow or undergo cell death, as compensatory pathways are inactivated. The second model is known as oncogenic shock [58]. The oncogenic shock model is based on the idea that a dominant addictive pathway simultaneously stimulates both pro- and anti- cell death signals. The oncogene addicted cells that are selected for during tumor evolution have more anti-cell death signals than pro-cell death signals, allowing them to survive. Inhibition of the dominant addictive pathway leads to a relative accumulation of pro-cell death signals, leading to cell death or cell cycle arrest. The third model for oncogene addiction is synthetic lethality [59]. This model describes a context in which a cancer cell's growth is driven by a dominant addictive pathway,

but also retains integrity of a compensatory pathway that is capable of sustaining cell growth and survival in the absence of signaling from the dominant addictive pathway. In this way, only simultaneous inhibition of both the dominant addictive pathway and the compensatory pathway will result in cell death or cell cycle arrest. Note that this model of oncogene addiction is conceptually different that the other two, since in this case, inhibition of both pathways is necessary for cell death. All of these models have support and may be at play to various degrees in different oncogene addiction contexts. In any case, many ongoing research programs are aimed at identifying signatures for oncogene addiction in various subtypes of cancer, and subsequently developing therapies that target the dominant pathway. Drugs that target molecular drivers of oncogene addicted cancer cells (rather than targeting all rapidly dividing cells) are likely to yield reduced side effects than cytotoxic conventional chemotherapy [9-11].

2.1.4 EGFR Addiction in NSCLC

One of the most widely studied examples of oncogene addiction is in a subset of non-small cell lung cancer (NSCLC) adenocarcinomas where tumors harbor an activating mutation in the proto-oncogene epidermal growth factor receptor (EGFR) [60,61]. Though EGFR is involved in many biological processes, one of the normal functions of EGFR is to respond to extracellular growth and survival cues through

ligand binding [62]. Normally, the EGFR pathway is tightly regulated by 1) the requirement of ligand binding to receptor in order to induce activation and 2) negative feedback shutting down EGFR signaling. Together, this regulation in normal cells can serve to maintain tissue homeostasis [63]. However, activating mutations in EGFR deregulate this tight control of signaling (NSCLC cells with activating mutations in EGFR are commonly referred to as "EGFR addicted" [60,61]) (Fig. 1.1). It is thought that EGFR variants harboring activating mutations tend to occupy a conformation similar to that of ligand-bound EGFR [64]. Occupation of this activated conformation allows for the transfer of phosphate groups from ATP to tyrosine residues (tyrosine kinase activity) in the cytosolic autophosphorylation domain of EGFR [65]. Adaptor proteins are then recruited to these phosphorylated sites, and subsequently various signaling pathway cascades are activated [65]. In particular, the activation of the RAS/RAF/MEK/ERK and PI3K/AKT/MTOR pathways are thought to be crucial to the survival and proliferation of EGFR addicted cells [66] (Fig. 1.1). Although involved in many processes, these pathways have been shown to stimulate anti-apoptotic signals and inhibit pro-apoptotic signals, and collectively these effects likely explain the addiction to constitutive EGFR signaling in NSCLC [66]. In particular, deletions in exon 19 and the point mutation L858R constitute the majority of activating mutations in EGFR addicted NSCLC, although other activating mutations in this context have been observed [61] (Fig. 2.1).

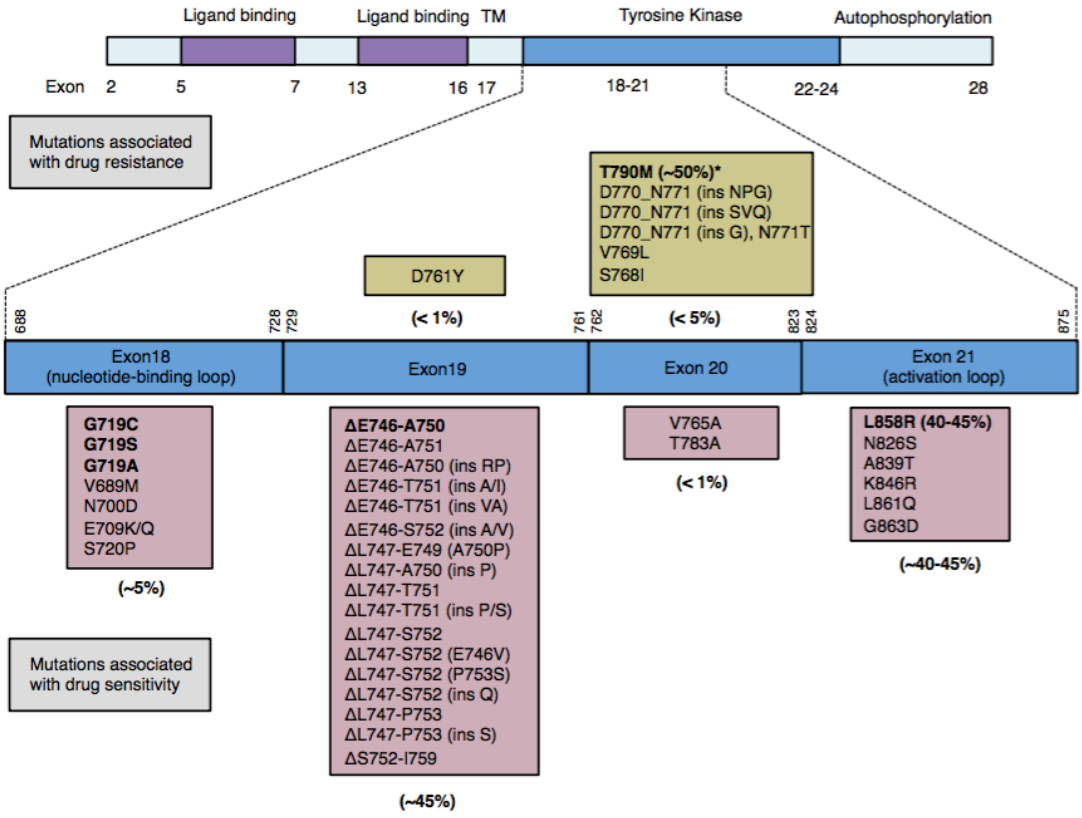


Figure 2.1: Erlotinib sensitivity and resistance-conferring mutations in EGFR

Representation of the domains of EGFR and listing of known mutations that confer either resistance or sensitivity to inhibition of EGFR signaling pathway in non-small cell lung cancer. Mutations occur in a "hotspot" in the tyrosine kinase domain between exons 18 and 21. Adapted from Sharma, et al., Nature Reviews Cancer 2007.

2.1.5 EGFR inhibition leads to strong cell death response in EGFR addicted NSCLC

After the elucidation of EGFR's role in growth, there was the observation that EGFR may play a role in cancer, as overexpression was observed in malignant gliomas, and overexpression of EGFR was found to correlate with poor prognosis in head and neck, ovarian, cervical, bladder, and esophageal cancers [67,68]. These observations lead to the development of EGFR inhibitors such as erlotinib. Erlotinib is a reversible competitive inhibitor: erlotinib competes with ATP for binding to EGFR tyrosine kinase pockets [69]. In competing with ATP, overall binding of ATP and subsequent tyrosine kinase activity is reduced. In 2004, the clinical observation was made that NSCLC patients that responded well to EGFR inhibitors correlated with activating EGFR mutations [70-72]. In this way, the inhibition of EGFR signaling with erlotinib causes a massive cell death response in EGFR addicted cells in NSCLC. Today, NSCLC tumors are routinely tested for the presence of EGFR mutations, and this information is used to guide whether or not erlotinib is likely to cause a treatment response in terms of reduction in tumor burden [73].

2.2 Cancer Evolution and the Emergence of Tumor Heterogeneity and Drug Resistance

I've introduced some key concepts in cancer regarding how mutations drive transformation, and how in some cases, a single mutation in the background of many mutations may confer oncogene addiction to cancer cells. Here I will discuss how mutations are utilized by cancer cell populations to continue expansion even under stressful tumor conditions, and how this results in an indefinite cycle of acquisition of new mutations, leading to tumors that are heterogeneous in terms of their genetic states. I will discuss how this property of tumors is thought to lead to drug resistance in the context of EGFR addicted NSCLC through expansion of rare cells with resistance-conferring mutations. Finally, I will discuss strategies that are currently being tested in EGFR addicted NSCLC whose collective goal is to limit resistance by targeting these rare resistant cells that exist before treatment is applied.

2.2.1 Selective pressures drive the emergence of mutational heterogeneity in cancer cell populations

As a tumor forms, cancer cells begin to experience various environmental stresses that arise as a consequence of rapid expansion of the neoplastic tissue. Normal mechanisms that ensure proper tissue formation and homeostasis are altered by the aberrant cancer-related signaling in transformed cells, and as a result, cancer cells reside in a chaotic and stressful extracellular environment [53]. Cells must

adapt to these, what I refer to as “endogenous selective pressures” in Figure 2.2. One example of an endogenous selective pressure is hypoxia, which occurs in regions of the tumor that are poorly perfused owing to a suboptimal distribution of blood vessels [74]. Other endogenous selective pressures might include destruction of cancer cells by the immune system [52], as well as the acidic environment cancer cells are exposed to as a result of increased metabolic activity [75]. In general, the environment is very stressful for cancer cells, and populations of cancer cells require a means of dealing with this dynamic and harsh microenvironment.

The tools that cancer cell populations use that allow for continued tumor growth include the acquisition of mutations that confer a selective advantage, as well as altered epigenetic states [52]. Owing to the mutations that contributed to transformation, cancer cells in general are more permissive to the acquisition of new mutations than normal cells, and as the population of cancer cells grows, new mutations can become acquired. Some of these mutations provide a selective advantage to cancer cells in a particular setting of stress, while others confer no advantage, yet do not disable the cell from dividing, thereby getting retained in the population; the latter are sometimes referred to as “passenger mutations” [76]. So, as any given cell lineage encounters various stresses in time and space, cells dynamically acquire new mutations that may or may not benefit them in the particular context at that time. In turn, a mutation that does not confer an advantage in one

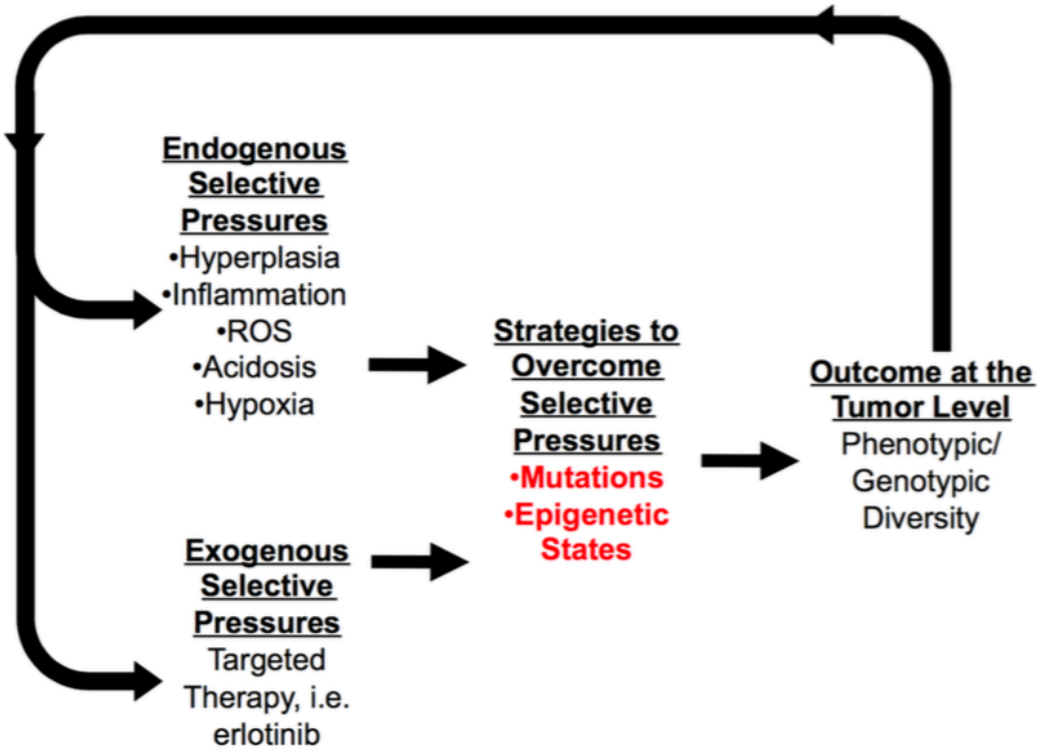


Figure 2.2: Selective pressures drive cancer cell evolution
Depiction of "endogenous selective pressures" typically experienced by cancer cell populations and "exogenous selective pressures" placed on these populations by anti-cancer compounds. At the level of the population, continued propagation is achieved through mutations and epigenetic states, resulting genotypic and phenotypic diversity between cancer cells.

selective pressure may confer an advantage in another setting (i.e. other "endogenous selective pressures" or "exogenous selective pressures" such as drug treatment) (Fig. 2.2) [76]. This results in populations of cancer cells that are composed

of a heterogeneous mixture of cells in different genetic states, in a constantly renewing cycle where more and more mutations are being acquired [53].

2.2.2 Resistance to erlotinib in EGFR addicted NSCLC: *fait accompli*

Because of the cyclical process of cells picking up mutations discussed in the last section, there ends up being a wide variety of mutations within a tumor. Therefore, when a drug treatment is applied, rare cells that harbor mutations that can confer resistance may exist, and expand even in the context of drug treatment [43]. Particularly in the case of erlotinib resistance in NSCLC, resistance to targeted therapies is considered to be a *fait accompli*, that is, eventual resistance is highly likely to occur [77].

2.2.3 On the role of pre-existing resistance mutations in tumors

I've mentioned that mutations are constantly being accumulated in tumors, and how this can lead to the existence of rare, pre-existing cells with resistance mutations [77]. This is the predominant model for the emergence of resistance to targeted therapies. The evidence to support this model has come from studies that characterize tumors before treatment and, through various techniques, are able to identify rare cells with resistance-conferring mutations [78,79]. Also, the literature has

many examples of mathematical modeling being used to support the model that pre-existing resistance mutants likely drive resistance in many cases [80]. In particular, the work of Bert Vogelstein suggests that rare resistant clones inevitably exist in human tumors, given the sheer number of cells in a given tumor [43,77].

2.2.4 Known mechanisms of resistance for erlotinib

Since the first cases of emergence of erlotinib resistance, groups have been molecularly characterizing resistant populations in hopes of finding resistance mechanisms. After years of intensive study from many groups, the characterized mechanisms that are clinically validated fall into two main classes: Target modification, and bypass signaling [81] (Fig. 2.3). Other types of mechanisms have been proposed in strictly cell culture/basic research settings [82-85], but for the purpose of this thesis, I will focus only on resistance mechanisms that have been clinically observed.

Target modification was the first class of mechanisms discovered. Clues came from literature on resistance to the targeted drug imatinib (aka Gleevec) in chronic myeloid leukemia (CML) that is driven by the fusion oncogene BCR-ABL. Analogous to EGFR addicted NSCLC, CML cells that harbor BCR-ABL have constitutively active pro-survival signaling, and are extremely sensitive to inhibitors

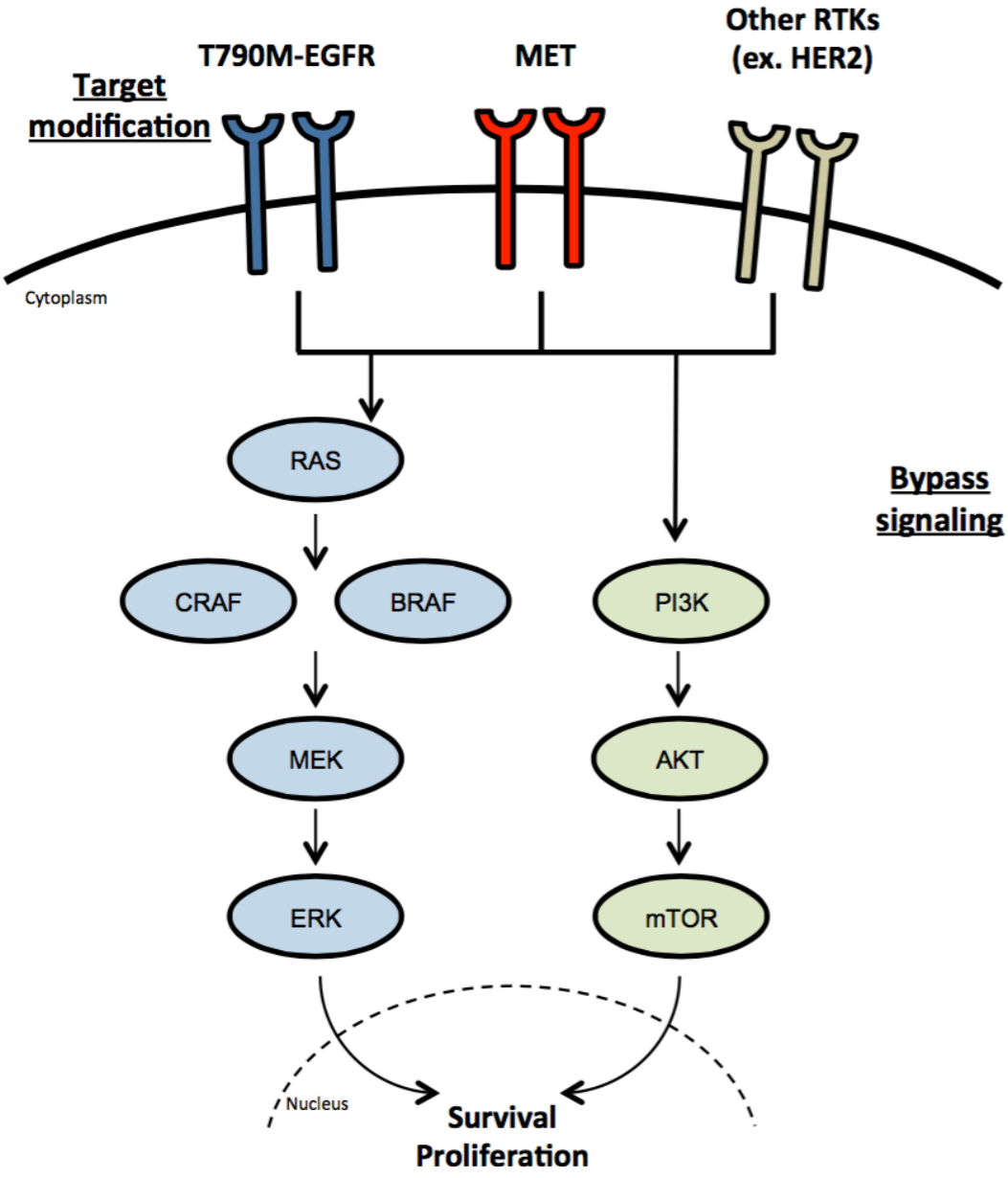


Figure 2.3: Target modification and bypass signaling in erlotinib resistance
Resistance is achieved through selection of cells containing a drug resistant variant of EGFR (most common resistance variant T790M-EGFR shown) or through deregulation of signaling in "bypass tracks" that are capable of driving pro-survival signaling.

that target BCR-ABL activity [86]. Resistance can occur when CML cells harboring a mutation in the "gatekeeper residue" are selected for [87]. The gatekeeper residue lies within the kinase pocket, and mutation results in steric hindrance of imatinib binding [87]. The mutation still allows the BCR-ABL variant to promote pro-survival signaling, and therefore, cells with this mutation have a selective advantage compared to other cells, and are able to expand [87]. The gatekeeper residue in EGFR is threonine 790 [87]. In the same way, EGFR addicted NSCLC cells that harbor a secondary mutation in EGFR, changing this threonine to methionine, are capable of erlotinib resistance (Fig 2.1) (Fig. 2.3) [88]. This "T790M-EGFR" mutation can be detected in ~50% of all cases of resistance to erlotinib in the EGFR addicted subtext [2], making it the most common form of resistance in this context (Fig. 2.4). Since it is so common, much effort has been put forth to develop drugs that are not limited by the steric hindrance in the kinase pocket caused by T790M-EGFR, but instead are irreversible inhibitors (rather than reversible like erlotinib) that inhibit EGFR signaling through irreversible binding to a site in the ATP pocket (steric hindrance preventing erlotinib from binding T790M-EGFR does not prevent these drugs from entering the ATP pocket due to differences in chemical structure) [89] . So much focus has been placed on designing T790M-EGFR inhibitors that a wave of "second generation EGFR inhibitors" is now starting to be replaced by "third generation T790M-EGFR inhibitors", that have the added feature of having less activity on wild type EGFR, allowing for less skin-related side effects [90] (second generation EGFR inhibitors are regarded by many

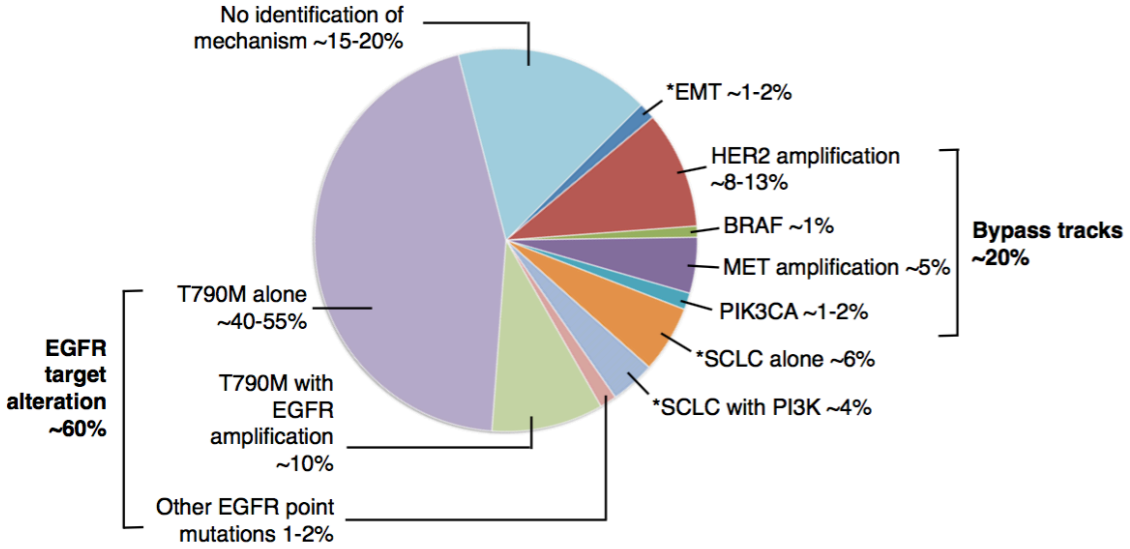


Figure 2.4: Summary of clinically-observed erlotinib resistance mechanisms
Summary of genes that, when mutated, are known to drive erlotinib resistance in the clinical setting. Exceptions include EMT (epithelial to mesenchymal transition) and SCLC (small cell lung cancer), which are phenotypic changes that have been observed in erlotinib resistance cases. Adapted from Camidge, et al., Nature Reviews 2014.

now to be limited by skin-related side effects, and the added specificity of third generation EGFR inhibitors is garnering much enthusiasm).

The second class of resistance is known as bypass signaling (Fig. 2.3). Bypass signaling refers to the situation where the original target (i.e. EGFR), is inhibited, but cells that are capable of utilizing another protein to achieve the same pro-survival signaling are selected for and allowed to expand. The ability to achieve

bypass signaling usually comes in way of a mutation, leading to constitutive or elevated levels of pro-survival signaling. For example, in erlotinib resistance, one such mechanism of bypass signaling resistance is amplification of the receptor tyrosine kinase MET (~5% of cases) [23] (Fig. 2.4). Increased copies of MET drive stimulation of the RAS/RAF/MEK/ERK and PI3K/AKT/MTOR pathways at sufficient levels to allow cells with MET amplification to gain a selective advantage and expand. Other notable clinically-validated bypass resistance mechanisms include activating mutations in PIK3CA (~2% of cases), and BRAF (~1% of cases), as well as amplification of HER2 (~10% of cases) [2] (Fig. 2.4). All of these mutations are capable of stimulating signaling of RAS/RAF/MEK/ERK and PI3K/AKT/MTOR pathways.

Of note, there have also been reports of phenotypic changes in erlotinib resistance that have been proposed to account for resistance, but the precise molecular mechanisms are unknown. These include the observation that in some cases, resistant tumors have undergone epithelial to mesenchymal transition (EMT) [91], or transformation to a different kind of lung cancer, small cell lung cancer (SCLC) (Fig. 2.4) [92]. These occur in a small fraction of cases (EMT ~1%, SCLC ~6%), and how these phenotypic changes contribute to resistance at the molecular level is still under investigation.

2.2.5 Current strategies for managing resistance

Given that resistance to targeted therapies is starting to be considered a *fait accompli* [43,77], there are two strategies that are currently being tested that are aimed at managing resistance. The first is called sequential treatment. With sequential treatment, the idea is that once resistance occurs, resistant tumors are analyzed in order to get a picture of what the molecular characteristics of the resistant cells are, and subsequently to guide what the next treatment should be. A strong proof of principle study on sequential treatment recently was published [24]; resistant tumors were sampled, and quickly developed into cell lines facilitated by use of feeder layer. The authors of this study then tested the resistant cell lines for sensitivity to a panel of targeted therapies that targeted various kinases, and they also sequenced the exome. Using the information garnered from this characterization, the authors were able to demonstrate a platform for finding an ideal next therapy for resistant tumors. Perhaps platforms like this will take root at other medical centers, leading to rationally-guided, sequential therapy programs, analogous to those seen in modern HIV treatment [93]. Also, this is currently being done in clinical trials in patients with EGFR addicted tumors that have developed resistance [2]. Typically, trials such as this involve the following pipeline: Tumors are re-biopsied at the time of resistance, and are tested for the presence of the T790M-EGFR secondary mutation or MET amplification (explained above), as 1) these are among the most common

mechanisms of resistance, and 2) these forms of resistance are actionable in that there are targeted therapies available to use on these tumors.

The second of resistance management involves the application of a drug bottleneck that seeks to targets both EGFR-addicted cells, as well as common resistance mechanisms upfront. Since T790M-EGFR and MET amplification are among the most common forms of resistance [2], such drug bottlenecks seek to target these mechanisms upfront. Therefore, such drug bottlenecks may consist of erlotinib and MET inhibitors for example, or the use of T790M-EGFR inhibitors with MET inhibitors [94]. Setting up drug bottlenecks in this way may be a more effective strategy than sequential treatment for resistance management. In studies by Bert Vogelstein's group, they show in a mathematical model that combination therapy may have a higher likelihood of tumor management than sequential treatment [95]. In reality, resistance may also emerge from combination therapy, and indeed, an initial combination therapy then subsequent sequential therapy may be necessary for management of resistance. Both approaches to resistance management rely heavily on having proper diagnostic signatures for assessing the molecular nature of resistant tumors, as well as having drugs for that resistance type. Therefore, continued characterization and more understanding of erlotinib resistance is needed in order for resistance management to be successful in EGFR addicted NSCLC.

2.2.6 A focus in current strategies on targeting pre-existing resistance mutants

Given what we've learned about the nature of cancer mutations and the inevitable resistance to erlotinib in EGFR addicted NSCLC, current strategies towards the goal of resistance management may result in longer lasting responses. However, the strategies currently being tested are largely predicated on the notion that resistance is derived solely from rare, pre-existing resistant mutants. Mechanisms of resistance that do not arise in this way, that instead arise from cancer cells surviving a drug challenge without the use of a genetic resistance mutation, in principle would undermine and confound the goal of managing the emergence of resistance. Even in a theoretically perfect situation, where all resistance mutations are targeted by a drug bottleneck, non-mutational resistance mechanisms that could allow cells to grow would then be capable of driving resistance. Identifying and understanding such mechanisms would then be of great importance.

2.3 On Drug Tolerant Persister States

I've discussed in the last section how heterogeneity in terms of genotype is constantly being generated in tumors. This inherent heterogeneity is thought to play

a significant role in the emergence of drug resistance, due to selection for rare, drug resistant mutants [96]. Consequently, current strategies in EGFR addicted NSCLC seek to limit resistance through targeting of the majority of cells with EGFR inhibition, while also targeting rare resistant mutants [20]. However, as mentioned above, the success of this strategy is predicated on the notion that selection of pre-existing resistance mutants represents the only path to drug resistance. In this section, I will go over recently proposed ideas about the role of what are known as “drug tolerant persisters” (or simply persisters), a non-genetic mechanism that allows for drug resistance [1]. Here I will discuss what is currently known about drug tolerant persisters, and how this form of drug resistance may confound efforts to target pre-existing resistance mutants. I will also introduce the proposed but not yet tested idea that cells may use the persister state to adapt to a drug challenge before eventually fixing their resistance phenotype in a genetic resistance mutation.

2.3.1 Persisters in microbial antibiotic resistance

The idea of “persisters” was first proposed in the field of microbial drug resistance in the 1940’s [97]. The idea behind persisters is that despite introduction of a drug bottleneck that kills the majority of cells, a small fraction of dormant cells will be able to survive this drug challenge without the use resistance mutations. Another characteristic of persisters is that upon the removal of drug, cells will resume growth

and their progeny will be drug sensitive. Thus, the persister state is thought to be a transient and reversible state. Finally, another proposed characteristic of persisters is that cells are randomly transitioning between a normal proliferating cell and a dormant persister cell capable of drug tolerance. Thus, persisters are thought to be a survival strategy at the population level, ensuring that the population will go on even in harsh conditions [97].

2.3.2 Persisters in Cancer

Often times, there are striking parallels between the nature of microbial pathogens and cancer. Of particular interest to this dissertation, in 2010, a group from Jeffrey Settleman's laboratory described a subpopulation of cancer cells with striking conceptual resemblance to bacterial persisters [1]. The authors note a clinical phenomenon known as the "retreatment response"; after a large response is seen, killing most tumor cells, occasionally if the patient is put on a "drug holiday", the resultant tumor that comes back will be sensitive to the same original drug [98].Inspired by this, they hypothesized that a reversible drug tolerant state analogous to bacterial persisters may exist in cancer cell populations, and developed a cell culture model system for studying what they termed "drug tolerant persisters" (DTPs) and "drug tolerant expanded persisters" (DTEPs). DTPs represent dormant cells that survive an extreme drug challenge (exceeding 100 times established IC50 values).

DTEPs are the name for DTPs that eventually resume growth in the presence of drug. They found that these populations were able to survive via insulin-like growth factor 1 (IGF1R) receptor signaling, which they proposed was linked to the modulation of the activity of histone modifier proteins, resulting in an altered chromatin state, leading to changes in gene expression that allow for growth in drug without the presence of a resistance mutation. Furthermore, they found that upon the removal of drug, that the resultant progeny of persister cells eventually reverted back to a drug sensitive state. Therefore, a population that shares characteristics of bacterial persisters exists in cancer.

2.3.3 Persisters represent a potential source of resistance not derived from pre-existing resistance mutants

The existence of persisters in cancer cell populations raises concerns about current strategies in managing the eventual drug resistance in EGFR addicted NSCLC. Persisters represent a mode of resistance that is not derived from the outgrowth of pre-existing cells with mutations that drive their resistance. Instead, persister mechanisms are a way for cells to survive the “problem” of a drug challenge without having a genetic “solution” upfront. Therefore, it is possible that persisters could drive resistance even in a theoretically ideal scenario where all pre-existing drug-resistance mutants are being targeted. Understanding cancer

persisters and their progeny therefore may be important towards the goal of optimizing strategies for limiting or preventing drug resistance.

2.3.4 Link between persisters and clinical resistance mutations?

Currently, it is not known to what degree persisters play a role in the clinic, as studies have only been performed in cell culture systems. The current, most prominently discussed model of drug resistance in EGFR addicted NSCLC is the expansion of pre-existing resistance mutants, since it has large support in the realms of both the basic science and clinical setting. How then does the persister-derived resistance model seen in the basic research setting reconcile with the clinically-validated model of expansion of pre-existing resistance mutants? Do these two models reconcile at all? Is the persister state in and of itself capable of driving drug resistance without mutation, or will resistance mutations eventually come to dominate the phenotypes of persister-derived lineages? Does the persister state represent an evolutionary dead end, not allowing for persister-dervied lineages to significantly drive resistance? Since cancer persisters are a relatively new observation, the answers to these questions remain outstanding. In this thesis, I show support for a model where the persister state is used as an intermediate growth strategy that eventually results in the fixation of a variety of clinically-observed resistance mutations.

CHAPTER THREE

Diverse Drug Resistance Mechanisms can Emerge from Drug Tolerant Cancer Persister Cells

3.1 Introduction

The emergence of diverse resistance mechanisms to targeted therapy is one of the foremost challenges in cancer today [99]. Within the same patient or even tumor, multiple mechanisms for drug resistance can coexist [96,100,101]. Random, resistance-conferring genetic events preceding drug treatment are an unquestionable means by which this diversity can occur [78,79,102]. Yet, understanding alternate routes by which cancer cell populations can arrive at resistance mechanisms is of key interest.

One recently proposed alternative route for acquiring resistance is *via* a drug-tolerant persister state (Fig 3.1) [1,97,103]. Across multiple cell lines, in response to a variety of strong drug challenges, small sub-populations of cells have been reported to survive by initially entering a persister state in which there is little to no population growth [1]. Crucially, after long-term treatment (weeks to months) in drug without appreciable growth, a fraction of persisters gain the ability to expand in drug-containing media. It has been hypothesized that survival and expansion through a

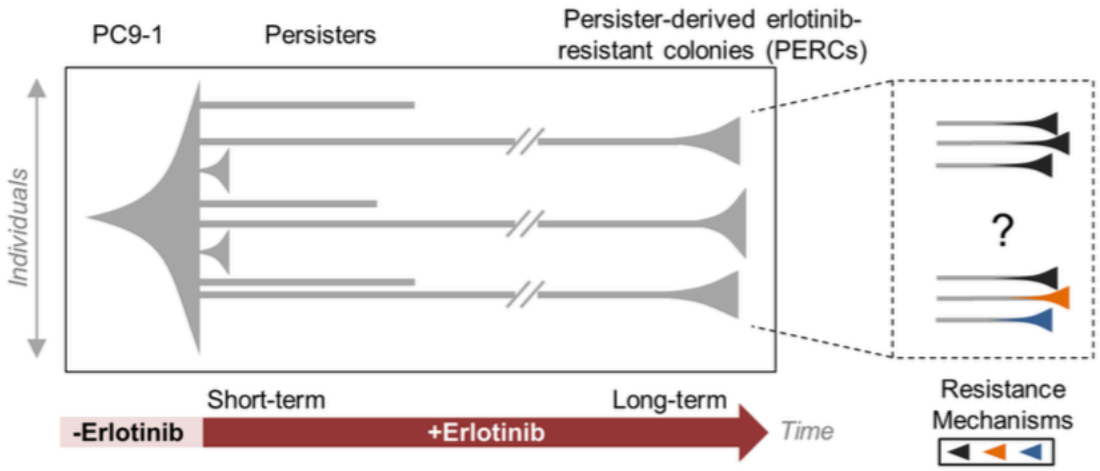


Figure 3.1: The emergence of PC9-1, persister-derived, erlotinib-resistant colonies (PERCs)
Schematic outline of the emergence of drug-resistant cancer cell populations (right), originating from a common clone (left), through the bottleneck of drug-tolerant, slow-growing persisters (middle gray lines). Vertical axis indicates population size; horizontal axis is time.

drug-tolerant state could be part of an initial strategy that mediates the acquisition of *bona fide*, genetically driven, resistance mechanisms [1]. However, the diversity of resistance mechanisms compatible with evolution from (or through) a persister bottleneck is unclear, and it is also unknown if cells can evolve from persister states to develop clinically validated mechanisms of resistance. Previous work examined pooled populations of drug-tolerant cells expanded from persisters [1] and did not

address this question. Does passage through the persister bottleneck in drug force cells into a single genetic/epigenetic state, or is the persister state a branching point from which multiple genetic resistance mechanisms can eventually emerge (Fig. 3.1)?

3.2 Results

3.2.1 Isolation of Persister-Derived Erlotinib-Resistant Colonies (PERCs)

To investigate this question, we chose as our model system the well-studied EGFR-addicted non-small cell lung cancer (NSCLC) cell line PC9 [104], where small numbers of cells enter a persister state to evade the strong selective pressure of high concentrations of the EGFR inhibitor erlotinib (2.5μM, ~100 x IC50) [1]. For our study, we followed the previously established procedure with two crucial changes (discussed next), which allowed us to focus on individual resistance solutions that emerged from persisters (Materials and Methods 4.1, Fig. 3.2).

First, to reduce pre-existing genetic heterogeneity, we established our persisters from a single, short-passage clonal parental cell line, PC9-1 (~20 doublings from the single, originating cell). Similar to previous observations about the generation of persisters, only a small fraction [1] (~0.5%) of PC9-1 cells were

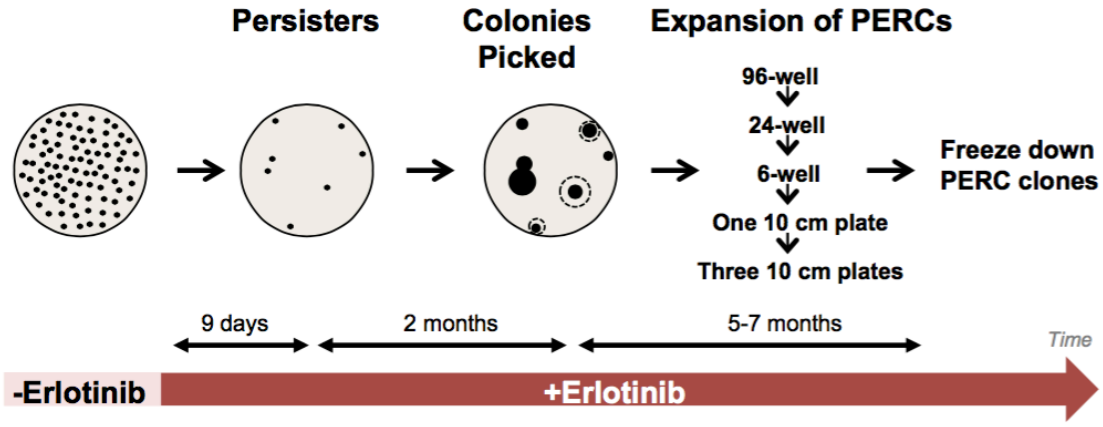


Figure 3.2: Schematic representation of PERC isolation process.
Schematic representation of PERC isolation process. 1 X 10^5 cells were plated on several 10 cm plates, allowed to stabilize overnight then treated with 2.5 μM erlotinib-containing media. After 9 days, drug-tolerant persisters remained. Sizable colonies were visible 2 months after initial persister stage, and spatially separated colonies were picked and transferred to individual wells of a 96-well plate. Over the course of ~7 ± 1.5 months, selected colonies were expanded by transfer to larger cell culture vessels.

observed to survive drug treatment (2.5 μM erlotinib). Our persisters were largely in a state of negligible growth during the first six weeks of observation. Second, to search for diversity not evident from pooled-population studies, we isolated small, recently expanded colonies that emerged ~2 months after seeding and expanded them in separate culture wells to eliminate growth competition. (Except when noted otherwise, colonies were cultured and assayed in 2.5 μM erlotinib.) Of the ~50 colonies originally isolated, 17 survived the expansion process (~7 ± 1.5 months to

generate three confluent 10cm plates; communication of unpublished results from Jeff Engelman's laboratory suggest that this time scale is consistent with the growth of persister-derived resistance rather than the more immediate expansion of intrinsically resistant cells that harbor resistance mutations before drug treatment). We refer to these PC9-1, persister-derived erlotinib-resistant colonies as PERCs. Both intra- and inter-colony heterogeneity in signaling and morphology was evident (Fig 3.3), consistent with previous observations of clonally derived populations [105].

3.2.2 PERCs Exhibit Stable Drug Resistance Indicating Fixation of Resistance Mechanisms

We tested whether our isolated PERCs were in the previously described, "meta-stable" state of drug resistance [1]. A functional signature of this state is eventual reversion to erlotinib sensitivity after an extended "drug holiday" [1] (30 passages). However, we observed no dramatic reversion to the erlotinib-sensitive level of the original parental PC9-1, even after continuously culturing the PERCs in erlotinib-free media for over 40 weeks (Fig 3.4; for our PERCs, 1 passage ≈ 1 week; Materials and Methods 4.1 and 4.2). Most PERCs had IC50s (50% viability at 2.5 μM; Fig 3.4) that were over a hundred fold greater than the IC50 of PC9-1 and remained stable between 20-40 weeks. The one apparent exception, PERC 3,

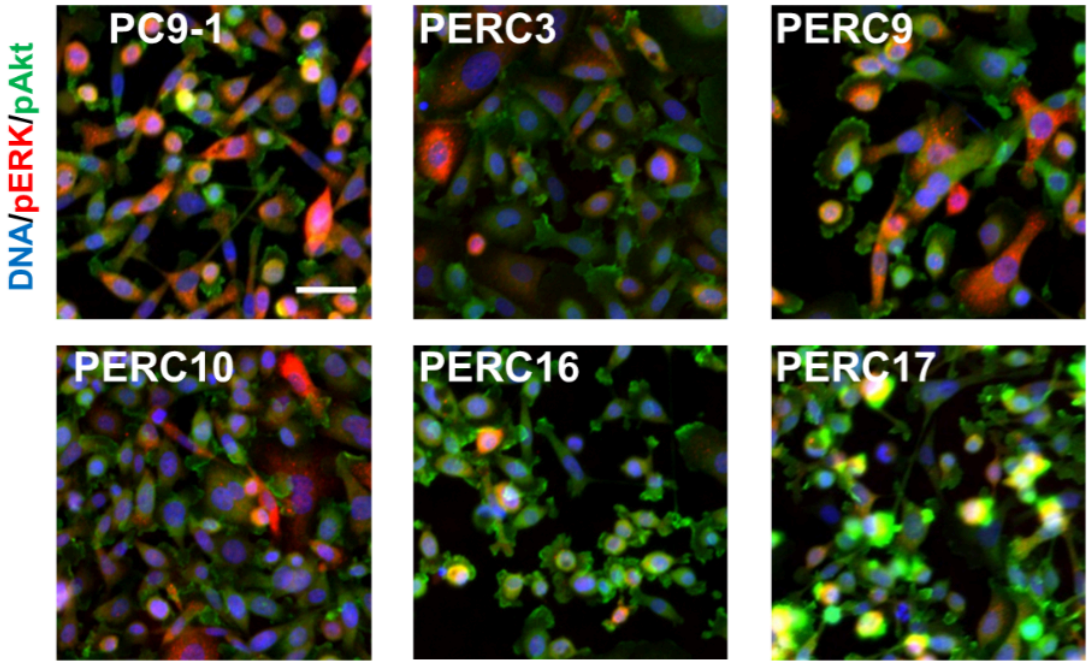


Figure 3.3: Diversity in signaling and morphology in select PERCs.
Sample fluorescence microscopy images of selected PERCs stained for DNA, pERK1(Y204)/pERK2(Y187) and pAKT(S473). Scalebar: 10 µm.

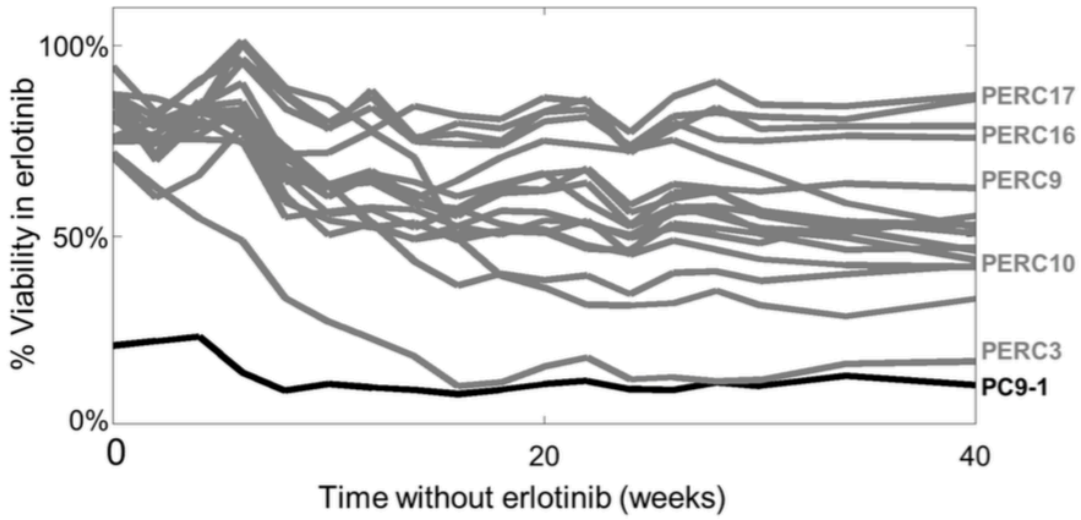


Figure 3.4: Evolution of PERC sensitivity to erlotinib after removal from drug treatment.
PERCs were grown in drug-free media and periodically retested over ~40 weeks for erlotinib sensitivity (2.5 μM erlotinib, 72 hr CellTiter-Glo assay; Materials and Methods 4.3). Black: PC9-1; gray: PERCs.

exhibited a slow-growth response [106], rather than cell death response like PC9-1, after being re-treated with erlotinib (Fig. 3.5). As our PERCs appeared not to be in the previously described state, we wondered what drug-resistance mechanisms our PERCs had acquired.

3.2.3 Use of Drug Response Profiling to Identify Putative Mechanisms of Resistance

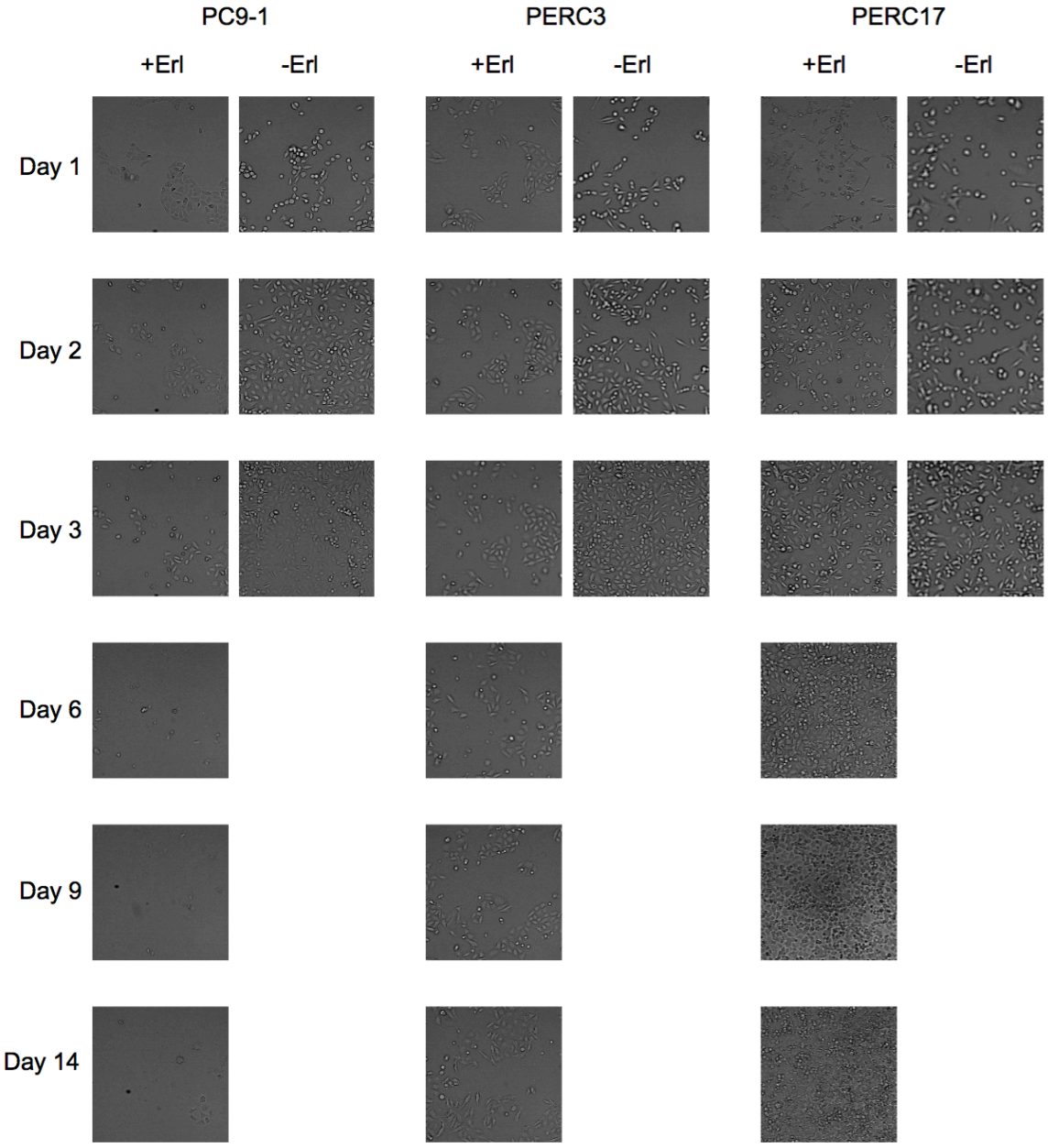

PC9-1
PERC3
PERC17
+Erl
-Erl
+Erl
-Erl
+Erl
-Erl
Day 1
Day 2
Day 3
Day 6
Day 9
Day 14

Figure 3.5: Slow-growth response of PERC3 to erlotinib after long-term drug holiday

Bright field images of PERC3 after long-term growth in drug-free media and subsequent re-treatment with 2.5 μM erlotinib (rows indicate specified days after erlotinib treatment and columns are cell line/drug combinations). Visual inspection reveals that the ratio of cells +/- erlotinib are comparable for PC9-1 and PERC3 after 3 days of drug treatment (and both ratios are lower than for PERC17) in agreement with the CellTiter-Glo result (Fig. 3.4). However, in subsequent days with erlotinib treatment, while the PC9-1 population is extinguished, PERC3 continues to grow (albeit slower than the other PERCs; non-treated plates become overgrown by day 6, data not shown).

To investigate erlotinib-resistance mechanisms present in our 17 PERCs, we performed a large-scale drug screen [24]. This allowed us to scan for therapeutic vulnerabilities among our PERCs that were absent in PC9-1 and thereby identify pathway or target alterations that conferred resistance. We assayed the sensitivities of our PERCs to a panel of 560 anticancer compounds (Methods 4.3; Appendix A). To search broadly for potential vulnerabilities, the panel included a diverse collection of compounds, including kinase inhibitors affecting multiple cancer-related pathways and drugs targeted to the specific erlotinib-resistance-conferring T790M-EGFR mutation [107-110], as well as chemotherapy and epigenetic drugs (Fig. 3.6, middle panel). All 560 compound assays were performed over a 6-fold dosage range, in duplicate and for all 17 PERCs and control PC9-1.

We focused on identifying PERCs whose drug responses were strongly altered from PC9-1. There are a number of approaches to access drug sensitivity from dose-response curves [4]; here, we chose to compute a score based on signed-area differences between drug-response curves of PERCs *vs.* PC9-1 that took into account experimental variability (Fig. 3.6 right, Pimasertib example; Materials and Methods 4.3, 4.7 and 4.8). The PERCs displayed diverse patterns of drug responses (Fig. 3.6, top panel). A few broad trends were noticeable. As compared with PC9-1, PERCs were generally resistant to EGFR inhibitors (as might be expected), Aurora Kinase Inhibitors and chemotherapeutics. Further, some

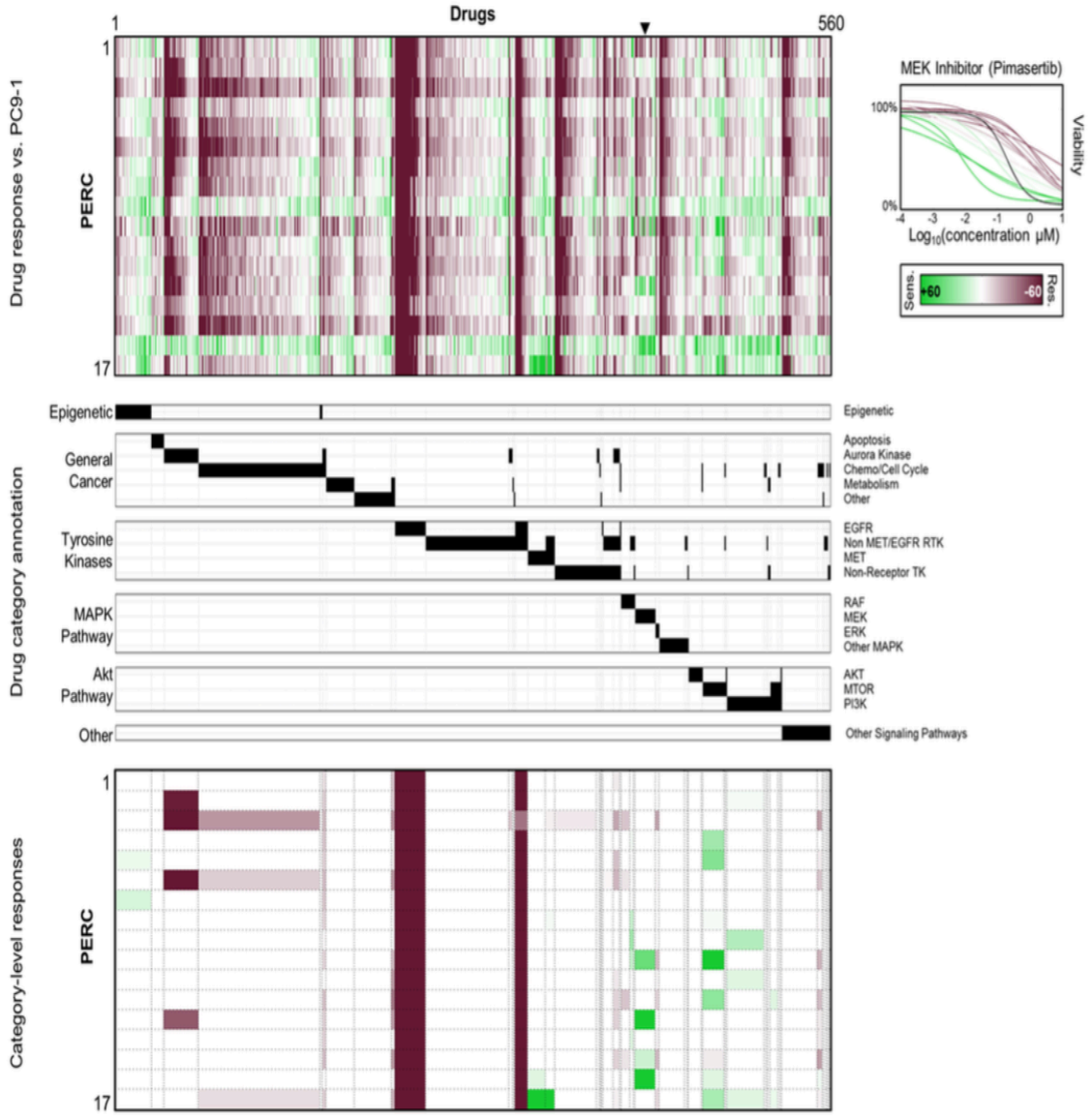

Drugs
1
560
Drug response vs. PC9-1
PERC
1
17
MEK Inhibitor (Pimasertib)
100%
0%
Viability
-4
-3
-2
-1
0
1
Log10(concentration µM)
Sens.
+60
-60
Res.
Drug category annotation
Epigenetic
General Cancer
Tyrosine Kinases
MAPK Pathway
Akt Pathway
Other
Epigenetic
Apoptosis
Aurora Kinase
Chemo/Cell Cycle
Metabolism
Other
EGFR
Non MET/EGFR RTK
MET
Non-Receptor TK
RAF
MEK
ERK
Other MAPK
AKT
MTOR
PI3K
Other Signaling Pathways
Category-level responses
PERC
1
17

Figure 3.6: Identification of PERC drug-resistance mechanisms *via* drug screening for therapeutic vulnerabilities

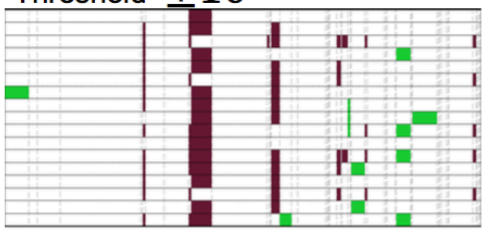
Response of PERCs vs. PC9-1 to a diverse drug library.
(Top) Heatmap: drug-response scores of the PERCs relative to PC9-1 (rows) for 560 anti-cancer compounds (columns). Scores are based on signed-area differences between drug-response curves of PERCs *vs.* PC9-1 (Materials and Methods 4.7). (Top right) Example of drug-response curves (Pimasertib; black triangle in top heatmap) for PC9-1 (black) vs PERCs (response curves are colored according to scores). Green/red: relative drug sensitivity/resistance of PERC compared to PC9-1.
(Middle) Annotation of drugs (columns) to specific drug categories (rows).
(Bottom) Heatmap: enrichment of PERCs (rows) for strong response to specific drug categories (columns). Category-level response scores are based on hyper-geometric test for varying drug-response scores (Materials and Methods 4.9, Fig. 3.7; colormap as in top panel).

PERCs developed broad resistance (e.g. PERC3) or sensitivity (e.g. PERC16) to drugs belonging to multiple drug classes.

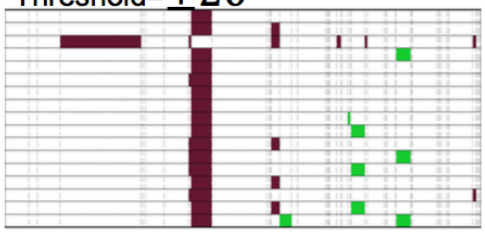
We searched for robust evidence of specific PERC vulnerabilities within drug categories. It is not to be expected that a PERC would respond similarly to every drug within the same category. Thus, to search for vulnerabilities, we developed a category-level response score to search for evidence of sensitivities to a larger-than-expected fraction of drugs in each defined category (Fig. 3.6, bottom panel; Materials and Methods 4.9; Fig. 3.7). There was no single category for which all 17 PERCs were vulnerable. However, we identified specific vulnerabilities of: PERC17 to MET drugs (including SGX-523, INCB28060, and JNJ-38877605); PERCs 10,13,16 to MEK inhibitors (including Selumetinib, Pimasertib, and PD0325901); and PERCs 4,5,10,17 to mTOR drugs (including Rapamycin and Everolimus). Taken together, our drug screen identified mechanistically distinct and clinically observed vulnerabilities, suggesting that our PERCs evolved multiple strategies to escape erlotinib treatment.

3.2.4 Whole-Exome Sequencing Reveals *Bona Fide* Genetically-Driven Erlotinib Resistance Mechanisms in PERCs

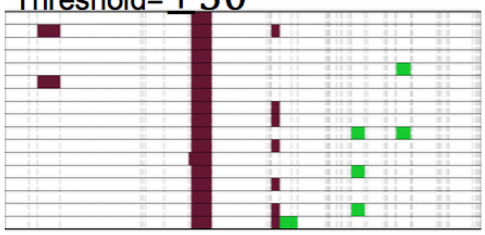
Threshold=±10



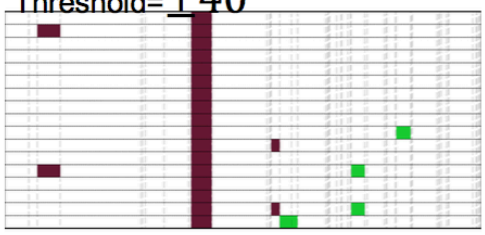
Threshold=±20



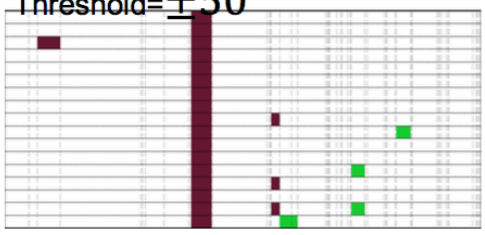
Threshold=±30



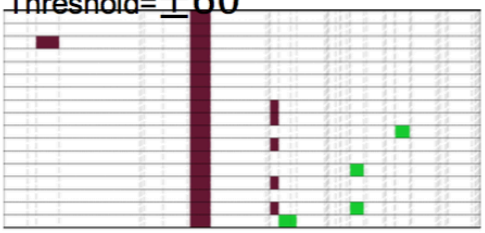
Threshold=±40



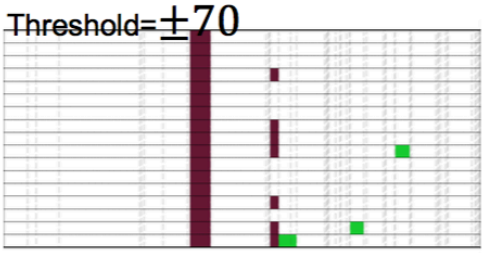
Threshold=±50



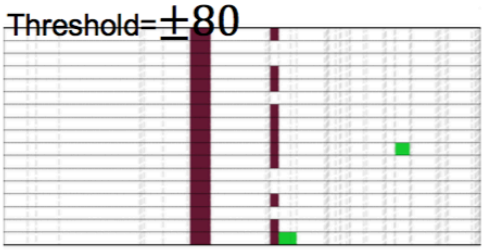
Threshold=±60



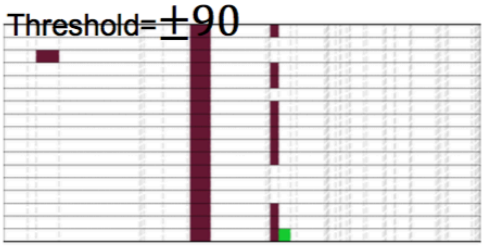
Threshold=±70



Threshold=±80



Threshold=±90



Threshold=±100

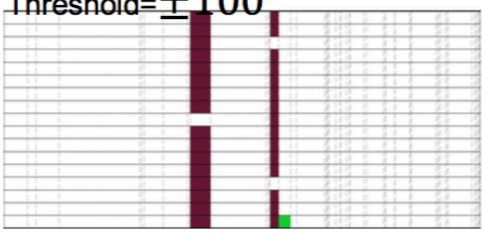


Figure 3.7: Enrichment for strong response in drug categories as a function of threshold above which response is considered strong.
A PERC is deemed to show enrichment for strong response in a drug category if a higher than expected (Bonferroni corrected hyper-geometric p-value<0.01) number of drugs elicit a "strong response"; strong response is defined in terms of the drug-response score for a drug/PERC combination exceeding a specified threshold. Shown here is the effect of varying this threshold; each panel corresponds to a specified threshold value (each row is a PERC and columns are drugs). All drugs in a category are colored green/red for a PERC if the drug category is deemed to elicit strongly increased/decreased sensitivity as defined above.

We next sought to use genetics as a way to corroborate predicted therapeutic vulnerabilities as well as to identify mechanisms that were not detected by our initial analysis of the drug screen. From our exome sequencing data, we identified genetic changes between each PERC and the parent PC9-1[111] (Fig. 3.8a, Materials and Methods 4.4, 4.10). The derivation of the PERCs from a single, clonal parent offered a unified basis to identify and interpret genetic changes (below we report on amplification > 2.5x compared to PC9-1).

First, we searched for the most commonly observed mechanism of erlotinib resistance found in the clinic—the T790M mutation in EGFR [109,110]. Based on the sequence data, we found this mutation present in PERCs 1,4-9 (Figs. 3.8a, Fig. 3.10; Appendix B). This caused us to reevaluate our analysis of the drug screen. While all PERCs became more resistant to EGFR drugs when compared to PC9-1 (Fig. 3.6), comparison of PERCs to each other revealed that those harboring a T790M mutation showed an increased, albeit partial, sensitivity to T790M-targeting drugs (including Afatinib, Dacomitinib, and WZ3146[107]; Fig. 3.8b, Figs. 3.9 and 3.10). (Unpublished results from Jeff Engelman's laboratory, showing reduced sensitivity of late-emerging clones with T790M mutations arising after drug treatment, are consistent with our results). We further confirmed the presence of a MET amplification [23,101] in PERC 17, which showed exquisite sensitivity to MET inhibitors and apoptosis from siRNA MET knockdown (Fig. 3.8c-d; Figs. 3.9, 3.11). Thus, MET

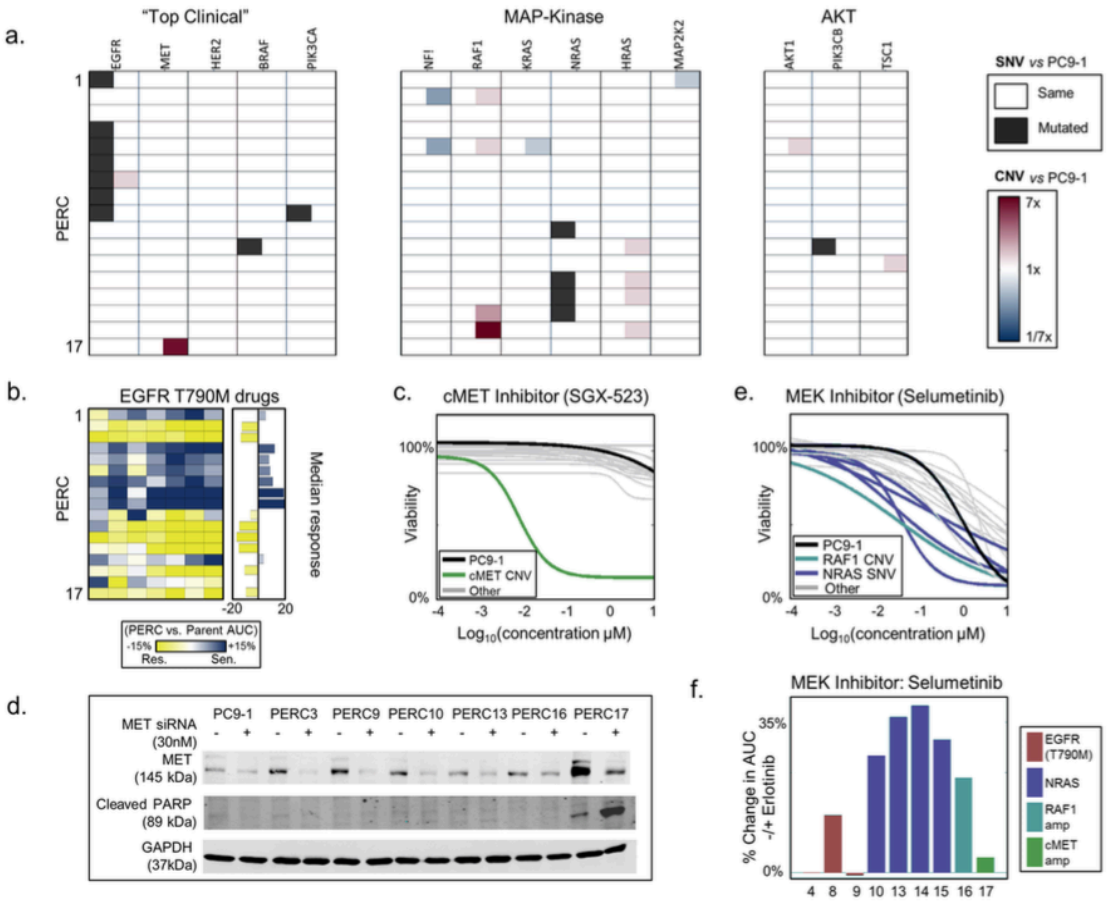

a.
"Top Clinical"
MAP-Kinase
AKT
EGFR
MET
HER2
BRAF
PIK3CA
NF1
RAF1
KRAS
NRAS
HRAS
MAP2K2
AKT1
PIK3CB
TSC1
PERC
1
17
SNV vs PC9-1
Same
Mutated
CNV vs PC9-1
7x
1x
1/7x
b.
EGFR T790M drugs
Median response
(PERC vs. Parent AUC)
-15%
+15%
Res.
Sen.
c.
cMET Inhibitor (SGX-523)
Viability
100%
0%
PC9-1
cMET CNV
Other
Log10(concentration μM)
e.
MEK Inhibitor (Selumetinib)
PC9-1
RAF1 CNV
NRAS SNV
Other
d.
MET siRNA (30nM)
PC9-1
PERC3
PERC9
PERC10
PERC13
PERC16
PERC17
MET (145 kDa)
Cleaved PARP (89 kDa)
GAPDH (37kDa)
f.
MEK Inhibitor: Selumetinib
% Change in AUC -/+ Erlotinib
35%
0%
EGFR (T790M)
NRAS
RAF1 amp
cMET amp

Figure 3.8: Assessment of PERC drug-resistance mechanisms *via* genetics and specific perturbations

a. Comparison of genetic alterations in PERCs (rows) for selected genes implicated in erlotinib resistance (columns). Black: presence of a non-synonymous single nucleotide variant (SNV) *vs.* PC9-1; Red/blue: copy number variations (CNV) corresponding to amplification/deletions of genetic regions (Materials and Methods 4.4, 4.10) *vs.* PC9-1. Panels show genes most commonly implicated in erlotinib resistance in the clinic (left), MAP-Kinase pathway (middle) and AKT pathway (right).

b-f. Corroboration of genetic information with predicted vulnerabilities.

b. Response among PERCs to drugs targeting the EGFR T790M mutation: (Left) Heatmap: relative sensitivity among PERCs (rows) to drugs targeting the EGFR T790M mutation (columns). Drug response is assessed by deviation of the AUC of a PERC from the mean AUC among all PERCs (Materials and Methods 4.11). Blue/yellow: relative drug sensitivity/resistance of PERC compared to other PERCs. (Right) Bar plot showing median response across drugs.

c,e. Dose-response curves of the PERCs screened for specified compounds (**c,** SGX-523 targeting cMET; **e,** Selumetinib targeting MEK). In each plot PC9-1 is black, lines with relevant mutations are colored as indicated, and other PERCs are gray.

d. Transient siRNA-mediated knockdown of MET in PC9-1 and a subset of PERCs. MET knockdown in PERC17 (only cell line with MET amplification) induces increased cleavage of PARP, a marker for apoptosis. GAPDH: loading control.

f. Drug responses (concentrations as in panel **e**) of various PERCs to the MEK inhibitor Selumetinib were measured +/- erlotinib. Bars indicate the strength of MEK bypass based on a previously proposed criterion: the extent to which the area under this drug response curve is changed by the presence of erlotinib. PERCs with mutations in NRAS and RAF1 (both upstream of MEK) show stronger evidence for the use of MEK as a bypass mechanism for erlotinib.

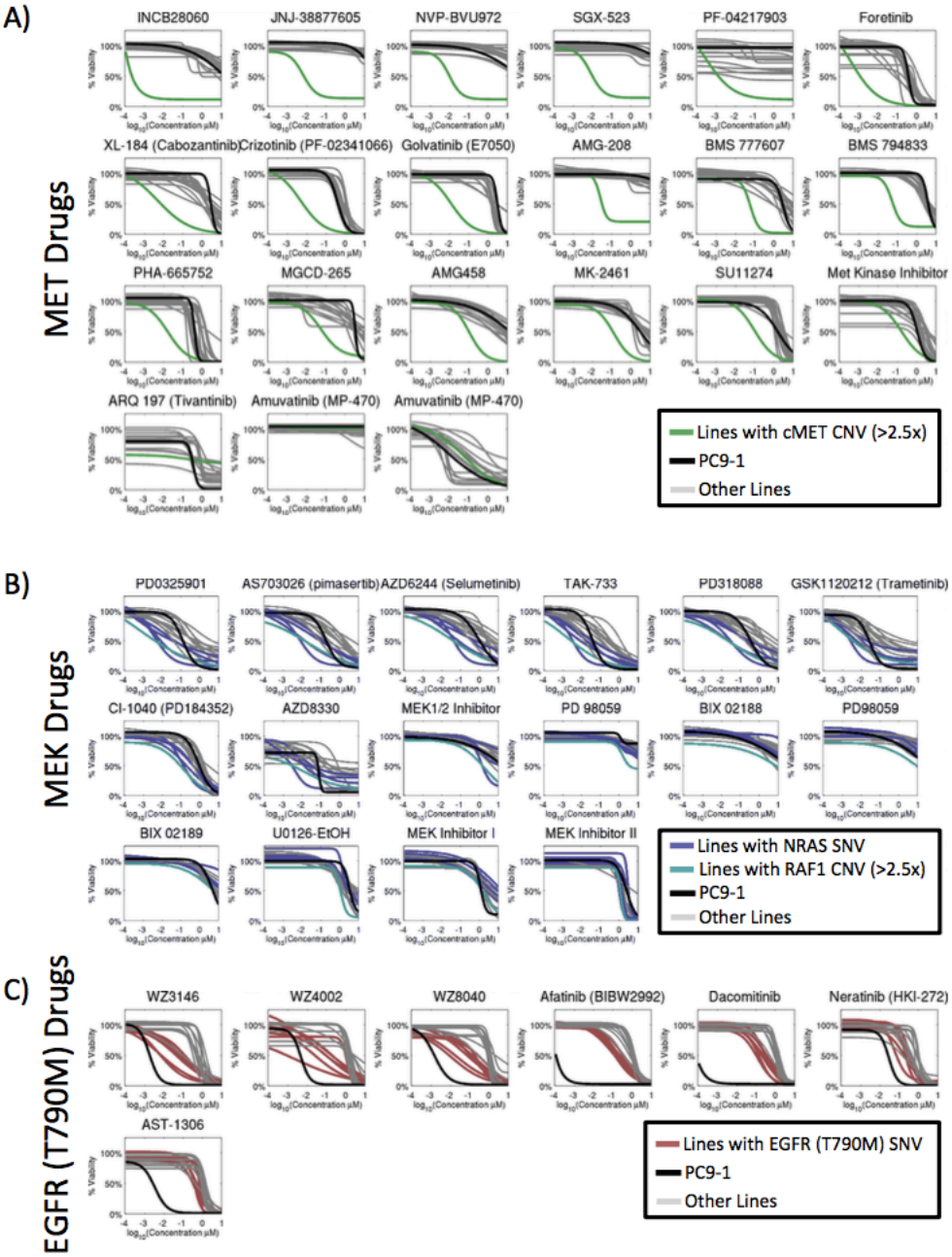
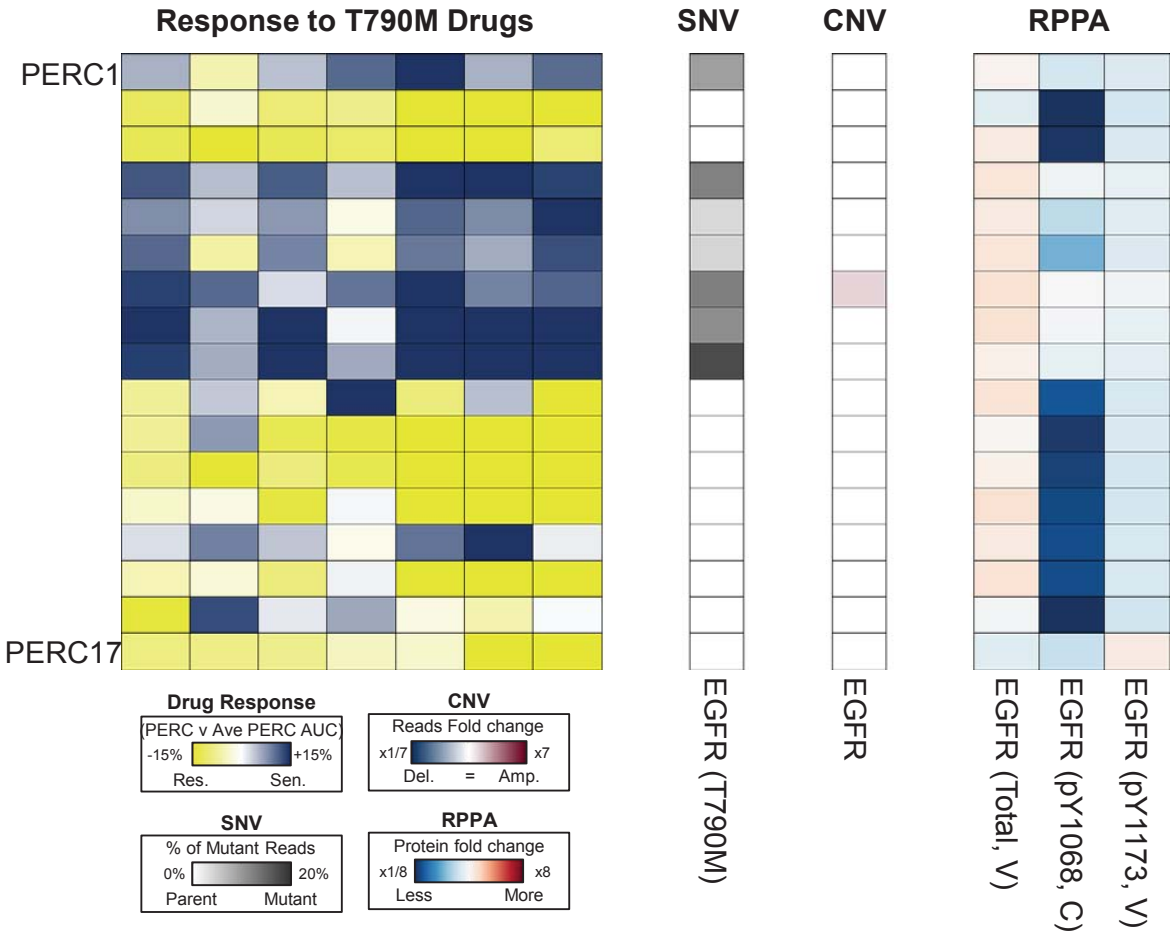
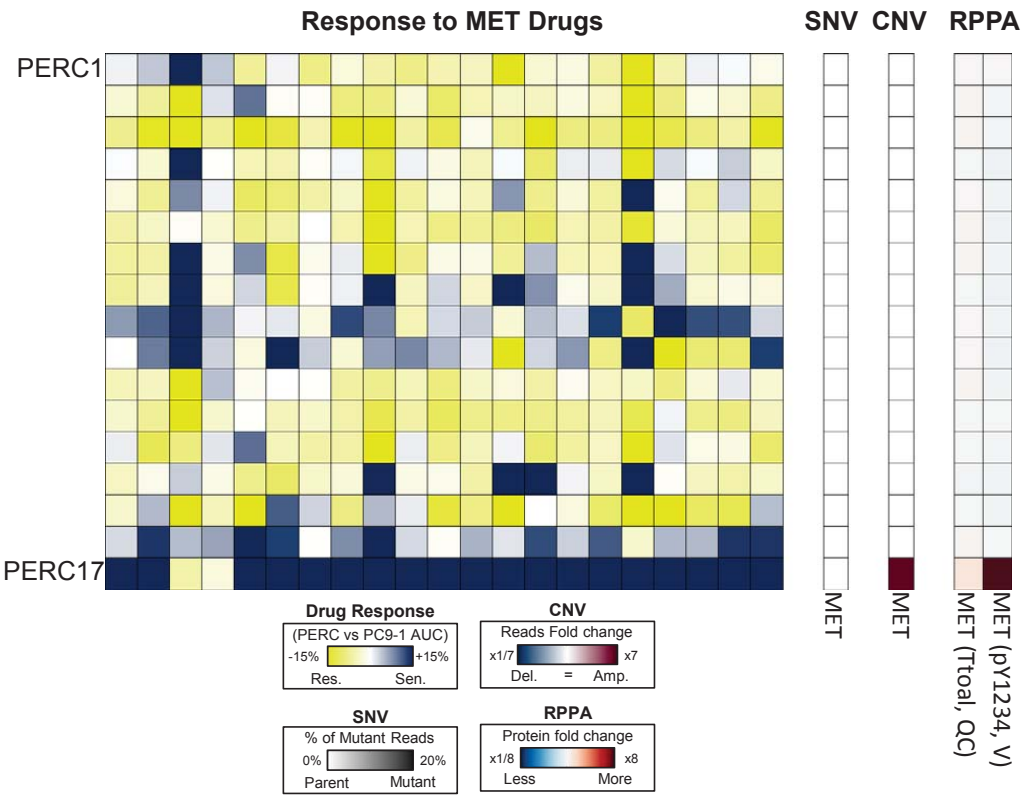
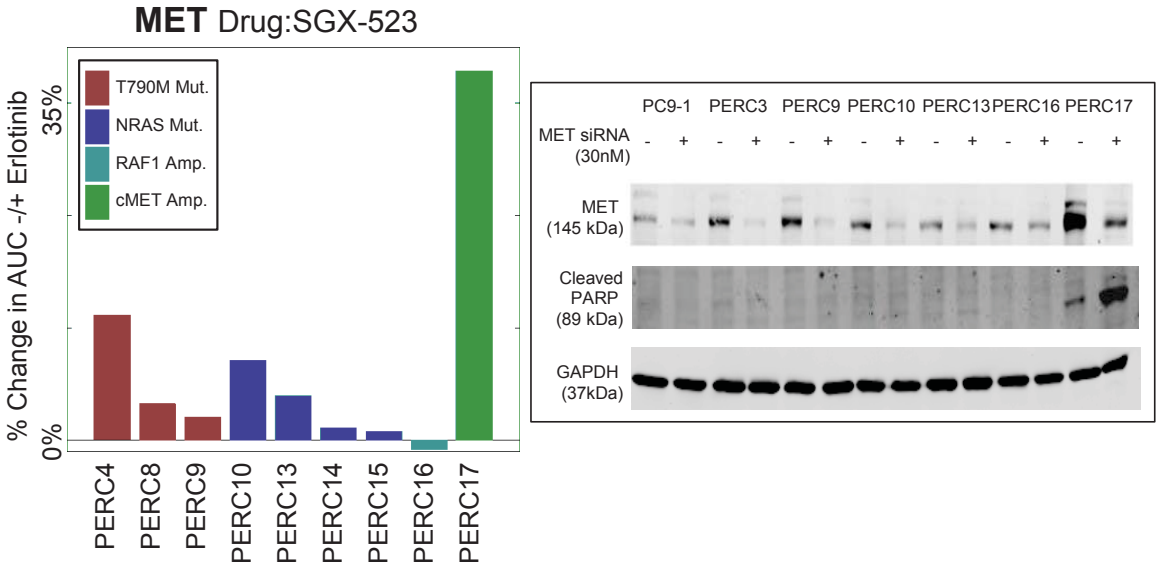
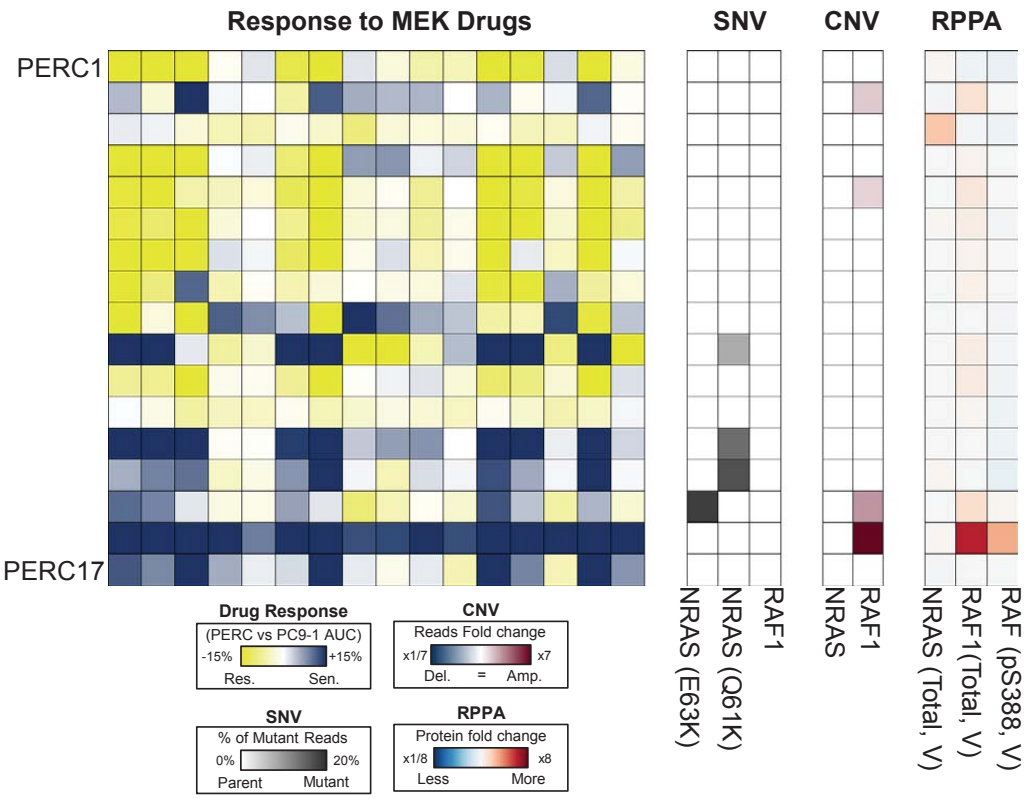

A)
MET Drugs
INCB28060
JNJ-38877605
NVP-BVU972
SGX-523
PF-04217903
Foretinib
XL-184 (Cabozantinib)
Crizotinib (PF-02341066)
Golvatinib (E7050)
AMG-208
BMS 777607
BMS 794833
PHA-665752
MGCD-265
AMG458
MK-2461
SU11274
Met Kinase Inhibitor
ARQ 197 (Tivantinib)
Amuvatinib (MP-470)
Amuvatinib (MP-470)
Lines with cMET CNV (>2.5x)
PC9-1
Other Lines
B)
MEK Drugs
PD0325901
AS703026 (pimasertib)
AZD6244 (Selumetinib)
TAK-733
PD318088
GSK1120212 (Trametinib)
CI-1040 (PD184352)
AZD8330
MEK1/2 Inhibitor
PD 98059
BIX 02188
PD98059
BIX 02189
U0126-EtOH
MEK Inhibitor I
MEK Inhibitor II
Lines with NRAS SNV
Lines with RAF1 CNV (>2.5x)
PC9-1
Other Lines
C)
EGFR (T790M) Drugs
WZ3146
WZ4002
WZ8040
Afatinib (BIBW2992)
Dacomitinib
Neratinib (HKI-272)
AST-1306
Lines with EGFR (T790M) SNV
PC9-1
Other Lines

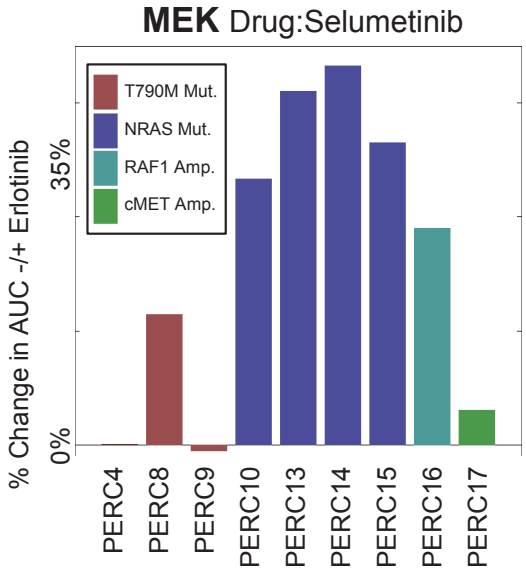
Figure 3.9: PERCs drug response curves to MET, MEK and T790M targeting drugs.
Each plot represents a single drug (as in Figs. 3.8.c,e). x-axis: drug concentration; y-axis: titer-glo intensity with respect to a DMSO control. The PC9-1 curves are show in black. A) MET Drugs: PERC 17 which has a cMET amplification is marked in green, while the other PERCs are marked in gray. B) MEK Drugs: Lines with mutations upstream of MEK are colored. PERCs 10,13,14 & 15 which have an NRAS mutation are marked in blue. PERC 16, which is the only PERC with a >2.5 fold RAF1 amplification is in Cyan. All other lines are marked in gray. C) T790M drugs: PERCs 1,4,5,6,7,8,9 with the T790M mutation are marked in red. All other lines are marked in gray.


Response to T790M Drugs
SNV
CNV
RPPA
PERC1
PERC17
Drug Response
(PERC v Ave PERC AUC)
-15%
+15%
Res.
Sen.
CNV
Reads Fold change
x1/7
x7
Del.
=
Amp.
SNV
% of Mutant Reads
0%
20%
Parent
Mutant
RPPA
Protein fold change
x1/8
x8
Less
More
EGFR (T790M)
EGFR
EGFR (Total, V)
EGFR (pY1068, C)
EGFR (pY1173, V)


Response to MET Drugs
SNV
CNV
RPPA
PERC1
PERC17
MET
MET
MET (pY1234, Y)
MET (Ttoal, QC)
Drug Response
(PERC vs PC9-1 AUC)
-15%
+15%
Res.
Sen.
CNV
Reads Fold change
x1/7
x7
Del.
=
Amp.
SNV
% of Mutant Reads
0%
20%
Parent
Mutant
RPPA
Protein fold change
x1/8
x8
Less
More


MET Drug:SGX-523
T790M Mut.
NRAS Mut.
RAF1 Amp.
cMET Amp.
% Change in AUC -/+ Erlotinib
35%
0%
PERC4
PERC8
PERC9
PERC10
PERC13
PERC14
PERC15
PERC16
PERC17
PC9-1
PERC3
PERC9
PERC10
PERC13
PERC16
PERC17
MET siRNA (30nM)
MET (145 kDa)
Cleaved PARP (89 kDa)
GAPDH (37kDa)


Response to MEK Drugs
SNV
CNV
RPPA
PERC1
PERC17
NRAS (E63K)
NRAS (Q61K)
RAF1
NRAS
RAF1
NRAS (Total, V)
RAF1(Total, V)
RAF (pS388, V)
Drug Response
(PERC vs PC9-1 AUC)
-15%
+15%
Res.
Sen.
CNV
Reads Fold change
x1/7
x7
Del.
=
Amp.
SNV
% of Mutant Reads
0%
20%
Parent
Mutant
RPPA
Protein fold change
x1/8
x8
Less
More


MEK Drug:Selumetinib
T790M Mut.
NRAS Mut.
RAF1 Amp.
cMET Amp.
% Change in AUC -/+ Erlotinib
35%
0%
PERC4
PERC8
PERC9
PERC10
PERC13
PERC14
PERC15
PERC16
PERC17

Figures 3.10-3.12: Summary drug, genetic and RPPA data supporting identified drug vulnerabilities.
Drug data and genetic annotation are as in Figs. 3.6 and 3.8. RPPA is described in Methods; confidence annotation for probes, provided by MD Anderson, indicate: V (validated), C (caution) and QC (validated for use in cell lines, but not tissue samples).

amplification is a *bona fide* resistance mechanism for PERC17. To our knowledge, a MET amplification has never previously been reported for the parent (PC9) line. Together, these two mechanisms, T790M and MET, account for over half of clinically observed resistance mechanisms to first generation EGFR inhibitors [2] (e.g. erlotinib and gefitinib).

We next examined genetic changes in the MAPK pathway, one of the most frequently mutated pathways associated with erlotinib resistance [112]. We observed point mutations in NRAS for PERCs 10,13,14 (Q61K) and PERC15 (E63K), two mutational events that have been implicated in erlotinib resistance (Figs. 3.8a, 3.9, 3.12) [113]. We also observed amplification in RAF1 in PERC16 (Figs. 3.8a, 3.9, 3.12), a genetic alteration that has not been reported in lung cancer, but that has been characterized as a driver mutation in other cancer types [114]. We used our genetic data to revisit our drug screen, and found that PERCs 10,13,16 were sensitive to MEK drugs (including Selumetinib), consistent with their upstream mutations (Figs. 3.6, 3.8.e, 3.9, 3.12, 3.14) [115]. PERCs 14,15 did not display this sensitivity across all drugs in our initial analysis of the drug screen (Fig. 3.6); however, re-examination of response curves, overlaid with genetic data, revealed all NRAS and RAF1 mutants had evident MEK sensitivities (Fig. 3.8e, 3.12). Further testing revealed that all PERCs carrying NRAS and RAF1 mutations had higher sensitivity to co-treatment

with erlotinib and MEK inhibitors than either alone, suggesting a role for MEK in "bypass" signaling [24,116] (Fig. 3.8f).

As might be expected, not every genetic mechanism was found to correspond to drug vulnerability. For example, we observed a mutation in PIK3CA (E542K); this mutation is implicated in driving constitutive signaling to AKT [117], which was not corroborated with drug sensitivities (Fig. 3.8a, 3.6). PERC3 also stood out as having nearly 3 fold more mutations than any other PERC, potentially due to mutation in the DNA polymerase gene PolN [118]; Fig. 3.13). Further, not every drug vulnerability was found to correspond to an obvious genetic mechanism. For example, we observed mTOR sensitivity observed in PERC 10 for which we could not find any obvious genetic basis (Fig. 3.6, 3.8a). Nevertheless, in total we discovered pharmacological and genetic (as well as corroborating reverse phase protein array (RPPA) evidence [119]; Materials and Methods 4.6, 4.11, Figs. 3.10-3.12) mechanisms of erlotinib resistance in 13 of our 17 PERCs (Fig. 3.14).

3.3 Discussion

Cancer therapy has traditionally been focused on eliminating fast-growing cells. Here, we focused on drug-resistant cancer populations that emerge from a persister state in which cells show little to no growth for weeks to months in drug

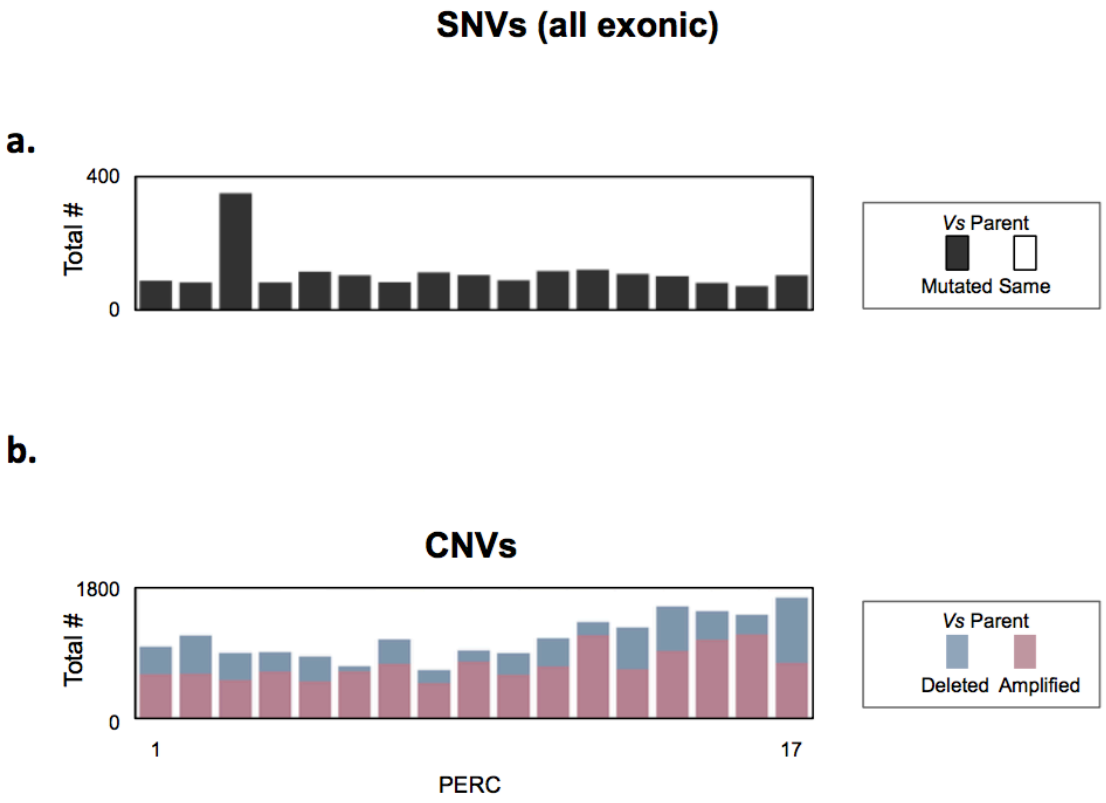


Figure 3.13: Abnormally high number of SNV mutations for PERC3.
Bar plots show the total number or exonic SNVs (**a**) or CNVs (**b**) per PERC

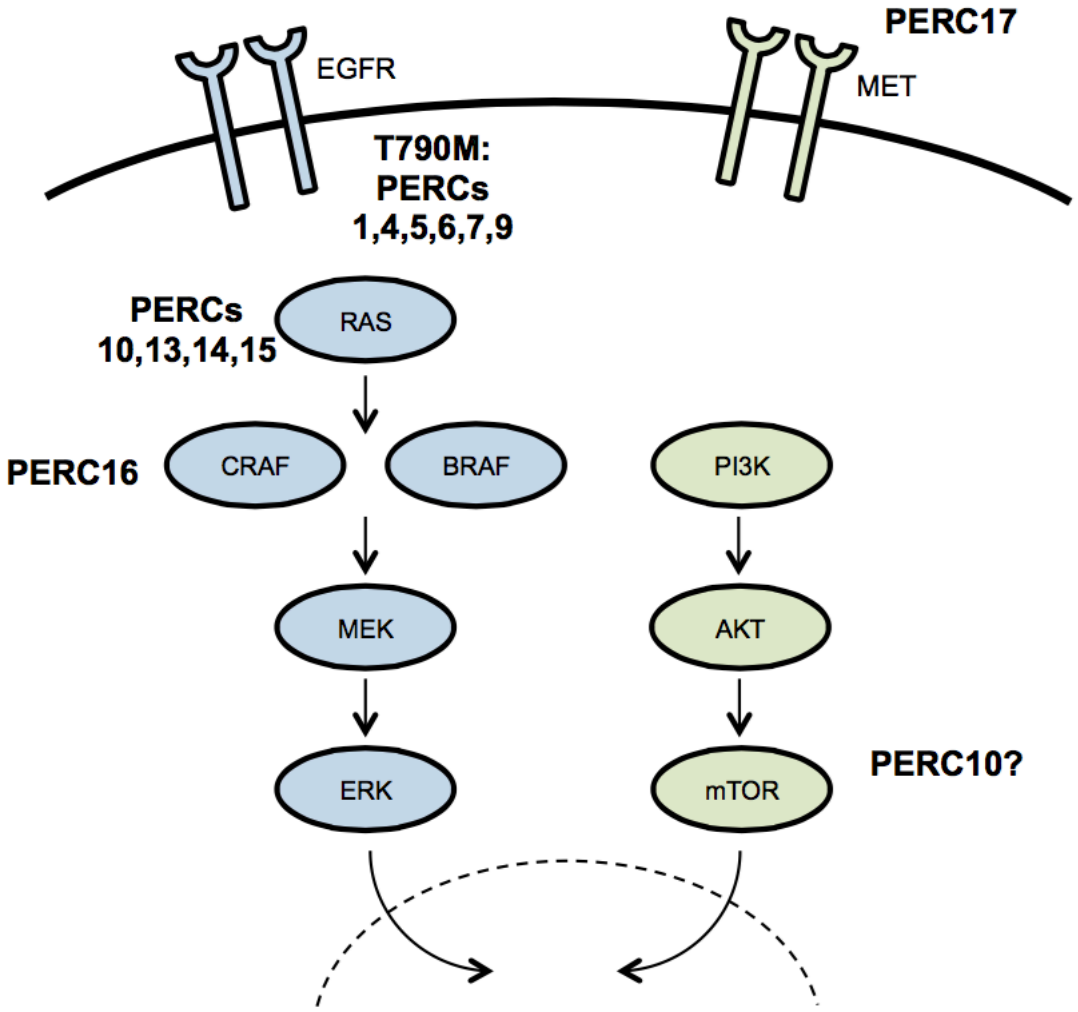


Figure 3.14: Summary of drug resistance mechanism across our PERCs.
PERC17 harbored a MET amplification. PERCs 1,4,5,6,7,9 harbored the T790M mutation in EGFR. PERCs 10,13,14,15 harbored mutations in NRAS. PERC16 harbored a C-RAF (RAF1) amplification. PERC10 showed drug-sensitivity to mTOR drugs, which could not be explained with genetic information.

treatment. We found that diverse, clinically observed drug-resistance mechanisms can emerge from persisters, derived from a single, recent ancestor cell and grown under the same selective pressure. Persisters, which are a small subpopulation of the bulk cancer population, are difficult to study in a clinical setting, and there is no known molecular signature of having passed through this state clinically. Cell culture provided a relevant model to investigate heterogeneity of drug-resistance mechanisms. We did not need to alter physiological conditions or microenvironment to arrive at diverse, clinically observed drug-resistance solutions.

The diversity of resistance mechanisms we observed, covering multiple, frequently observed clinically mechanisms, suggests that passage through the persister state is not a limiting factor in the emergence of drug-resistance heterogeneity. Though our study does not directly address when or how resistance arose, we believe it is unlikely that our observed resistance mechanisms were pre-existing at the time of drug treatment: resistant cells would have had to emerge *de novo* within 20 generations without selective pressure from a single cell and then not expand appreciably for ~6 weeks in drug. We suspect, as previously conjectured [1], that persisters provide a reservoir of cells from which an initial drug-tolerance phenotype can ultimately be fixed into a drug-resistance genotype [120]. (The ability of resistance mutations to arise after treatment is further demonstrated by the unpublished study from Jeff Engelman's laboratory that was previously introduced).

Our studies, in a single instance of evolution, provide motivation for further studies of the timing, diversity and mechanisms by which drug resistance can arise from (or through) drug-tolerant cells in different growth conditions, selective pressures and cancer types.

Our work suggests yet a new layer of complexity for treating cancer. Diverse drug-resistance mechanisms can arise from pre-existing mutations before treatment (as has been extensively studied [78,79,102] as well as from slow-growing persisters after long-time treatment (which we study here). In fact, both mechanisms may contribute to clinically observed drug-resistance heterogeneity [1]. Certainly, eliminating, modulating or even anticipating, the range of drug-resistance solutions that can emerge from the persister state will help guide the treatment of cancer.

CHAPTER 4

Materials and Methods

Experimental methods

4.1 Cell line derivation/cell culture conditions

4.1.1 Media conditions

Two types of media were used in this study. First, “erlotinib-free media” was composed of RPMI 1640 (Corning #10 040 CM) supplemented with 5% Fetal Bovine Serum (Life Technologies #16140-071) and 1% Antibiotic-Antimycotic (Life Technologies #15240-062). Second, “erlotinib media” is composed of erlotinib-free media and 2.5 μM erlotinib. Unless otherwise stated, all experiments with PC9 and PC9-1 were performed in “erlotinib-free media” and PERCs were performed in “erlotinib media.”

4.1.2 The generation of clonal cell line PC9-1 in erlotinib-free media

The “EGFR-addicted” non-small cell lung cancer cell line PC9 was used in this study, acquired from ATCC as part of the NCI60 panel. PC9 harbors a deletion in exon 19 of EGFR (ΔE746-A750). 100,000 PC9 cells were seeded on a 10 cm plate. At this low cell density, most cells were isolated from one another. PC9 clonal colonies were selected (colonies that were well-separated from others to maximize the chance of being clonally derived were chosen) and transferred to a new 96 well plate. These clones were then rapidly expanded from the 96 well plate to a 24 well plate to a 6 well plates to one 10 cm plate. The process of generating a confluent plate for each of the clonal population took ~2 weeks. Four vials of each clone were frozen down using all cells from the single confluent 10 cm plate. We designated one of these clones PC9-1 and used it for all subsequent experiments.

4.1.3 The generation of PC9-1-derived PERCs in erlotinib media

PERCs were derived by seeding 100,000 PC9-1 cells onto five 10 cm plates (two rounds of drug treatment/isolation were performed, each round with one thawed-out PC9-1 vial). Cells were allowed to stabilize overnight, then media was replaced with erlotinib media. Note that erlotinib media was used for the whole duration of the PERC generation time (2 months before isolation and ~7 ± 1.5 months after isolation) and changed regularly (~every 2-3 days). Most cells died, leaving a few, isolated and very slow-growing cells, called persisters, on the plates. Expansion from persisters was only observed after ~6 weeks of Erlotinib-treatment. Clearly separated colonies (~50) were

then isolated and transferred to a new 96 well plate between 6-8 weeks of drug treatment. Colonies were then expanded from 96 well plates to 24 well plates to 6 well plates to one confluent 10 cm plate then finally to three confluent 10 cm plates, changing media every 3 days. Note that plate transfers were performed only when the cells were grown to confluence. Nine vials of PERCs at 4th passage were frozen down using all cells from the three confluent 10 cm plates. Note that to obtain sufficient cells for large-scale experiments of drug screening, exome sequencing and RPPA assays, PERCs and PC9-1 cells had to be expanded ~6 further passages.

4.2 Reversion

Long-term reversion experiments were performed by maintaining established PERCs in erlotinib-free media. PERCs and PC9-1 were then probed for their responses to erlotinib periodically over the course of 40 weeks. Percent viability (relative to vehicle-treated cells) of PERCs treated with 2.5 μM erlotinib was determined after 72 hours using CellTiter-Glo assays.

4.3 Compound Screen

The primary screen was performed at the UT Southwestern High-Throughput Screening (UTSW-HTS) Core Facility. For the primary screen, a custom library was

constructed using the following libraries (summarized in Fig. 3.6): Kinase Inhibitor Screening Library (96-well) (Selleckchem, Cat.#L1200), Epigenetic Compound Library (96-well) (Selleckchem, Cat.#L1900), Apoptosis Compound Library (96-well) (Selleckchem, Cat.#3300), InhibitorSelect™ 384-Well Protein Kinase Inhibitor Library I (EMD Calbiochem, Cat.#539743, Batch#D00105831), and the NCI Oncology Set (Plates 4762 and 4763). Cell lines were each seeded in 384-well plates at an empirically determined optimal seeding density, which was defined as the seeding density that resulted in vehicle-treated cells being 70-80% confluent at the end of the experiment, and allowed to adhere overnight. Compounds and negative controls were added using a BIOMEK liquid handling robot on the second day, resulting in a final DMSO concentration of 0.5%, and six, ten-fold dilutions of compound doses from 10 μM-100 pM. Cells were then incubated for 96 hours at 37ºC and 5% CO2. Next, media was removed and 25 µl of CellTiter-Glo diluted 1:5 with passive lysis buffer (Promega) was added using a Multidrop Reagent Dispenser. Plates were incubated for 10 mins at RT with shaking and read on an Envision plate reader (Perkin Elmer). Percent viability calculation was performed by UTSW-HTS using the following formula: Percent Viability $= 100\times \frac{\text{SampleRawValues}}{\text{medianDMSOControl}}$.

A small-scale “bypass” experiment was performed at the Small Molecule Discovery Center at UCSF using the same protocol described above. Selected PERCs

were treated with +/- erlotinib conditions and either SGX-523 (MET drug) or Selumetinib (MEK drug).

4.4 Exome-seq

Genomic DNA was extracted from confluent 15 cm plates using the QIAamp DNA Micro Kit (Qiagen #56304). The user-developed protocol for "Purification of genomic DNA from cultures cells using the QIAamp DNA Micro Kit" was followed, except that lysis and ethanol precipitation steps were scaled up 2-fold, and samples were RNAse-treated. Samples were submitted to Beijing Genome Institute (BGI) for quality control, library preparation, and whole exome sequencing. Only samples that were found to be "Qualified (A level)" in the quality control phase were allowed to proceed. "Qualified (A level)" samples were defined as samples where: 1) the total quantity was over 6 μg; 2) a single band of DNA that was greater than 20 kb with no degradation was detectable by agarose gel electrophoresis; 3) sample concentration was >37.5 ng/μl, and 4) OD260/280 = 1.8~2.0. A 150-200 bp insert library (Agilent SureSelect Human All Exon v4 kit) was used for library construction. Sequencing was performed at BGI on an Illumina HiSeq 2000 sequencer with a paired-end 100 bp read length, and 100X coverage per sample.

4.5 Antibodies/Transient knockdown

The following antibodies were used in this study: MET (Cell Signaling #4560), GAPDH (Santa Cruz #sc-47724), Cleaved PARP (Cell Signaling #9541), phospho-ERK1 (Y204)/phosphor-ERK2 (Y187) (Cell Signaling #5726), and phospho-AKT(S473) (Cell Signaling #4060). Transient knockdown of MET was achieved using SignalSilence MET siRNA I (Cell Signaling #6618), with SignalSilence Control siRNA (Cell Signaling #6568) as a negative control. Cells were seeded in a 6 well plate, allowed to adhere overnight, then transfected using Lipofectamine RNAiMax transfection reagent (30nM siRNA). Whole cell lysates were collected 72 hours post-transfection using RIPA buffer supplemented with PMSF, sodium orthovanadate, and a protease inhibitor cocktail (Santa Cruz #sc-24948). SDS-PAGE immunoblots were performed, and data was collected using the LI-COR Odyssey infrared imaging system.

4.6 Reverse Phase Protein Array (RPPA)

Cells were seeded onto 6-well plates at a density of 1.5 X 10^5 cells/well and cultured for 24 hours before lysed. Cells were lysed using the protocol outlined by the MD Anderson Functional Proteomics Core Facility, where RPPA was performed. Total protein concentration in lysates was determined by performing a BCA assay, and samples were adjusted to a concentration of 1-1.5 μg/μl. Samples were then denatured using the SDS sample buffer recommended by the core facility, boiled for 5 minutes, then stored at -80^{o}C before being shipped to MD Anderson on dry ice. Samples were

then serially diluted and arrayed onto nitrocellulose-coated slides. Slides were then probed with the core facility's collection of antibodies (Listed on website: CoreStdAbList 1_21_2014.xls), and signal was generated using a DAB colorimetric reaction-based system. Background subtraction and spot density determination was done using MiroVigene software. The relative concentration of each protein of interest was defined using the "Super Curve Fitting" method developed by MD Anderson's Functional Proteomics Core Facility.

Analytical methods (developed with Dr. Satwik Rajaram, postdoctoral fellow in Altschuler and Wu Lab):

4.7 Drug response score

In the following description, the notation $V_{c,r}^{n,d}$ will be used to denote the percentage viability of PERC n (=1 to 17) treated with drug d (=1 to 560) at concentration c (= 1 to 6) and replicate measurement r (=1 to 2).

The goal was to identify PERCs whose drug response differed strongly and reproducibly from that of PC9-1. Area Under the Curve (AUC), $A_{n,d} = \sum_{c=1,r}^{6} V_{c,r}^{n,d}$ was used to quantify changes. The score used for change reflected the degree to which

AUC for response curves of PC9-1 and PERCs were distinguishable given a notion of experimental variability (described in detail below).

A measure of variability was constructed by considering the distribution of AUCs when systematically sampling from the replicate measurements; at each of the 6 concentrations c, one of the two replicates was chosen, giving rise to $2^6 = 32$ possible response curves and 32 corresponding AUCs. For every PERC n and drug d, the AUC set: $S(n,d) = \{A^1_{n,d}, \dots, A^{32}_{n,d}\}$ was constructed.

The parent line PC9-1 acts as a control which to compare to, and was therefore assayed twice for each drug (each with two replicates). These two replicate assays are denoted here by PC9-1r_1 and PC9-1r_2 and define a corresponding AUC set for PC9-1 by combining the corresponding AUC sets: $S(PC91,d) = \{A^1_{\text{PC9-1r}_1,d}, \dots, A^{32}_{\text{PC9-1r}_1,d}, A^1_{\text{PC9-1r}_2,d}, \dots, A^{32}_{\text{PC9-1r}_2,d}\}$.

Then the drug response for PERC n and drug d was characterized in terms of its difference from the PC9-1 response as quantified by the test statistic of the student t test (which measures the likelihood that two sets have the same mean):

$$\text{Response}(n,d) = \text{t-statistic}\big(S(n,d), S(PC9-1,d)\big).$$

4.8 Smoothing drug response curves

The mean (across replicates) dose response curves were fit to the sigmoidal form[121]

$$f(c,\beta) = \beta_1 + \frac{\beta_2}{1 + \exp\left(-\frac{\beta_3 - c}{\beta_4}\right)}$$

Where *c* represents the concentration and β_1 ... β_4 are the parameters to be fit, subject to the constraints that $\beta_1 > 0$, $\beta_2 > 0$ and $\beta_4 > 0$.

4.9 Category-level response scores

It was not to be expected that every drug in a class would be equally effective on a PERC. Therefore, a statistical measure to prioritize specific PERC/drug category combinations was developed for further testing. Broadly speaking, the confidence that observed response (either towards resistance or sensitivity) is biologically meaningful can be broken up into two components:

1) Strength of response: Drugs which elicit extremely large deviation (as measured by the response score measured above) are more likely to identify a biological vulnerability. However it is unclear what constitutes large enough deviation threshold to be a biologically meaningful hit.

2) Enrichment of response: When many drugs within a drug category display a strong response, there is a gain of confidence that the results are not caused by individual errant measurements. This can be quantified using a p-value that measures if many more hits than would be expected by chance are seen.

This analysis is guided by the principle that, a few (albeit statistically significant) number of drugs exhibiting a large response are more likely to be indicative of vulnerability than a larger number of drugs showing a comparatively smaller response. Therefore, confidence in each PERC/drug category combination is ordered by the highest response threshold at which there is still enrichment (at a desired level of statistical significance) for hits. In practice this is implemented in two steps:

1) Calculation of p-values at a threshold: At a given response threshold, enrichment p-values are calculated to determine whether each PERC/category is enriched enough for hits to be significant.

2) Threshold scan: thresholds were scanned over to determine, for each category, at what threshold it stops being significant.

The final category-level response score of a PERC to a drug category is the highest response threshold at which the category is significant (categories that never achieve significance have a category response score of 0).

4.9.1 Calculation of p-values at a threshold

At a given threshold T a drug is said to be a sensitivity hit for a PERC if its t-test response is higher than T (or lower than $-T$ for resistance). It was calculated whether the PERC was enriched for hits in a category using a hypergeometric test (corrected for multiple hypothesis testing). The corresponding *p*-value was calculated as the probability of observing an equal, or greater, number of hits in a category of the same size under an appropriate hyper-geometric null model of hits being distributed randomly across categories.

To apply the p-value calculation above to the data, drug categories and the null distribution must first be defined. First, drug categories were defined based on a literature-based, hand-curated drug annotation. All drugs in the same drug category (dashed vertical lines in Fig 3.6 middle) have the same drug annotation (row in Fig 3.6 middle). For example, drugs that targeted only Akt and those that targeted Akt and PI3K would constitute two different categories. Second, in defining the null distribution, it was important to account for the property that certain PERCs would be generally more sensitive (or resistant) across all drug categories due to general resistance mechanisms, rather than specific resistance mechanisms. Thus, it was necessary to find a means of avoiding getting multiple hit categories in these PERCs solely as a result of such global effects. So, in calculating p-values using a hyper-geometric

distribution, the number of hits in a category (as selected from number of drugs in a category) was compared to the total number of hits across all categories (as selected from of all 560 drugs) for that PERC. To ensure we had enough evidence to support hits, only p-values supported by at least 3 hits were counted, and Bonferroni correction was applied to account for multiple hypothesis testing.

4.9.2 Threshold scan

Next, the threshold T was varied from 1 to 200 (i.e. sensitive hit if response $> T$ and resistant hit if response $< -T$), and for each PERC/drug category combination it was determined whether it is significant at 0.01 level (after Bonferroni correction) as described above. It should be noted that the significance is not a monotonic function of threshold; the categories deemed significant at various thresholds are shown in Fig. 3.7. For each PERC/category, the highest threshold at which it is significant is its final category response score. In this case, PERC17/MET is the only category significant even at a threshold of 100, and thus has the highest level of confidence.

4.10 Exome Seq Analysis

Data was processed and aligned to the reference genome hg19 by BGI using BWA[122] ALN. Somatic SNVs (compared to PC9-1) were called using MuTect[123] with

default parameters. Somatic CNVs (compared to PC9-1) were called using ExomeCNV[124] with default parameters to provide a specificity and sensitivity of 99.99%. CNVs with read ratios in the range 0.6 < ratio <1.4 were filtered out.

Variants called reflect a difference in the genetic state between PC9-1 and the PERCs. These can either arise from: a) evolution of the PERCs in drug, or b) from the evolution of PC9-1 as it was being expanded to perform sequencing. Events of type b) are expected to be rare and likely to manifest as differences from PC9-1 that are common to all PERCs. As the focus of this study was on the evolution of PERCs, such events were dropped in Fig 3.8a (in practice only a single NRAS deletion common to all PERCs was omitted).

4.11 EGFR drug-response score

The AUC for all 17 PERCs for the EGFR drugs were calculated on the smooth curves described above. If $\mathrm{A_{n,d}}$ denotes the AUC for the nth PERC when treated with EGFR drug d, then the drug response was measured in terms of the percentage difference from the mean AUC (across all lines) for the drug

$$r_{n,d} = 100\times\frac{\mathrm{A_{n,d}} - \mu_d}{\mu_d}$$

Where $\mu_d = \frac{1}{17}\sum_{n=1}^{17}\mathrm{A_{n,d}}$.

4.12 RPPA Analysis

The output of the experiment is a matrix of intensities, each row being a cell line (PERC) and each column and antibody. This data is analyzed as follows:

1) Normalize: The intensity data (which is in log 2) is linearized (by raising to power 2) and then each row (cell-line) is divided by its median, then each column (antibody) by its median. The values are then converted back to log 2.

2) Replicates: There are multiple replicates for each cell line. These are averaged to generate cell line profiles.

3) For each cell line/antibody, the level of the corresponding antibody for PC9-1 is subtracted out.

4) Quality: Antibodies marked as Validated (V), Caution (C) or (QC) were used. (V): Pearson correlation coefficient between in-house RPPA data and western blot data is > 0.7. (C): Pearson correlation coefficient between in-house RPPA data and western blot data is < 0.7. (QC): Antibody is suitable for cell line analysis but not tissue sample analysis (Total MET antibody Pearson correlation coefficient between in-house RPPA data and western blot data is > 0.84 for cell line samples).

CHAPTER FIVE

Conclusions and Recommendations

5.1 Overview

In this thesis, we show that in a cell culture model of EGFR addiction, there is a link between drug-tolerant persister states and acquisition of several clinically observed mutations that drive resistance. This suggests a model where persister states are utilized by cells to survive a severe drug challenge (without a resistance mutation), allowing for growth and eventual fixation of resistance mutations. While the ability of persisters to acquire a variety of resistance mutations was not verified *in vivo* in this study, the linking of persister states to clinically-relevant resistance mutations calls for further investigation into the contribution of persisters to drug resistance *in vivo* relative to the expansion of rare, pre-existing resistance mutants. We observed the acquisition of several different resistance mutations between clones; *in vivo*, it could be that lineages from spatially-distinct persister clones could acquire distinct resistance mutations. This could, in principle, contribute to the development of a resistant tumor heterogeneous in terms of resistance mutations. It would then be practical to inhibit outgrowth of resistant cells deriving from persisters before the development of heterogeneity (Fig 5.1).

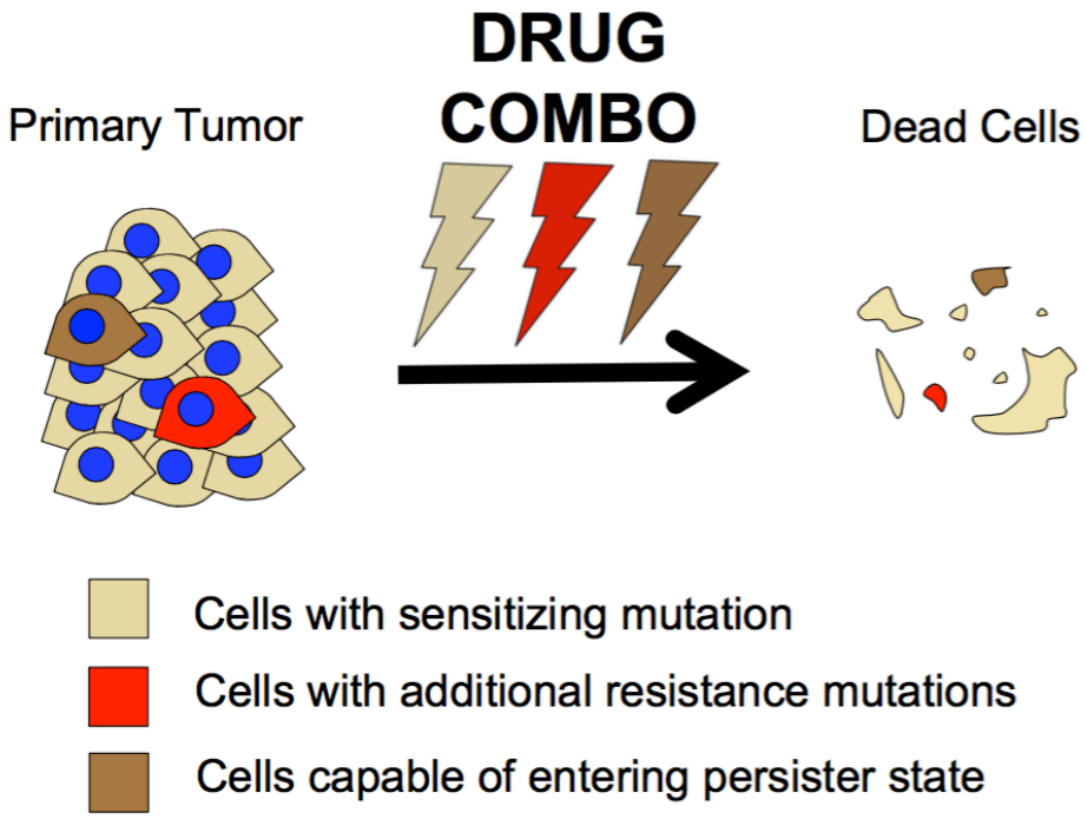


Figure 5.1: A proposed strategy to manage resistance
Representation of a primary tumor composed of 1) cells with a mutation that sensitizes to a targeted therapy (such as erlotinib in EGFR addiction), 2) pre-existing cells with mutations that confer drug resistance (such as T790M-EGFR), and 3) cells capable of surviving the drug bottleneck designed to target cells listed in 1) and 2) through a persister state. If persister states significantly contribute to clinical erlotinib resistance, drugs targeting the molecular basis of persister states may be used upfront to prevent evolution deriving from persister states that could eventual end in fixation of a variety of resistance mutations.

To conclude this thesis, I will discuss the new questions raised by the work presented here and the current challenges in answering these questions. Finally, I will end with a discussion on how drugs that prevent the outgrowth of persister-derived lineages could be incorporated into ongoing strategies to limit resistance.

5.2 New questions

5.2.1 *In vivo* relevance

To date, the contribution of persisters to drug resistance *in vivo* has not been determined. It could be that the expansion of pre-existing mutants completely dominates. However, if persister do play a role in *in vivo* drug resistance, the results of this dissertation predict that if given time, lineages derived from the persister state may fix a variety of different resistance mutations, thereby contributing to the development of resistant tumors heterogeneous for drug resistance mechanisms.

The current challenge in validating the evolution of persisters *in vivo* lies mainly in the lack of understanding of the biology driving cells to utilize persister states for surviving a drug challenge. Further characterization may lead to the development of a strategy to either monitor persisters *in vivo*, or perform lineage-tracing experiments. In the next section, I will discuss unanswered questions in the

biology of persisters, and introduce potential aspects that could be potentially exploited therapeutically.

5.2.2 Characterization of persisters

To date, few studies have been conducted on persisters in cancer. In their molecular characterization, Sharma et al. implicate increased IGF1R signaling in persisters, and linked this to modulation of the activity of the histone demethylase KDM5A. Global changes in methylation of Histone H3K4 were proposed to be due to the increased activity of KDM5A, and the authors discuss how this may be responsible for the altered chromatin state that distinguishes the persister subpopulation from the parental population. Presumably, this altered chromatin state allowed for differences in gene expression that in turn contributed to the process of persister survival and growth. The authors note in their discussion that an altered chromatin state that allows for persister growth could potentially be achieved through other chromatin modifiers besides KDM5A.

Despite some clues about the biology of persisters outlined by Sharma et al., the model proposed leaves several questions unanswered. Firstly, the initiation of the process is unclear. Are cells in a persister state initially, or are they induced by the stress of a drug challenge? On the one hand, due to stochastic differences in

gene expression, cells could randomly and transiently harbor molecular features that confer them with the capability of surviving a drug challenge and growing in the presence of drug. On the other hand, the stress of the drug challenge could induce signaling changes that only a small number of cells are capable of. If the persister state is induced by the stimulus of drug treatment, at what dose are the changes in chromatin necessary? Other studies in the literature that have examined responses to lower doses of erlotinib, and have characterized molecular changes that can contribute to the ability of cells to adapt and grow despite the presence of a low dose of drug [125].However, whether cells that adapt to these less stringent selective pressures possess an altered chromatin state is unclear. These unanswered questions highlight one of the most important questions in cancer biology; how do selective pressures contribute to resultant drug resistance mechanisms?

Secondly, more extensive molecular characterization is warranted. The current persister model is conceptually comprised of four components: 1) Signaling events that are coupled to alteration of the activity of chromatin modifiers, 2) Chromatin modifiers altering the chromatin state, 3) Transcription factors whose activity could be relatively altered due to changes in chromatin, and 4) Target genes whose increased or decreased expression could contribute to persister survival and growth. Each component represents area of potential therapeutic intervention, but

requires a much more detailed understanding of molecular mechanisms in persisters.

Thirdly, more elucidation of the evolution of persisters is needed. For example, the degree of heterogeneity between persister clones and whether this contributes to mutations that eventually get fixed are unknown. In this study, we took steps to minimize heterogeneity between persisters by using a clonal population (PC9-1) to derive persisters. Ideally, single cell exome sequencing would be performed on individual persister clones to determine if cells are truly isogenic. Although this approach may be currently limited by the technology of single cell next-generation sequencing, this area is rapidly advancing, and it may be possible to answer this question one day. If there are subtle differences in genotype between persisters, do these differences guide the evolution towards certain resistance mechanisms? What is the degree of heterogeneity in terms of epigenetic states between persister clones, and does this contribute to the evolution? Or, is the evolution completely random? Furthermore, does the presence of drug accelerate the evolution in any way?

Finally, does the path to acquisition of resistance mutations confer differences in the biology of resistant cells? Recent work suggests that cells that harbored the T790M-EGFR resistance mutation before treatment are more sensitive to T790M-

EGFR inhibitors compared to cells that evolved this mutation through the persister state (unpublished work from Jeffrey Engelman's lab). Indeed, although we observed an increased sensitivity to T790M inhibitors in T790M-positive cells, the separation was modest compared to the increased sensitivity to MET inhibitors seen in PERC17, which harbored a MET amplification. Perhaps similar differences between persister-derived and pre-existing resistant cells occur in the context of other resistance conferring mutations.

5.3 Concluding Remarks

As discussed in this thesis, cancer is characterized by constant evolution. This results in significant heterogeneity in primary tumors, leading to selection for rare, pre-existing cells with resistance–conferring mutations. Drug-tolerant persisters potentially complicate this picture, allowing for growth of cells without necessitating a resistance mutation upfront. Work presented in this thesis suggests that persisters can acquire a variety of clinically-relevant mutations, raising the possibility that persisters could contribute to the emergence of heterogeneity in resistant patient tumors. If persister-derived resistance is found to significantly contribute to clinical resistance, the work presented here would call for efforts to develop drugs that can inhibit the expansion of persisters (Fig 5.1). In this way, inhibition of persister growth

could compliment ongoing strategies to target pre-existing resistance mutants through prevention of the evolution of *de novo* resistance mutations.

APPENDIX A
Annotation of Drug Library

This appendix contains the names of all drugs used to probe functional response in PERCs, including the targets of each drug (provided by the manufacturer). The third column contains annotation of the assigned drug class used in Figure 3.6 (middle).

Drug_Name	Drug_Target	Assigned_Drug_Class
2-Methoxyestradiol	HIF	Other
3-Methyladenine	PI3K	PI3K
A66	PI3K	PI3K
A-674563	Akt, CDK, PKA	AKT,Chemotherapy/Cell Cycle,Metabolism
A-769662	AMPK	Metabolism
ABT-199 (GDC-0199)	Bcl-2	Apoptosis
ABT-263 (Navitoclax)	Bcl-2	Apoptosis
ABT-737	Bcl-2	Apoptosis
AEE788 (NVP-AEE788)	EGFR, Flt, VEGFR, HER2	EGFR,Other RTK
Afatinib (BIBW2992)	EGFR, HER2	EGFR,Other RTK
AG 1024	IGF-1R	Other RTK
AG 112	EGFR	EGFR
AG 1295	PDGFR	Other RTK
AG 1296	PDGFR	Other RTK
AG 1478	EGFR	EGFR
AG 490	EGFR	EGFR
AG 9	Negative Control	Other
AG-1024	IGF-1R	Other RTK
AG-1478 (Tyrphostin AG-1478)	EGFR	EGFR
AG-490	JAK, EGFR	EGFR,Non-Receptor Tyrosine Kinase
AGL 2043	PDGFR	Other RTK
Akt Inhibitor IV	AKT	AKT
Akt Inhibitor V, Triciribine	AKT	AKT
Akt Inhibitor VIII, Isozyme-Selective, Akti-1/2	AKT	AKT
Akt Inhibitor X	AKT	AKT
Aloisine A, RP107	CDK, GSK-3	Chemotherapy/Cell Cycle,Other

		Signalling
Aloisine, RP106	CDK, GSK-3	Chemotherapy/Cell Cycle,Other Signalling
Alsterpaullone	CDK	Chemotherapy/Cell Cycle
Alsterpaullone, 2-Cyanoethyl	CDK	Chemotherapy/Cell Cycle
Altretamine	Alkylating agent	Chemotherapy/Cell Cycle
AMG 900	Aurora Kinase	Aurora Kinase
AMG 900	Aurora Kinase	Aurora Kinase
AMG-208	c-Met	MET
AMG458	c-Met	MET
Aminopurvalanol A	CDK	Chemotherapy/Cell Cycle
AMPK Inhibitor, Compound C	AMPK	Metabolism
Amuvatinib (MP-470)	Flt, PDGFR, c-Kit, c-Met, c-RET	MET,Other RTK
Amuvatinib (MP-470)	Flt, PDGFR, c-Kit, c-Met, c-RET	MET,Other RTK
Apatinib (YN968D1)	VEGFR	Other RTK
Apoptosis Activator 2	Caspase	Apoptosis
AR-42 (HDAC-42)	HDAC	Epigenetic
ARQ 197 (Tivantinib)	c-Met	MET
ARRY334543	EGFR	EGFR
Arry-380	HER2	Other RTK
AS-252424	PI3K	PI3K
AS-604850	PI3K	PI3K
AS-605240	PI3K	PI3K
AS703026 (pimasertib)	MEK	MEK
AST-1306	EGFR	EGFR
AT101	Bcl-2	Apoptosis
AT7519	CDK	Chemotherapy/Cell Cycle
AT7867	Akt, S6 kinase	AKT,MTOR
AT9283	Aurora Kinase, Bcr-Abl, JAK	Aurora Kinase,Non-Receptor Tyrosine Kinase
AT9283	Aurora Kinase, Bcr-Abl, JAK	Aurora Kinase,Non-Receptor Tyrosine Kinase
ATM Kinase Inhibitor	ATM	Chemotherapy/Cell Cycle
ATM/ATR Kinase Inhibitor	ATM, ATR	Chemotherapy/Cell Cycle
Aurora A Inhibitor I	Aurora Kinase	Aurora Kinase
Aurora Kinase Inhibitor II	Aurora Kinase	Aurora Kinase
Aurora Kinase Inhibitor	Aurora Kinase, Lck, Bmx, IGF-	Aurora Kinase,Non-Receptor

III	1R, Syk	Tyrosine Kinase,Other RTK
Aurora Kinase/Cdk Inhibitor	Aurora Kinase, CDK	Aurora Kinase,Chemotherapy/Cell Cycle
Axitinib	VEGFR, PDGFR, c-Kit	Other RTK
AZ 960	JAK	Non-Receptor Tyrosine Kinase
AZ628	Raf	RAF
Azacitidine (Vidaza)	DNA Methyltransferase	Epigenetic
AZD2014	mTOR	MTOR
AZD4547	FGFR	Other RTK
AZD5438	CDK	Chemotherapy/Cell Cycle
AZD6244 (Selumetinib)	MEK	MEK
AZD7762	Chk	Chemotherapy/Cell Cycle
AZD8055	mTOR	MTOR
AZD8330	MEK	MEK
AZD8931	EGFR, HER2	EGFR,Other RTK
Barasertib (AZD1152-HQPA)	Aurora Kinase	Aurora Kinase
Barasertib (AZD1152-HQPA)	Aurora Kinase	Aurora Kinase
Baricitinib (LY3009104,incb28050)	JAK	Non-Receptor Tyrosine Kinase
BAY 11-7082	IKK	Other Signalling
Bcr-abl Inhibitor	Bcr-Abl	Non-Receptor Tyrosine Kinase
Belinostat (PXD101)	HDAC	Epigenetic
BEZ235 (NVP-BEZ235)	mTOR, PI3K	MTOR,PI3K
BGJ398 (NVP-BGJ398)	FGFR	Other RTK
BI 2536	PLK	Chemotherapy/Cell Cycle
BI6727 (Volasertib)	PLK	Chemotherapy/Cell Cycle
BIBF1120 (Vargatef)	VEGFR, PDGFR, FGFR	Other RTK
BIRB 796 (Doramapimod)	p38 MAPK	Other MAPK
Bisindolylmaleimide I	GSK-3	Other Signalling
Bisindolylmaleimide IV	PKC	Metabolism
BIX 02188	MEK	MEK
BIX 02189	MEK	MEK
BIX01294	Histone Methyltransferase	Epigenetic
BKM120 (NVP-BKM120)	PI3K	PI3K
Bleomycin Sulfate	Intercalation	Chemotherapy/Cell Cycle
BMS 777607	c-Met	MET
BMS 794833	c-Met, VEGFR	MET,Other RTK
BMS-265246	CDK	Chemotherapy/Cell Cycle
BMS-599626 (AC480)	EGFR, HER2	EGFR,Other RTK

Bohemine	CDK	Chemotherapy/Cell Cycle
Bortezomib	Proteasome inhibitors	Chemotherapy/Cell Cycle
Bosutinib (SKI-606)	Src	Non-Receptor Tyrosine Kinase
BPIQ-I	EGFR	EGFR
Brivanib (BMS-540215)	VEGFR	Other RTK
Brivanib alaninate (BMS-582664)	VEGFR	Other RTK
BS-181 HCl	CDK	Chemotherapy/Cell Cycle
Busulfan	Alkylating agent	Chemotherapy/Cell Cycle
BX-795	PDK-1	Metabolism
BX-912	PDK-1	Metabolism
BYL719	PI3K	PI3K
Cabazitaxel	N/A	Other
CAL-101 (GS-1101)	PI3K	PI3K
Carboplatin	DNA crosslinker	Chemotherapy/Cell Cycle
carfilzomib	N/A	Other
Carmustine	Alkylating agent	Chemotherapy/Cell Cycle
Casein Kinase I Inhibitor, D4476	Casein Kinase	Other Signalling
Casein Kinase II Inhibitor III, TBCA	Casein Kinase	Other Signalling
CAY10505	PI3K	PI3K
CCT128930	Akt	AKT
CCT129202	Aurora Kinase	Aurora Kinase
CCT129202	Aurora Kinase	Aurora Kinase
CCT137690	Aurora Kinase	Aurora Kinase
CCT137690	Aurora Kinase	Aurora Kinase
Cdc2-Like Kinase Inhibitor, TG003	CLK	Other
Cdk/Crk Inhibitor	CDK, CRK, GSK-3	Chemotherapy/Cell Cycle,Other,Other Signalling
Cdk1 Inhibitor	CDK	Chemotherapy/Cell Cycle
Cdk1 Inhibitor, CGP74514A	CDK	Chemotherapy/Cell Cycle
Cdk1/2 Inhibitor III	CDK	Chemotherapy/Cell Cycle
Cdk1/5 Inhibitor	CDK	Chemotherapy/Cell Cycle
Cdk2 Inhibitor III	CDK	Chemotherapy/Cell Cycle
Cdk2 Inhibitor IV, NU6140	CDK	Chemotherapy/Cell Cycle
Cdk4 Inhibitor	CDK	Chemotherapy/Cell Cycle
Cdk4 Inhibitor II, NSC 625987	CDK	Chemotherapy/Cell Cycle
Cdk4 Inhibitor III	CDK	Chemotherapy/Cell Cycle

Cediranib (AZD2171)	VEGFR, Flt	Other RTK
Celecoxib	Other	Other
CEP33779	JAK	Non-Receptor Tyrosine Kinase
cFMS Receptor Tyrosine Kinase Inhibitor	FMS	Other RTK
CH5424802	ALK	Other RTK
Chelerythrine Chloride	PKC	Metabolism
CHIR-124	Chk	Chemotherapy/Cell Cycle
CHIR-98014	GSK-3	Other Signalling
Chk2 Inhibitor II	Chk	Chemotherapy/Cell Cycle
Chlorambucil	Alkylating agent	Chemotherapy/Cell Cycle
CI-1033 (Canertinib)	EGFR, HER2	EGFR,Other RTK
CI-1040 (PD184352)	MEK	MEK
CI994 (Tacedinaline)	HDAC	Epigenetic
Cisplatin	DNA crosslinker	Chemotherapy/Cell Cycle
Cladribine	Antimetabolite (Purine)	Chemotherapy/Cell Cycle
Compound 52	Cdc	Chemotherapy/Cell Cycle
Compound 56	EGFR	EGFR
CP 673451	PDGFR	Other RTK
CP-724714	EGFR, HER2	EGFR,Other RTK
Crenolanib (CP-868596)	PDGFR	Other RTK
Crizotinib (PF-02341066)	c-Met, ALK	MET,Other RTK
CUDC-101	HDAC	Epigenetic
CX-4945 (Silmitasertib)	PKC	Metabolism
CYC116	Aurora Kinase, VEGFR	Aurora Kinase,Other RTK
CYC116	Aurora Kinase, VEGFR	Aurora Kinase,Other RTK
Cyclophosphamide	Alkylating agent	Chemotherapy/Cell Cycle
Cyt387	JAK	Non-Receptor Tyrosine Kinase
Dabrafenib (GSK2118436)	Raf	RAF
Dacomitinib (PF299804,PF-00299804)	EGFR	EGFR
Dactinomycin	Intercalation	Chemotherapy/Cell Cycle
Danusertib (PHA-739358)	Aurora Kinase, Bcr-Abl, FGFR, Src, c-RET	Aurora Kinase,Non-Receptor Tyrosine Kinase,Other RTK
Danusertib (PHA-739358)-1	Aurora Kinase, Bcr-Abl, FGFR, Src, c-RET	Aurora Kinase,Non-Receptor Tyrosine Kinase,Other RTK
Danusertib (PHA-739358)-2	Aurora Kinase, Bcr-Abl, FGFR, Src, c-RET	Aurora Kinase,Non-Receptor Tyrosine Kinase,Other RTK
Dasatinib	TK	Non-Receptor Tyrosine Kinase
Dasatinib (BMS-354825)	Src, Bcr-Abl, c-Kit	Non-Receptor Tyrosine Kinase,Other RTK

Daunorubicin HCl	Topo II + intercalation	Chemotherapy/Cell Cycle
DCC-2036 (Rebastinib)	Bcr-Abl	Non-Receptor Tyrosine Kinase
Decitabine	Antimetabolite, DNA Methyltransferase	Chemotherapy/Cell Cycle,Epigenetic
Decitabine	Antimetabolite, DNA Methyltransferase	Chemotherapy/Cell Cycle,Epigenetic
Deforolimus (Ridaforolimus)	mTOR	MTOR
Desmethyl Erlotinib (CP-473420)	EGFR	EGFR
Diacylglycerol Kinase Inhibitor II	Diacylglycerol Kinase	Metabolism
Dinaciclib (SCH727965)	CDK	Chemotherapy/Cell Cycle
DMBI	PDGFR	Other RTK
DMSO-1	Other	Other
DMSO-2	Other	Other
DMSO-3	Other	Other
DNA-PK Inhibitor II	DNA-PK	Chemotherapy/Cell Cycle
DNA-PK Inhibitor III	DNA-PK	Chemotherapy/Cell Cycle
DNA-PK Inhibitor V	DNA-PK	Chemotherapy/Cell Cycle
Docetaxel	Microtubule disassembly	Chemotherapy/Cell Cycle
Dovitinib (TKI-258)	FLT3	Other RTK
Dovitinib Dilactic acid (TKI258 Dilactic acid)	FLT3	Other RTK
Doxorubicin HCl	topo II + intercalation	Chemotherapy/Cell Cycle
Droxinostat	HDAC	Epigenetic
E7080 (Lenvatinib)	VEGFR	Other RTK
EGFR Inhibitor	EGFR	EGFR
EGFR/ErbB-2 Inhibitor	EGFR, HER2	EGFR,Other RTK
EGFR/ErbB-2/ErbB-4 Inhibitor	EGFR, HER2, HER4	EGFR,Other RTK
ENMD-2076	Flt, Aurora Kinase, VEGFR	Aurora Kinase,Other RTK
Entacapone	Histone Methyltransferase	Epigenetic
Entinostat (MS-275, SNDX-275)	HDAC	Epigenetic
Enzastaurin (LY317615)	PKC	Metabolism
ERK Inhibitor II, Negative control	ERK	ERK
ERK Inhibitor II, FR180204	ERK	ERK
ERK Inhibitor III	ERK	ERK
Erlotinib HCl	EGFR	EGFR
Etoposide	Topoisomerase inhibitor	Chemotherapy/Cell Cycle

Everolimus (RAD001)	mTOR	MTOR
EX 527	Sirtuin	Metabolism
Fascaplysin, Synthetic	CDK	Chemotherapy/Cell Cycle
FG-4592	HIF	Other
Flavopiridol HCl	CDK	Chemotherapy/Cell Cycle
Flt-3 Inhibitor	FLT3	Other RTK
Flt-3 Inhibitor II	FLT3	Other RTK
Flt-3 Inhibitor III	FLT3	Other RTK
Fludarabine Phosphate	Antimetabolite	Chemotherapy/Cell Cycle
Fluorouracil (5-FU)	Antimetabolite (Pyrimidine)	Chemotherapy/Cell Cycle
Foretinib (GSK1363089, XL880)	c-Met, VEGFR	MET,Other RTK
G? 6976	GSK-3	Other Signalling
G? 6983	PKC	Metabolism
GDC-0068	Akt	AKT
GDC-0879	Raf	RAF
GDC-0941	PI3K	PI3K
GDC-0980 (RG7422)	mTOR, PI3K	MTOR,PI3K
Gefitinib (Iressa)	EGFR	EGFR
Gemcitabine HCl	Antimetabolite (Pyrimidine)	Chemotherapy/Cell Cycle
Golvatinib (E7050)	c-Met	MET
GSK J4 HCl	Histone demethylases	Epigenetic
GSK1059615	PI3K, mTOR	MTOR,PI3K
GSK1070916	Aurora Kinase	Aurora Kinase
GSK1070916	Aurora Kinase	Aurora Kinase
GSK1120212 (Trametinib)	MEK	MEK
GSK1838705A	IGF-1, ALK	Other RTK
GSK1904529A	IGF-1R	Other RTK
GSK2126458	PI3K, mTOR	MTOR,PI3K
GSK-3 Inhibitor IX	GSK-3, CDK	Chemotherapy/Cell Cycle,Other Signalling
GSK-3 Inhibitor X	GSK-3	Other Signalling
GSK-3 Inhibitor XIII	GSK-3	Other Signalling
GSK-3b Inhibitor I	GSK-3, FLT3	Other RTK,Other Signalling
GSK-3b Inhibitor II	GSK-3	Other Signalling
GSK-3b Inhibitor VIII	GSK-3	Other Signalling
GSK-3b Inhibitor XI	GSK-3	Other Signalling
GSK3b Inhibitor XII, TWS119	GSK-3	Other Signalling
GSK461364	PLK	Chemotherapy/Cell Cycle
GSK690693	Akt	AKT

GTP-14564	PKC	Metabolism
H-89, Dihydrochloride	Chk, GSK-3	Chemotherapy/Cell Cycle,Other Signalling
HA 1077, Dihydrochloride Fasudil	PKA, ROCK	Metabolism,Other
HA14-1	Bcl-2	Apoptosis
Herbimycin A, Streptomyces sp.	PKA	Metabolism
Hesperadin	Aurora Kinase	Aurora Kinase
HMN-214	PLK	Chemotherapy/Cell Cycle
IC261	Casein Kinase	Other Signalling
IC-87114	PI3K	PI3K
IGF-1R Inhibitor II	IGF-1R, GSK-3, CDK	Chemotherapy/Cell Cycle,Other RTK,Other Signalling
IKK-2 Inhibitor IV	IKK, Casein Kinase	Other Signalling
Imatinib (Gleevec)	PDGFR	Other RTK
Imatinib Mesylate	PDGFR, c-Kit, Bcr-Abl	Non-Receptor Tyrosine Kinase,Other RTK
IMD 0354	IKK	Other Signalling
INCB28060	c-Met	MET
Indirubin	GSK-3	Other Signalling
Indirubin Derivative E804	IKK	Other Signalling
Indirubin-3?-monoxime	CDK, Src	Chemotherapy/Cell Cycle,Non-Receptor Tyrosine Kinase
INK 128 (MLN0128)	mTOR	MTOR
IOX2	HIF	Other
IRAK-1/4 Inhibitor	IRAK, IGF-1R	Other RTK,Other Signalling
Irinotecan HCl	Topoisomerase inhibitor	Chemotherapy/Cell Cycle
Isogranulatimide	FMS, c-Kit, FLT3	Other RTK
ITF2357 (Givinostat)	HDAC	Epigenetic
JAK Inhibitor I	IRAK, JAK	Non-Receptor Tyrosine Kinase,Other Signalling
JAK3 Inhibitor II	JAK, Tyk	Non-Receptor Tyrosine Kinase
JAK3 Inhibitor IV	JAK	Non-Receptor Tyrosine Kinase
JAK3 Inhibitor VI	JAK	Non-Receptor Tyrosine Kinase
JNJ 26854165 (Serdemetan)	p53	Chemotherapy/Cell Cycle
JNJ-26481585	HDAC	Epigenetic
JNJ-38877605	c-Met	MET
JNJ-7706621	Aurora Kinase, CDK	Aurora Kinase,Chemotherapy/Cell Cycle

JNJ-7706621	Aurora Kinase, CDK	Aurora Kinase,Chemotherapy/Cell Cycle
JNK Inhibitor II	JNK, JAK	Non-Receptor Tyrosine Kinase,Other MAPK
JNK Inhibitor IX	JNK	Other MAPK
JNK Inhibitor V	JNK	Other MAPK
JNK Inhibitor VIII	JNK	Other MAPK
JNK Inhibitor, Negative Control	JNK	Other MAPK
K-252a, Nocardiopsis sp.	PKA, PKC, PKG	Metabolism,Other
Kenpaullone	GSK-3, CDK, Lck	Chemotherapy/Cell Cycle,Non-Receptor Tyrosine Kinase,Other Signalling
Ki8751	VEGFR, c-Kit, PDGFR	Other RTK
KN-62	CaM Kinase	Other
KN-93	CaM Kinase	Other
KRN 633	VEGFR, PDGFR	Other RTK
Ku-0063794	mTOR	MTOR
KU-55933	ATM	Chemotherapy/Cell Cycle
KU-60019	ATM	Chemotherapy/Cell Cycle
KW 2449	Flt, Bcr-Abl, Aurora Kinase	Aurora Kinase,Non-Receptor Tyrosine Kinase,Other RTK
KX2-391	Src	Non-Receptor Tyrosine Kinase
Lapatinib Ditosylate (Tykerb)	EGFR, HER2	EGFR,Other RTK
LAQ824 (NVP-LAQ824, Dacinostat)	HDAC	Epigenetic
Lck Inhibitor	Lck	Non-Receptor Tyrosine Kinase
LDN193189	TGF-beta/Smad	Other RTK
Lenalidomide (Revlimid)	TNF-alpha	Other Signalling
Linifanib (ABT-869)	PDGFR, VEGFR	Other RTK
Linsitinib (OSI-906)	IGF-1R	Other RTK
Lomustine; CCNU	Alkylating agent	Chemotherapy/Cell Cycle
LY 294002	PI3K	PI3K
LY 303511- Negative control	Negative Control	Other
LY2228820	p38 MAPK	Other MAPK
LY2603618 (IC-83)	Chk	Chemotherapy/Cell Cycle
LY2784544	JAK	Non-Receptor Tyrosine Kinase
LY294002	PI3K	PI3K
M344	HDAC	Epigenetic
Masitinib (AB1010)	c-Kit, PDGFR, FGFR, FAK	Other,Other RTK
MC1568	HDAC	Epigenetic

MEK Inhibitor I	MEK	MEK
MEK Inhibitor II	MEK	MEK
MEK1/2 Inhibitor	MEK	MEK
Melphalan	Alkylating agent	Chemotherapy/Cell Cycle
Mercaptopurine	Antimetabolite (Purine)	Chemotherapy/Cell Cycle
Met Kinase Inhibitor	c-Met	MET
Methotrexate	Antimetabolite (Folic Acid)	Chemotherapy/Cell Cycle
MGCD-265	c-Met, VEGFR, Tie-2	MET,Other RTK
Milciclib (PHA-848125)	CDK	Chemotherapy/Cell Cycle
Mitomycin C	Intercalation	Chemotherapy/Cell Cycle
Mitoxantrone	topo II + intercalation	Chemotherapy/Cell Cycle
MK-2206 2HCl	Akt	AKT
MK-2461	c-Met	MET
MK2a Inhibitor	MK2	Other
MK-5108 (VX-689)	Aurora Kinase	Aurora Kinase
MLN8054	Aurora Kinase	Aurora Kinase
MLN8237 (Alisertib)	Aurora Kinase	Aurora Kinase
MNK1 Inhibitor	MNK1	Other MAPK
Mocetinostat (MGCD0103)	HDAC	Epigenetic
Motesanib Diphosphate (AMG-706)	VEGFR, PDGFR, c-Kit	Other RTK
Mubritinib (TAK 165)	HER2	Other RTK
Necrostatin-1	TNF-alpha	Other Signalling
Neratinib (HKI-272)	HER2, EGFR	EGFR,Other RTK
NF-kB Activation Inhibitor	NFKB	Other Signalling
Nilotinib (AMN-107)	Bcr-Abl	Non-Receptor Tyrosine Kinase
NSC-207895 (XI-006)	p53, Mdm2	Chemotherapy/Cell Cycle
NU7441 (KU-57788)	DNA-PK, PI3K	Chemotherapy/Cell Cycle,PI3K
Nutlin-3	Mdm2	Chemotherapy/Cell Cycle
NVP-ADW742	IGF-1R	Other RTK
NVP-BGT226	PI3K	PI3K
NVP-BHG712	VEGFR, Src, Raf, Bcr-Abl	Non-Receptor Tyrosine Kinase,Other RTK,RAF
NVP-BSK805	JAK	Non-Receptor Tyrosine Kinase
NVP-BVU972	c-Met	MET
NVP-TAE226	FAK	Other
Obatoclax mesylate (GX15-070)	Bcl-2	Apoptosis
ON-01910	PLK	Chemotherapy/Cell Cycle
OSI-027	mTOR	MTOR

OSI-420	EGFR	EGFR
OSI-930	c-Kit, VEGFR	Other RTK
OSU-03012	PDK-1	Metabolism
Oxaliplatin	DNA crosslinker	Chemotherapy/Cell Cycle
p38 MAP Kinase Inhibitor	p38 MAPK	Other MAPK
p38 MAP Kinase Inhibitor III	p38 MAPK	Other MAPK
PAC-1	Caspase	Apoptosis
Paclitaxel	Microtubule disassembly	Chemotherapy/Cell Cycle
Palomid 529	PI3K	PI3K
Pazopanib HCl	VEGFR, PDGFR, c-Kit	Other RTK
PCI-24781	HDAC	Epigenetic
PCI-32765 (Ibrutinib)	Src	Non-Receptor Tyrosine Kinase
PCI-34051	HDAC	Epigenetic
PD 0332991 (Palbociclib) HCl	CDK	Chemotherapy/Cell Cycle
PD 158780	EGFR	EGFR
PD 169316	p38 MAPK	Other MAPK
PD 174265	EGFR	EGFR
PD 98059	MEK	MEK
PD0325901	MEK	MEK
PD153035 HCl	EGFR	EGFR
PD173074	FGFR	Other RTK
PD318088	MEK	MEK
PD98059	MEK	MEK
PDGF Receptor Tyrosine Kinase Inhibitor II	PDGFR	Other RTK
PDGF Receptor Tyrosine Kinase Inhibitor III	PDGFR	Other RTK
PDGF Receptor Tyrosine Kinase Inhibitor IV	PDGFR, Lck, Src, Fyn,Bcr-Abl	Non-Receptor Tyrosine Kinase,Other RTK
PDGF RTK Inhibitor	PDGFR	Other RTK
PDK1/Akt/Flt Dual Pathway Inhibitor	PDK-1, FLT3	Metabolism,Other RTK
Pelitinib (EKB-569)	EGFR	EGFR
Pemetrexed	Antimetabolite (Folic Acid)	Chemotherapy/Cell Cycle
Pentostatin	Antimetabolite (Purine)	Chemotherapy/Cell Cycle
PF-00562271	FAK	Other
PF-03814735	Aurora Kinase	Aurora Kinase
PF-03814735	Aurora Kinase	Aurora Kinase
PF-04217903	c-Met	MET

PF-04691502	mTOR, PI3K, Akt	AKT,MTOR,PI3K
PF-05212384 (PKI-587)	mTOR, PI3K	MTOR,PI3K
PFI-1	Epigenetic Reader Domain	Epigenetic
PH-797804	p38 MAPK	Other MAPK
PHA-665752	c-Met	MET
PHA-680632	Aurora Kinase	Aurora Kinase
PHA-680632	Aurora Kinase	Aurora Kinase
PHA-767491	CDK	Chemotherapy/Cell Cycle
PHA-793887	CDK	Chemotherapy/Cell Cycle
Phenformin HCl	AMPK	Metabolism
PHT-427	Akt	AKT
PI 3-Kg Inhibitor	PI3K	PI3K
PI 3-Kg Inhibitor II	PI3K	PI3K
PI-103	ATM, ATR, DNA-PK, PI3K, mTOR	Chemotherapy/Cell Cycle,MTOR,PI3K
PI-103	ATM, ATR, DNA-PK, PI3K, mTOR	Chemotherapy/Cell Cycle,MTOR,PI3K
PI3K/HDAC Inhibitor I	HDAC	Epigenetic
Piceatannol	Syk	Non-Receptor Tyrosine Kinase
Pifithrin-?	p53	Chemotherapy/Cell Cycle
PIK-293	PI3K	PI3K
PIK-294	PI3K	PI3K
PIK-75	PI3K, DNA-PK	Chemotherapy/Cell Cycle,PI3K
PIK-90	PI3K	PI3K
PIK-93	PI3K, VEGFR	Other RTK,PI3K
Pipobroman	Alkylating agent	Chemotherapy/Cell Cycle
PKCb Inhibitor	PKC	Metabolism
PKCbII/EGFR Inhibitor	EGFR	EGFR
PKI-402	PI3K	PI3K
PKR Inhibitor	PKR	Chemotherapy/Cell Cycle
PKR Inhibitor, Negative Control	Negative Control	Other
Plicamycin	Intercalation	Chemotherapy/Cell Cycle
PLX-4720	Raf	RAF
Pomalidomide	TNF-alpha	Other Signalling
Ponatinib (AP24534)	Bcr-Abl, VEGFR, FGFR, PDGFR, Flt	Non-Receptor Tyrosine Kinase,Other RTK
PP1 Analog II, 1NM-PP1	Fyn	Non-Receptor Tyrosine Kinase
PP-121	DNA-PK, mTOR, PDGF	Chemotherapy/Cell Cycle,MTOR,Other RTK
PP242	mTOR	MTOR
PP3	EGFR	EGFR

Pralatrexate	N/A	Other
Procarbazine HCl	Alkylating agent	Chemotherapy/Cell Cycle
Purvalanol A	CDK	Chemotherapy/Cell Cycle
Quercetin (Sophoretin)	PI3K, PKC, Sirtuin, Src	Metabolism,Non-Receptor Tyrosine Kinase,PI3K
Quercetin (Sophoretin)	PI3K, PKC, Sirtuin, Src	Metabolism,Non-Receptor Tyrosine Kinase,PI3K
Quizartinib (AC220)	Flt	Other RTK
R406	Syk, Flt	Non-Receptor Tyrosine Kinase,Other RTK
R406 (free base)	Flt, Syk	Non-Receptor Tyrosine Kinase,Other RTK
R788 (Fostamatinib)	Syk	Non-Receptor Tyrosine Kinase
R935788 (Fostamatinib disodium, R788 disodium)	Syk	Non-Receptor Tyrosine Kinase
Raf265 derivative	VEGFR, Raf	Other RTK,RAF
Rapamycin	mTOR	MTOR
Rapamycin (Sirolimus)	mTOR	MTOR
Regorafenib (BAY 73-4506)	c-Kit, Raf, VEGFR	Other RTK,RAF
Resveratrol	Sirtuin	Metabolism
Rho Kinase Inhibitor III, Rockout	Rho Kinase	Other
Rho Kinase Inhibitor IV	Rho Kinase, ROCK	Other
RITA (NSC 652287)	p53	Chemotherapy/Cell Cycle
Ro-32-0432	PKC	Metabolism
Rocilinostat (ACY-1215)	HDAC	Epigenetic
ROCK Inhibitor, Y-27632	ROCK	Other
Romidepsin	N/A	Other
Roscovitine (Seliciclib, CYC202)	CDK	Chemotherapy/Cell Cycle
Ruxolitinib (INCB018424)	JAK	Non-Receptor Tyrosine Kinase
SAR131675	VEGFR	Other RTK
Saracatinib (AZD0530)	Src, Bcr-Abl	Non-Receptor Tyrosine Kinase
SB 202190	p38 MAPK	Other MAPK
SB 202190	p38 MAPK	Other MAPK
SB 202474, Negative control for p38 MAPK inhibition studies	Negative Control	Other
SB 203580	p38 MAPK	Other MAPK
SB 203580	p38 MAPK	Other MAPK

SB 216763	GSK-3	Other Signalling
SB 218078	Chk, CDK	Chemotherapy/Cell Cycle
SB 415286	GSK-3	Other Signalling
SB 431542	TGF-beta/Smad	Other RTK
SB 525334	TGF-beta/Smad	Other RTK
SB220025	p38 MAPK	Other MAPK
SB590885	Raf	RAF
SB939 (Pracinostat)	HDAC	Epigenetic
SC-68376	p38 MAPK	Other MAPK
Scriptaid	HDAC	Epigenetic
Semaxanib (SU5416)	VEGFR	Other RTK
SGX-523	c-Met	MET
Sirtinol	Sirtuin	Metabolism
SKF-86002	p38 MAPK	Other MAPK
SNS-032 (BMS-387032)	CDK	Chemotherapy/Cell Cycle
SNS-314	Aurora Kinase	Aurora Kinase
SNS-314 Mesylate	Aurora Kinase	Aurora Kinase
Sodium Phenylbutyrate	HDAC	Epigenetic
Sorafenib (Nexavar)	VEGFR, PDGFR, Raf	Other RTK,RAF
Sotrastaurin (AEB071)	PKC	Metabolism
SP600125	JNK	Other MAPK
Sphingosine Kinase Inhibitor	SK1	Other
Src Kinase Inhibitor I	Src, Lck	Non-Receptor Tyrosine Kinase
SRT1720	Sirtuin	Metabolism
Staurosporine	PKC	Metabolism
Staurosporine, N-benzoyl-	PKC, PDGFR, VEGFR, Syk, FLT3, CDK, PKA, c-Kit, c-Fgr, Src, VEGFR, EGFR	Chemotherapy/Cell Cycle,EGFR,Metabolism,Non-Receptor Tyrosine Kinase,Other RTK
Staurosporine, Streptomyces sp.	PKC, PKA, PKG,MLCK, CaM Kinase	Metabolism,Other
STO-609	CaM Kinase	Other
SU11274	c-Met	MET
SU11652	PDGFR, VEGFR	Other RTK
SU6656	Yes, Lyn, Fyn, Src	Non-Receptor Tyrosine Kinase
SU9516	CDK	Chemotherapy/Cell Cycle
Sunitinib Malate (Sutent)	VEGFR, PDGFR, c-Kit, Flt	Other RTK
Syk Inhibitor	Syk	Non-Receptor Tyrosine Kinase
Syk Inhibitor II	Syk	Non-Receptor Tyrosine Kinase
Syk Inhibitor III	Syk	Non-Receptor Tyrosine Kinase

TAE684 (NVP-TAE684)	ALK	Other RTK
TAK-285	EGFR	EGFR
TAK-733	MEK	MEK
TAK-901	Aurora Kinase	Aurora Kinase
TAK-901	Aurora Kinase	Aurora Kinase
Tandutinib (MLN518)	Flt	Other RTK
Telatinib (BAY 57-9352)	VEGFR, PDGFR, c-Kit	Other RTK
Temozolomide	Alkylating agent	Chemotherapy/Cell Cycle
Temsirolimus (Torisel)	mTOR	MTOR
Teniposide	Topoisomerase inhibitor	Chemotherapy/Cell Cycle
Tenovin-1	Mdm2	Chemotherapy/Cell Cycle
TG 100713	PI3K	PI3K
TG100-115	PI3K	PI3K
TG101209	Flt, JAK, c-RET	Non-Receptor Tyrosine Kinase,Other RTK
TG101209	Flt, JAK, c-RET	Non-Receptor Tyrosine Kinase,Other RTK
TG101348 (SAR302503)	JAK	Non-Receptor Tyrosine Kinase
TGF-b RI Inhibitor III	TGF-beta/Smad, p38 MAPK	Other MAPK,Other RTK
TGF-b RI Kinase Inhibitor	TGF-beta/Smad, p38 MAPK	Other MAPK,Other RTK
TGX-221	PI3K	PI3K
Thalidomide	TNF-alpha	Other Signalling
Thiazovivin	ROCK	Other
Thioguanine	Atimetabolite (Purine)	Chemotherapy/Cell Cycle
Thiotepa	Alkylating agent	Chemotherapy/Cell Cycle
Tideglusib	GSK-3	Other Signalling
Tie2 kinase inhibitor	Tie-2	Other RTK
Tivozanib (AV-951)	VEGFR, c-Kit, PDGFR	Other RTK
Tofacitinib (CP-690550, Tasocitinib)	JAK	Non-Receptor Tyrosine Kinase
Tofacitinib citrate (CP-690550 citrate)	JAK	Non-Receptor Tyrosine Kinase
Topotecan HCl	Topoisomerase inhibitor	Chemotherapy/Cell Cycle
Torin 1	mTOR	MTOR
Torin 2	mTOR	MTOR
TPCA-1	IKK	Other Signalling
Tpl2 Kinase Inhibitor	Tpl Kinase	Other Signalling
Trichostatin A (TSA)	HDAC	Epigenetic
Triciribine (Triciribine phosphate)	Akt	AKT
TSU-68 (SU6668)	VEGFR, PDGFR , FGFR	Other RTK
Tubastatin A HCl	HDAC	Epigenetic

TW-37	Bcl-2	Apoptosis
TWS119	GSK-3	Other Signalling
Tyrphostin AG 879 (AG 879)	HER2	Other RTK
U0126-EtOH	MEK	MEK
Uracil mustard	Alkylating agent	Chemotherapy/Cell Cycle
Valproic acid sodium salt (Sodium valproate)	HDAC	Epigenetic
valrubicin	topo II + intercalation	Chemotherapy/Cell Cycle
Vandetanib (Zactima)	VEGFR	Other RTK
Vatalanib 2HCl (PTK787)	VEGFR, c-Kit, Flt	Other RTK
VEGF Receptor 2 Kinase Inhibitor I	VEGFR	Other RTK
VEGF Receptor 2 Kinase Inhibitor II	VEGFR, PDGFR	Other RTK
VEGF Receptor 2 Kinase Inhibitor III	VEGFR, FLT3	Other RTK
VEGF Receptor 2 Kinase Inhibitor IV	VEGFR	Other RTK
VEGF Receptor Tyrosine Kinase Inhibitor II	VEGFR, Flt, c-Kit	Other RTK
VEGFR Tyrosine Kinase Inhibitor IV	VEGFR, EphB2, PDGFR, c-Kit, Tie	Other RTK
Vemurafenib (PLX4032)	Raf	RAF
Vinblastine Sulfate	Microtubule assembly	Chemotherapy/Cell Cycle
Vincristine Sulfate	Microtubules assembly	Chemotherapy/Cell Cycle
Vinorelbine Tartrate	microtubule assembly	Chemotherapy/Cell Cycle
Vismodegib	N/A	Other
Vorinostat (SAHA)	HDAC	Epigenetic
VX-680 (MK-0457, Tozasertib)	Aurora Kinase	Aurora Kinase
VX-702	p38 MAPK	Other MAPK
WAY-600	mTOR	MTOR
WHI-P154	JAK	Non-Receptor Tyrosine Kinase
Wortmannin	PI3K	PI3K
Wortmannin	PI3K	PI3K
WP1066	JAK	Non-Receptor Tyrosine Kinase
WP1130	DUB, Bcr-Abl	Non-Receptor Tyrosine Kinase,Other
WYE-125132	mTOR	MTOR
WYE-354	mTOR	MTOR
WYE-687	mTOR	MTOR
WZ3146	EGFR	EGFR

WZ4002	EGFR	EGFR
WZ8040	EGFR	EGFR
XL147	PI3K	PI3K
XL-184 (Cabozantinib)	VEGFR, c-Met, Flt, Tie-2, c-Kit	MET,Other RTK
XL765	PI3K, mTOR	MTOR,PI3K
Y-27632 2HCl	ROCK	Other
YM155	IAP	Apoptosis
YM201636	PI3K	PI3K
ZM 336372	Raf	RAF
ZM-447439	Aurora Kinase	Aurora Kinase
ZM-447439	Aurora Kinase	Aurora Kinase
ZSTK474	PI3K	PI3K

APPENDIX B
Summary of Notable SNV and CNV Events in PERCs

Listing of single nucleotide variation (SNV) and copy number variation (CNV) events in genes implicated in erlotinib resistance that were observed in PERCs. For SNVs, the identity of the mutated gene product is listed. For CNVs, the change relative to PC9-1 is listed.

CellLine	SNVs	CNVs
PERC1	EGFR(p.T790M)	
PERC2		RAF1(x1.5315),NF1(x0.37639)
PERC3		
PERC4	EGFR(p.T790M)	
PERC5	EGFR(p.T790M)	RAF1(x1.4251),NF1(x0.4061),KRAS(x0.58593),AKT1(x1.6197)
PERC6	EGFR(p.T790M)	
PERC7	EGFR(p.T790M)	EGFR(x1.4414)
PERC8	EGFR(p.T790M)	
PERC9	PIK3CA(p.E542K),EGFR(p.T790M)	
PERC10	NRAS(p.Q61K)	
PERC11	PIK3CB(p.E563K),BRAF(p.G466A)	HRAS(x1.5675)
PERC12		TSC1(x1.6241)
PERC13	NRAS(p.Q61K)	HRAS(x1.5983)
PERC14	NRAS(p.Q61K)	HRAS(x1.5779)
PERC15	NRAS(p.E63K)	RAF1(x2.2914)
PERC16		RAF1(x22.1433),HRAS(x1.5933)
PERC17		MET(x6.4958)

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