Characterizing resistance and sensitivity to targeted therapies in patient-derived models of EGFR mutant lung cancer

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Characterizing resistance and sensitivity to targeted therapies in patient-derived models of

*EGFR* mutant lung cancer

A dissertation presented

by

Pınar Özden Eser

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Biological and Biomedical Sciences

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Characterizing resistance and sensitivity to targeted therapies in patient-derived models of 

EGFR mutant lung cancer

Abstract

Advanced non-small cell lung cancer (NSCLC) continues to be an incurable family of thoracic malignancies that is chronically managed with chemotherapy, targeted therapy, and immunotherapy. While the discovery of driver oncogenes and the advent of targeted therapies in the last decade have vastly improved disease management for patients whose tumors harbor druggable mutations, the clinical efficacy of targeted therapies remains limited by the development of acquired drug resistance. One proto-oncogene that is mutated in 10-15% of NSCLC is the epidermal growth factor receptor (EGFR). Activating EGFR mutations drive constitutive receptor phosphorylation, which in turn activates downstream kinase cascades promoting cell proliferation and survival. Tumors harboring sensitizing EGFR mutations respond to EGFR tyrosine kinase inhibitors (TKI), before ultimately becoming refractory to therapy. Resistance to targeted EGFR TKIs predominantly occurs through reactivation of EGFR signaling, either by acquisition of secondary EGFR mutations that enable the receptor to evade drug binding and remain active, or through compensatory upregulation of bypass signaling pathways that reactivate canonical EGFR-downstream signaling. MET kinase is a proto-oncogene that is
commonly amplified and/or overexpressed and activated in EGFR-driven tumors to confer TKI resistance through bypass signaling.

Here, we develop and characterize novel models of drug resistance in EGFR mutant backgrounds in an effort to pinpoint novel therapeutic opportunities for EGFR-driven tumors progressed on first-line therapy with EGFR tyrosine kinase inhibitors. In Chapter 2, CRISPR-Cas9 genome engineering is applied to introduce drug resistance mutations into EGFR in inhibitor-naïve EGFR-dependent cell line models, creating a platform to test novel targeted inhibitors and rational combination therapies. The focus of Chapter 3 is the identification and characterization of a novel subset of EGFR-mutant, MET upregulated lung cancers from patient-derived specimens that exhibit a complete switch away from EGFR addiction to MET oncogene dependency. Finally, in Chapter 4, we observe glucocorticoid associated modulation of drug sensitivity in a subset of NSCLC lines, and examine the biological contexts underlying this glucocorticoid response. Through development and characterization of novel patient-derived models of EGFR-driven NSCLC, we hope to contribute to a deeper understanding of the molecular processes underlying resistance and sensitivity to targeted therapies in NSCLC.
# Table of Contents

Abstract ................................................................................................................................. v
Table of Contents ...................................................................................................................... vii
Acknowledgements .................................................................................................................. ix
List of Figures .......................................................................................................................... xiii
List of Tables ............................................................................................................................ xv
List of Abbreviations ................................................................................................................ xvi

**Chapter 1: Introduction** ................................................................................................... 1
  Copyright Disclosure ............................................................................................................. 2
  1.1. Non-small cell lung cancer classification and treatment ............................................. 2
  1.2. EGFR and ERBB family signaling pathways ............................................................... 5
  1.3. Development of resistance to EGFR-targeted therapies through acquisition of secondary and tertiary EGFR mutations ................................................................. 9
  1.4. Development of resistance to EGFR-targeted therapies through MET kinase amplification ............................................................................................................................. 17
  1.5. Preclinical models of driver oncogenes and drug resistance in NSCLC ................. 19

**Chapter 2: Modeling point mutations implicated in drug resistance using CRISPR/Cas9-mediated genome engineering** ................................................................. 24
  Attributions .......................................................................................................................... 25
  2.1. Introduction .................................................................................................................... 25
  2.2. Introducing EGFR inhibitor resistance mutations using CRISPR-Cas9 genome engineering ........................................................................................................................... 28
  2.3. Using a CRISPR-engineered model of EGFR del19/T790M/C797S to predict assess the efficacy of experimental therapeutic regimens against triple mutant EGFR ............. 36
  2.4. Characterizing the development and biology of the predicted drug resistance mutation T790I in cell line models of NSCLC ................................................................. 42
  2.5. Discussion of ongoing and future directions ................................................................ 45

**Chapter 3: Identification and characterization of MET kinase dependency in EGFR-mutant, MET-overexpressing NSCLC** .......................................................................... 47
  Attributions: .......................................................................................................................... 48
  3.1. Introduction .................................................................................................................... 48
  3.2. Identification of a switch to MET dependency in a subset of EGFR mutant, MET amplified NSCLC .................................................................................................................. 51
3.3. Exogenous ERBB ligand treatment induces crizotinib resistance in MET-dependent cell line models

3.4. Assessing the predictive potential of activated EGFR-HER3 versus MET-HER3 dimers as an indicator of single oncogene MET dependency in EGFR-mutant, MET-amplified models of NSCLC

3.5. Ectopic overexpression of activating mutant EGFR induces resistance to single-agent crizotinib in MET-dependent cell lines

3.6. DFCI161 cells develop EGFR-mediated acquired resistance to crizotinib

3.7. Discussion

Chapter 4: Identification of a steroid-induced switch in drug sensitivity in EGFR-mutant MET-overexpressing NSCLC models

Chapter 5: Discussion and Conclusions

Appendices

Appendix I: Supplemental Materials for Chapter 2

Appendix II: Supplemental Materials for Chapter 3

Appendix III: Supplemental Materials for Chapter 4

Materials and Methods

References:
Acknowledgements

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For my parents.
List of Figures

Figure 1-1: Canonical EGFR signaling ........................................................................................................7
Figure 1-2: Progression of EGFR inhibitor resistance through acquisition of resistance mutations .........................................................................................................................13
Figure 2-1: CRISPR Study Design ..................................................................................................................27
Figure 2-2: Validation of drug resistance and mutational profiles of CRISPR-edited PC9 cells ..........30
Figure 2-3: Quantification of mutant allele enrichment in CRISPR-edited H3255 cells .................33
Figure 2-4: Prevalence of spontaneous mutations in CRISPR edited H3255 cells ......................33
Figure 2-5: In vivo assessment of the efficacy of the EGFR-inhibitory antibody necitumumab against PC9 cells harboring T790M and C797S drug resistance mutations .........................................................39
Figure 2-6: Heterogeneity in the drug response of PC9 T790M/C797S xenografts to osimertinib and necitumumab treatment ..................................................................................................................41
Figure 2-7: Preliminary characterization of EGFR T790I in cis with activating EGFR mutation as a drug resistance mechanism ..................................................................................................................44
Figure 3-1: Three new patient-derived models of EGFR mutant MET-amplified NSCLC ..........51
Figure 3-2: Patient-derived cell line and xenograft models exhibit sensitivity to MET inhibitors ..........................................................................................................................................................53
Figure 3-3: Patient-derived cell line and xenograft models exhibit MET-dependent modulation of downstream kinase signaling ..........................................................................................................................56
Figure 3-4: Hypothesized basis of MET oncogene dependency versus EGFR-MET codependency may rely on differential dimerization with and activation of HER3 ................................................................................60
Figure 3-5: EGFR activation through treatment with exogenous ERBB family ligands is sufficient to induce MET inhibitor resistance through downstream pathway reactivation ..................................................................................63
Figure 3-6: Assessment of the prevalence of activated EGFR-HER3 versus MET-HER3 dimers as a potential predictor of MET dependency ........................................................................................................69
Figure 3-7: Ectopic overexpression of activating mutant EGFR is sufficient to induce EGFR/MET codependency in MET-dependent models of EGFR-mutant NSCLC ........................................................................72
Figure 3-8: Treatment with DNA methyltransferase inhibitor decitabine induces crizotinib resistance in DFCI81 and DFCI161 cell lines ................................................................................................................74
Figure 3-9: DFCI161 cells develop crizotinib resistance through activation of EGFR signaling .77
Figure 4-1: Observation of a culture media dependent switch in drug sensitivity of DFCI81 and DFCI161 cell lines ......................................................................................................................................................89
Figure 4-2: Activation of downstream signaling cascades in DFCI161 cells established in RPMI-1640 versus ACL-4 media ............................................................................................................................................90
Figure 4-3: Hydrocortisone in ACL-4 media induces A10-associated drug sensitivity phenotype .................................................................93

Figure 4-4: Glucocorticoid-mediated downregulation of MET expression and activation may be sufficient to induce GC-associated drug sensitivity phenotype in DFCI161 cells .........................95

Figure 4-5: Dexamethasone induces cell cycle arrest and senescence-associated properties..97

Figure 4-6: Dexamethasone treatment of mice bearing patient-derived DFCI161 xenografts did not induce any change in the drug sensitivity of the tumors ..........................................................99

Figure 5-1: Predicted clinical benefits of first-line MET inhibitor monotherapy for patients whose EGFR-mutant tumors exhibit a switch to MET oncogene dependency .................106

Supplemental Figure I-1: CRISPR construct design .................................................................110

Supplemental Figure I-2: Rate of spontaneous mutation in CRISPR-edited PC9 cells ........111

Supplemental Figure II-1: DFCI81 and DFCI161 cells are unique in their sensitivity to single-agent crizotinib treatment ........................................................................................................113

Supplemental Figure II-2: DFCI81 and DFCI161 cells are unique in their sensitivity to single-agent crizotinib treatment ........................................................................................................114

Supplemental Figure II-3: DFCI307 xenografts exhibit MET-dependent activation of HER3 and EGFR-downstream signaling ........................................................................................................115

Supplemental Figure II-4: Expression of growth factors and receptor tyrosine kinases in DFCI81 and DFCI161 cell line models ...................................................................................................................................116

Supplemental Figure II-5: EGFR involvement in ERBB ligand-induced crizotinib resistance...117

Supplemental Figure II-6: Ectopic overexpression of wild type EGFR in DFCI81 cells is sufficient to induce EGFR-MET codependency following ERBB ligand treatment .......................118

Supplemental Figure II-7: Prevalence of EGFR-HER3 dimers is unaffected by ligand treatment .................................................................................................................................119

Supplemental Figure II-8: p85-HER3 dimerization dynamics ....................................................119

Supplemental Figure II-9: Mini-screen of Epigenetic Modifiers in DFCI81 and DFCI161 cells 120

Supplemental Figure II-10: Decitabine induces EGFR-mediated resistance to crizotinib in DFCI81 cells ........................................................................................................................................123

Supplemental Figure II-11: Characterization of crizotinib resistant DFCI161 populations and clones ...........................................................................................................................................124

Supplemental Figure II-12: Identification of alternative mechanisms of resistance development in DFCI81 cells ..................................................................................................................125

Supplemental Figure III-1: Media-associated modulation of drug sensitivity in EGFR mutant, MET-dependent cell lines ..................................................................................................................126

Supplemental Figure III-2: Dexamethasone-associated switches in drug sensitivity are transient and reversible ..........................................................................................................................128
List of Tables

Table 1-1: ERBB Family Ligands ..................................................................................................................8
Table 1-2: EGFR targeted therapy in lung cancer and resistance development (65-72) ..............11
Table 1-3: Non-Small Cell Lung Cancer Cell Line Models ........................................................................20
Table 1-4: Preclinical models of sensitivity and resistance to targeted therapy in NSCLC ..........22
Table 2-1: CRISPR Cell Line Creation ........................................................................................................28
Table 2-2: EGFR Codon 790, Nucleotides 2368-2370 ..............................................................................43
Table 3-1: Patient-derived cell lines and xenograft models being characterized .......................49
Table 3-2: Prevalence of MET dependency in patient-derived models .............................................81
Table 4-1: Composition of ACL-4 Media: RPMI-1640 + Factors (156) .................................................86
Supplemental Table I-1: Frequencies of Donor Sequence Integration versus Spontaneous
Mutation in Post-Selection CRISPR Models .......................................................................................112
Supplemental Table III-1: Media-dependent drug sensitivity profile appears to be unique to
MET-dependent models ......................................................................................................................127
List of Abbreviations

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EPR: Epiregulin

ERBB: Avian erythroblastosis oncogene B

GC: Glucocorticoid

GR: Glucocorticoid receptor

HER: Human epidermal growth factor receptor

HGF: Human growth factor

MET: Mesenchymal-epithelial transition factor

NSCLC: Non-small cell lung cancer

NRG: Neuregulin

RTK: Receptor tyrosine kinase

TKI: Tyrosine kinase inhibitor
Chapter 1: Introduction
Copyright Disclosure

Portions of this Chapter are adapted from the following publication:


1.1. Non-small cell lung cancer classification and treatment

Despite the advent of precision therapies, lung cancer remains the leading cause of cancer deaths worldwide (1,2). The two histological classes of lung cancer are small cell lung cancer (SCLC), accounting for nearly 20% of lung cancers, and non-small cell lung cancer (NSCLC), which comprises the remaining 80-85% (3,4). NSCLC is further subdivided into three subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (5,6). Clinical standard of care treatments for lung cancer are determined by disease stage, histopathological subtype, and tumor mutational profile (7,8). Conventional standard of care therapies for late-stage metastatic NSCLC include cytotoxic chemotherapy, targeted therapy, and immunotherapy (8,9).

The clinical treatment of NSCLC is predicated on tumor stage, histopathology, genomic alterations, and immune status. Stage I, stage II, and some locally advanced stage III NSCLCs are surgically resectable with full lobectomy as the preferred approach, and mediastinal lymphadenectomy recommended for stage III patients (3). Following surgery, stage II and III patients may be administered adjuvant chemotherapy and/or radiotherapy (10,11). Patients with advanced stage III and metastatic stage IV disease often receive no survival benefit from surgical intervention (12-14). Late stage lung cancers are instead treated with first-line systemic therapy, including chemotherapy, targeted therapy, and/or immunotherapy (15,16). Unfortunately, while chemotherapy, targeted therapy, and immunotherapy interventions significantly prolong
progression-free survival and overall survival, they are not curative, with the vast majority of patients ultimately developing recurrent and/or refractory disease. Because the clinical treatment of NSCLC is limited by the development of drug resistance, the new frontier for thoracic oncology and translational medicine has become the elucidation and targeting of drug resistance mechanisms in an effort to establish novel combinatorial therapeutic approaches that translate into improved patient prognosis and survival.

Tumors become treatment refractory through a diverse array of mechanisms, which are often contingent upon the underlying genomics of the cancer cells and the therapeutic regimen received. NSCLC subtypes are heterogeneous, with different histological subtypes exhibiting distinct drug susceptibilities. The mainstay cytotoxic chemotherapy for squamous cell carcinomas—which exhibit elevated expression of thymidylate synthase and an increased dependency on pyrimidine synthesis compared to their non-squamous counterparts—is combination treatment with gemcitabine (a synthetic pyrimidine nucleoside analog) plus cisplatin (a DNA crosslinking agent) (3,17). By contrast, the standard of care chemotherapeutic regimen for non-squamous NSCLC with no actionable driver mutations is cisplatin plus pemetrexed (a folate antimetabolite). Patients generally respond transiently to these cytotoxic regimens with median progression-free survival benefit of five months and a median OS increase of eleven months to one year on platinum doublet chemotherapy (17). Resistance to cytotoxic as well as targeted therapies may either be endogenous to a patient’s tumor (de novo resistance) or develop over the course of drug exposure (acquired resistance) (18).

In addition to limited duration of treatment response, chemotherapy is poorly tolerated due to the indiscriminate toxicity to many rapidly-proliferating cell types. Precision approaches
to NSCLC treatment are rapidly expanding as an alternative to cytotoxic chemotherapy for cancers bearing targetable genetic alterations. In contrast to chemotherapy, precision therapies are better tolerated, inducing significantly fewer off-target side effects due to selective targeting of cancer cells, thereby offering tangible improvements to quality of life compared to their cytotoxic counterparts (19,20). Targeted therapies additionally confer a significant survival benefit on patients whose tumors harbor targetable lesions, resulting in a median overall survival increase from 7.9 months in 2002, when the standard of care for all patients was cytotoxic chemotherapy, to 27.3 months in 2015, once targeted therapies were in routine clinical use (21).

Precision treatments target the functions of aberrantly mutated or hyperactivated oncogenes specific to cancer cells. This specificity is associated with reduction of off-target effects associated with chemotherapy—including hair loss, nausea, skin sensitivity, and renal sensitivity, among others—due to collateral damage to healthy cell and tissue types. Some next-generation targeted therapies are mutant-specific, with drug scaffolds designed to spare the functions of wild type proteins found in healthy tissues thereby mitigating ubiquitous oncogene targeting (22). However, even targeted therapies that lack mutant specificity are significantly better tolerated than cytotoxic chemotherapy because in oncogene dependency, cancer cells rely disproportionately on the function of a single mutant oncogene and are thereby disproportionately sensitive (compared to healthy cells) to the inhibition of that oncogene and perturbation of its downstream signaling (18,23).

A limitation of targeted therapies is that they only benefit a subset of patients whose tumors harbor targetable driver mutations. Some 30% of adenocarcinomas in Caucasian patients (higher in Asian patients), as well as a subset of squamous cell lung cancers, have identified driver
mutations, whereby their survival is dependent on the activity of a single oncogene (24,25). The most prevalent adenocarcinoma drivers are activating mutations in the Kirsten rat sarcoma viral oncogene homolog (KRAS), accounting for ~25% of driver mutations, epidermal growth factor receptor (EGFR) mutations, comprising ~10% of driver oncogene mutations in the Caucasian adenocarcinoma population (and ~40% of oncogenic alterations in East Asian NSCLC patient populations (25)), and oncogenic fusions in anaplastic leukemia kinase (ALK), occurring in ~5% of adenocarcinoma patients (24). Less common driver alterations, each accounting for <5% of adenocarcinomas, include oncogenic ROS1 fusions, activating HER2 mutations, oncogenic RET rearrangements, and activating human growth factor receptor (MET) lesions (24,26-28). While KRAS GTPase is not a therapeutically druggable target, precision therapies are available for tumors harboring mutations to receptor tyrosine kinases, including EGFR, ALK, ROS1, HER2, and MET mutant tumors (29-31). Unfortunately, however, targeted therapies are also not curative: as with chemotherapy, the efficacy of targeted therapies is limited by the development of drug resistance (18).

1.2. EGFR and ERBB family signaling pathways

EGFR, a member of the avian erythroblastosis oncogene B (ERBB) family of receptors, is a cell surface receptor tyrosine kinase (RTK) that promotes cell proliferation and survival in response to ligand binding (32-35). EGFR comprises an N-terminal extracellular domain, including structures for ligand binding that control dimerization, a single-pass transmembrane domain, an intracellular kinase domain, and an intracellular C-terminal tail (36). Following ligand binding, wild type EGFR associates with other receptors by either homodimerizing with another EGFR protein or heterodimerizing with a different receptor tyrosine kinase, often another ERBB family
ERBB dimerization results in ATP-mediated transphosphorylation of the C-terminal tail of one receptor, the acceptor, by the kinase domain of the other, the donor receptor (36,39). Although both donor and acceptor receptors in a dimer may be capable of kinase activity, the phosphorylation that occurs is always a result of acceptor phosphorylation by the donor due to the structural asymmetry of the dimers. In contrast to other ERBB family members, HER3 has low kinase activity; while EGFR, HER2, and HER4 receptors may act as donors or acceptors during dimerization, HER3 is always an acceptor (36). In reality, the dimer model is a simplified view: activated EGFR and ERBB oligomers often exist on the cell membrane as chains of activated EGFR, as well as other receptor tyrosine kinases, that transphosphorylate one other, amplifying and consolidating pro-proliferative signaling (40-42).

Once phosphorylated, EGFR and other ERBB family members create binding sites for scaffolding and adaptor proteins that contain Src homology (SH2) domains specialized for phosphoprotein binding (Figure 1-1) (36). One SH2 domain-containing adaptor protein that is recruited to phosphorylated ERBB receptors is Grb2 (43). While the SH2 domain of Grb2 mediates its interaction with phosphorylated tyrosine residues on activated ERBB receptors, among other RTKs, SH3 domains on the protein recruit the guanosine exchange factor Son of Sevenless (SOS) and the scaffolding protein Grb2-associated binder 1 (Gab1) to the plasma membrane (43). Once localized to the membrane, SOS activates membrane-bound KRAS GTPase, which in turn initiates a protein kinase cascade culminating in the phosphorylation and activation of ERK1/2, a driver of cell proliferation (43). Upon recruitment to the plasma membrane, the scaffolding protein Gab1 is phosphorylated, creating docking sites for the PI3K regulatory subunit p85 (43,44). The p85 interaction with phosphorylated GAB1 recruits the p110 catalytic PI3K subunit to the membrane,
where it initiates a signaling cascade that ultimately drives phosphorylation and activation of Akt, a pro-survival signal (Figure 1-1) (43,45). In addition to its adaptor functions, Gab1 contains numerous docking sites to other proteins to the plasma membrane to be activated, serving to not only transmit but also amplify signaling from activated RTKs (46).

**Figure 1-1: Canonical EGFR signaling**

EGFR signaling is activated by ligand binding, which induces receptor homodimerization (with another EGF receptor), or heterodimerization with another ERBB3 family member. Dimerization results in receptor transphosphorylation, which creates binding sites to recruit scaffolding and adaptor proteins to the membrane. These adaptor proteins, in turn, recruit downstream kinases to the membrane to be activated. Two of the principal nodes through which EGFR-downstream signaling cascades are perpetuated are PI3K/Akt and MAPK/ERK signaling. Phosphorylation corresponds to activation for most EGFR-downstream pathways.
In both healthy cells and tumors, activation of wild type EGFR requires ligand engagement for kinase activation, dimerization, and transphosphorylation. As revealed by its name, the epidermal growth factor (EGF) is among the ligands associated with EGFR activation, although EGFR may also be activated by other ERBB family ligands, including epiregulin, epigen, and HB-EGF (47,48). These ligands, which are essential to the dimerization and thereby activation of EGFR, HER3, and HER4, are listed in Table 1-1, alongside their respective receptor binding repertoires. Notably, unlike the other ERBB family members, HER2 is constitutively active and lacks a ligand binding domain. HER2 signaling is restricted by its ability to dimerize with other ligand-bound ERBB family members, predominantly HER3, or form distinctive homodimers (36,49,50). As a safeguard against aberrant proliferative signaling through constitutively active HER2 homodimers, the level of HER2 protein expression is often low in healthy tissues, and the formation of HER2-HER2 homodimers is structurally unfavorable compared to HER2 dimerization with another liganded ERBB family member (48,50).

Table 1-1: ERBB Family Ligands (47,48)

<table>
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<tr>
<th>Ligand</th>
<th>Abbreviation</th>
<th>ERBB Receptors Bound</th>
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<tr>
<td>Epidermal Growth Factor</td>
<td>EGF</td>
<td>EGFR</td>
</tr>
<tr>
<td>Amphiregulin</td>
<td>AREG</td>
<td>EGFR</td>
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<tr>
<td>Epigen</td>
<td>EPGN</td>
<td>EGFR</td>
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<tr>
<td>Transforming Growth Factor α</td>
<td>TGFα</td>
<td>EGFR</td>
</tr>
<tr>
<td>Betacellulin</td>
<td>BTC</td>
<td>EGFR, HER4</td>
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<tr>
<td>Heparin Binding EGF-Like Growth Factor</td>
<td>HB-EGF</td>
<td>EGFR, HER4</td>
</tr>
<tr>
<td>Epiregulin</td>
<td>EREG, EPR</td>
<td>EGFR, HER4</td>
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<tr>
<td>Neuregulin 1</td>
<td>NRG1, HRG1</td>
<td>HER3, HER4</td>
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<tr>
<td>Neuregulin 2</td>
<td>NRG2, HRG2</td>
<td>HER3, HER4</td>
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<tr>
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<td>HER4</td>
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<tr>
<td>Neuregulin 4</td>
<td>NRG4, HRG4</td>
<td>HER4</td>
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Each ERBB ligand has distinct properties, not only in its spatiotemporal expression patterns and receptor binding repertoire, but also in its binding affinities, and activated receptor conformations as well as higher-order ERBB receptor organization induced (37,51). While the idiosyncrasies of the signaling cascades triggered by distinct ERBB ligand-receptor pairings remain poorly understood, it is important to appreciate that the repertoire or ERBB activating ligands expressed are distinct in their biological functions.

Activating EGFR mutations in NSCLC result in locking the kinase domain in an active conformation, rendering the receptors independent of ligand binding to assume an active conformation (36). While the general consensus is that mutant EGFR still requires dimerization for maximal activity, the barrier of ligand stimulation for activation is effectively removed in these receptors (52). It has also been reported that some activating EGFR mutants are able to signal independently of dimerization (53). Fortuitously, the constitutively active kinase domain conformation of these mutant EGFR receptors, the oncogenic drivers of a subset of NSCLC, renders them uniquely susceptible to inhibition by small molecule kinase inhibitors that engage the receptor in its active conformation (36,52).

1.3. Development of resistance to EGFR-targeted therapies through acquisition of secondary and tertiary EGFR mutations

Activating somatic mutations of the epidermal growth factor receptor (EGFR), which drive lung cancer by promoting constitutive, ligand-independent receptor activation, are detected in the tumors of 10-15% of Caucasian NSCLC patients (more prevalent in East Asian NSCLC populations) (54-57). Identified EGFR activating mutations include a missense mutation in codon 858 resulting in a substitution of arginine for leucine (L858R), short in-frame deletions of exon 19
—most commonly, a fifteen base pair deletion resulting in loss of codons 746-750, known as ELREA deletions due to the sequence of amino acids lost from the receptor (glutamic acid-leucine-arginine-glutamic acid-alanine)—and short in-frame exon 20 insertions (57,58). L858R missense mutations and exon 19 deletions account for approximately 85% of activating EGFR mutations. Unlike lung cancers harboring exon 20 insertions, tumors positive for EGFR L858R and EGFR del19 mutations often exhibit de novo sensitivity to targeted EGFR inhibitors (57). Consequently, the clinical standard of care for EGFR sensitizing mutant NSCLC is first-line treatment with EGFR inhibitors including erlotinib (Tarceva), gefitinib (Iressa), and osimertinib (Tagrisso) (54,56,59,60).

Initially EGFR-mutant tumors are highly sensitive to EGFR-targeted therapy, but the clinical success of EGFR inhibitors is limited by the development of drug resistance and tumor progression within a median of 12-14 months of treatment (61-63). Resistance to EGFR kinase inhibitors may occur through a diverse array of mechanisms that can be classified in three general categories, each of which converges on EGFR signaling reactivation (18). First, the targeted kinase (EGFR in this case) can be reactivated through secondary mutation. Second, EGFR-downstream pathways, such as ERK1/2 and Akt, may be reactivated to preserve cell survival, even in the absence of EGFR activation. Finally, bypass mechanisms can activate compensatory parallel pathways, such as other cell surface RTKs including MET and HER2 (Figure 1) (18), or mitigate cell dependency on EGFR signaling through activation of alternative pathways such as epithelial-to-mesenchymal transition (EMT), or histopathological transition to small cell lung cancer (64). Some examples of resistance mechanisms to EGFR targeted therapies observed in clinical and preclinical studies are listed in Table 1-2.
Table 1-2: EGFR targeted therapy in lung cancer and resistance development (65-72)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>First-Line Therapy</th>
<th>Drug resistance mechanism</th>
<th>Clinical Prevalence</th>
<th>Second-line Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR del19 EGFR L858R</td>
<td>Erlotinib Gefitinib</td>
<td>T790M mutation</td>
<td>50-60%</td>
<td>EGFR TKI (Osimertinib)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MET amplification and activation</td>
<td>5-20%</td>
<td>EGFR TKI + MET TKI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HER2 amplification</td>
<td>5-10%</td>
<td>EGFR &amp; HER2 TKI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCLC Transformation</td>
<td>5-15%</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other/Unknown</td>
<td>15-30%</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>EGFR del19 EGFR L858R Del19/T790M L858R/T790M</td>
<td>Osimertinib</td>
<td>C797S mutation</td>
<td>20-40%</td>
<td>EGFR TKI (Erlotinib)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other tertiary EGFR mutations</td>
<td>5-20%</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-T790M allele enrichment *</td>
<td>20-60%</td>
<td>EGFR TKI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MET amplification and activation</td>
<td>5-30%</td>
<td>EGFR TKI + MET TKI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCLC Transformation</td>
<td>~5%</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other/Unknown</td>
<td>~20</td>
<td>Chemotherapy</td>
</tr>
</tbody>
</table>

*Not mutually exclusive with other mechanisms of osimertinib resistance.

One mechanism of resistance to EGFR targeted therapies is mutational reactivation of the EGFR protein—a problem that may be combated by the development of next-generation targeted inhibitors. An example of this paradigm comes from the development of secondary and tertiary drug resistance mutations in the progression of EGFR mutant NSCLC (Figure 1-2). The most prevalent resistance mechanism, detected in 50-60% of patients following treatment with the first-generation EGFR inhibitors erlotinib and gefitinib, is a secondary EGFR missense mutation, T790M, which is always acquired in cis with the primary activating mutation. EGFR T790M is a classic gatekeeper mutation, which results in substitution of a bulkier methionine residue for the endogenous threonine at codon 790, increasing receptor ATP affinity and thereby
prohibiting binding of first-generation reversible EGFR inhibitors (57,73). Paradoxically, although the T790M gatekeeper mutation has been shown to enhance EGFR kinase activity and cellular transformation ability—even in the absence of cis EGFR activating mutations—EGFR T790M-harboring tumors and cell lines exhibit characteristically indolent growth in pre-clinical models of EGFR mutant NSCLC, and are associated with favorable clinical prognosis compared to tumors exhibiting other mechanisms of drug resistance (73). Activating + T790M-mutant EGFR is thought to be present at low allelic and clonal frequency in pre-treatment tumors (though often undetectable due to the low prevalence and indolent growth kinetics associated with the mutation), though it may also develop de novo during the course of clinical treatment (74,75). Regardless of its origin, T790M must be in cis with the activating driver mutation (L858R or del19) in order to confer drug resistance, and is enriched in the tumor population over the course of first-generation EGFR inhibitor treatment (76,77).
Drug resistance in EGFR-driven NSCLC may develop through the progressive accumulation of mutations in cis with the EGFR activating mutation (exon 19 deletions and the L858R point mutation). The most prevalent mechanism of resistance to EGFR inhibitors erlotinib and gefitinib is the gatekeeper mutation T790M. While the mutant-selective EGFR inhibitor osimertinib retains efficacy against T790M-mutant EGFR, the tertiary C797S mutation alters its covalent binding site, resulting in drug resistance and disease progression. In a clinical scenario where osimertinib replaces erlotinib as first-line therapy, C797S emerges alone, in the absence of T790M. C797S mutant EGFR retains sensitivity to first-generation EGFR inhibitors including erlotinib and gefitinib, which may in turn select for T790M mutant EGFR. Regardless of which class of EGFR inhibitor is used in first-line therapy, triple mutant EGFR (del19/T790M/C797S or L858R/T790M/C797S) is expected to remain the clinically untargetable endpoint upon progression.
Because T790M is the most prevalent resistance mechanism in EGFR-driven NSCLC, recent research in medicinal chemistry has been directed toward the clinical development of T790M-targeting EGFR inhibitors. Initial attempts to target T790M yielded the second-generation irreversible pan-ERBB inhibitors afatinib and dacomitinib, which covalently bind EGFR (78-81). Clinical use of afatinib and dacomitinib has been restricted by the dose-limiting toxicities associated with irreversibly inhibiting wild type EGFR (82,83). Furthermore, while afatinib and dacomitinib exhibit modestly improved binding and inhibition of T790M-mutant EGFR, the gatekeeper mutation nonetheless reduces drug efficacy. Preclinical models have also revealed amplification of the T790M-mutant EGFR allele as a mechanism of dacomitinib resistance (84). Nevertheless, pan-ERBB inhibitors have remained in clinical use, prescribed for cancers driven by aberrant HER2 activation, or tumors that develop HER2-mediated drug resistance (83,85).

As the limitations of second-generation pan-ERBB inhibitors for EGFR targeting were becoming evident, a screen of irreversible tyrosine kinase inhibitors yielded the third-generation inhibitor WZ4002, which exhibited a 30- to 100-fold increased potency against EGFR T790M and significantly reduced affinity for wild type EGFR compared to first- and second-generation inhibitors (86). Diminished binding to wild type EGFR by mutant-selective inhibitors is clinically appealing because it mitigates dose-limiting toxicities (86,87). Preclinical studies with WZ4002 led to the subsequent development of two clinical compounds, AZD9291 (osimertinib/Tagrisso) and CO-1686 (rocilitinib), which were shown in clinical trials to have significant activity in patients with EGFR T790M-harboring tumors, with a response rate of ~60% (86,88,89). Clinical development of rocilitinib was ultimately discontinued due to lack of efficacy and toxicities, including drug-induced diabetes, drug-induced cataracts, and cardiac arrhythmias (90). On the
other hand, osimertinib was ultimately approved for not only second-line therapy for EGFR-mutant cancers progressed on first-line erlotinib treatment, but was eventually approved for first-line treatment of inhibitor-naïve tumors (91). Osimertinib is now the preferred first-line standard of care therapy for patients with sensitizing EGFR-mutant NSCLC, due to its superior mutant selectivity, increased tolerability, enhanced potency, dramatically improved progression-free survival (with a median progression-free survival benefit of 18.9 months on first-line osimertinib, compared to 10.2 months on erlotinib) (21,60). Aggregate advancements in overall survival have yet to be quantified, as clinical trials are still ongoing (60).

Due to its mutant selectivity, first-line osimertinib treatment prohibits resistance development through the EGFR T790M gatekeeper mutation. Even so, both preclinical studies and clinical assessment have predicted the development of a distinct set of point mutations to confer resistance to osimertinib therapy. Among osimertinib resistance mutations is EGFR C797S, which may emerge either alone or in cis with EGFR T790M when osimertinib is administered as second-line therapy following T790M-mediated progression on first-line erlotinib treatment (92,93). Like afatinib, osimertinib is a covalent EGFR inhibitor, however unlike afatinib, osimertinib also requires covalent receptor engagement for its inhibitory activity (91). Both these classes of irreversible inhibitors bind to cysteine 797, such that mutation of this cysteine to a serine residue (C797S) prohibits covalent drug binding. While afatinib is still able to engage the receptor (albeit with reduced efficacy) in the absence of covalent binding, the activity of osimertinib is completely abolished by the presence of an EGFR C797S mutation, resulting in cancer cell drug resistance. Fortunately for patients who progress on first-line osimertinib through C797S mutation, this genetic lesion alone does not affect the ability of receptor binding
and inhibition by first-generation EGFR inhibitors, so gefitinib and erlotinib remain viable next-line therapeutic options in this context (Figure 1-2).

A number of other tertiary EGFR mutations have been observed to confer osimertinib resistance, including mutations at codons L718, L792, G796, and V802 (71,92,94,95). While the C797S mutation confers osimertinib resistance both in cis and in trans to the T790M mutation, these mutations have exclusively been observed in cis T790M when osimertinib is administered as second-line therapy (94). Osimertinib resistance mutations may occur either individually or concurrently in a tumor. In most cases, when there are multiple mutations associated with resistance to osimertinib present in the same tumor, sequencing has revealed the mutations existing in trans to each other, most likely pointing to intratumoral clonal heterogeneity over the course of osimertinib resistance development (71,94).

“Loss” of the EGFR T790M allele has also been associated with resistance in close to 50% of osimertinib resistant patient-derived specimens, depending on the cohort (71,92,94,95). While prevalent in acquired drug resistance, it is unlikely that T790M loss drives drug resistance. Instead, it is more likely that the drug resistant subclone harboring T790M was outgrown by other mechanisms over the course of osimertinib resistance progression (70-72). In the absence of erlotinib, there would be no selective pressure to promote T790M survival, so detectible T790M in the tumor population would be outgrown and lost.

While T790M-mutant EGFR retains osimertinib sensitivity, and C797S-mutant EGFR retain sensitivity to gefitinib and erlotinib, triple mutant EGFR (del19/T790M/C797S, or L858R/T790M/C797S) remains clinically incurable. Ultimately, triple-mutant EGFR is expected to emerge—whether acquired through sequential treatment with first- then third-generation
inhibitors, or sequential treatment with third- followed by first-generation inhibitors—ultimately restricting the clinical efficacy of existing EGFR kinase inhibitors. The current standard of care for triple EGFR-mutant NSCLC is chemotherapy, or investigational EGFR-targeted therapies including antibodies such as cetuximab and necitumumab (96,97). Allosteric inhibitors are another class of EGFR inhibitor that present a viable alternative to kinase inhibitors. Allosterics have shown promise in pre-clinical investigation, they require further optimization to rival the single-agent efficacy of small molecule kinase inhibitors (98). While allosteric EGFR have been shown to effectively inhibit triple mutant EGFR, they are only able to bind if the receptor has the L858R activating mutation, and unable to engage EGFR del19 due to constriction of the binding pocket as a result of a shortened protein (98).

1.4. Development of resistance to EGFR-targeted therapies through MET kinase amplification

There is significant crosstalk between signaling pathways downstream of EGFR and other cell surface receptors, including HER2/3, MET, and IGF1R. This redundancy between signaling cascades gives rise to compensatory mechanisms, which can facilitate EGFR pathway reactivation and resistance to EGFR-targeted therapies (99-102). Crosstalk between signaling cascades downstream of EGFR and other cell surface receptor tyrosine kinases (RTKs) MET, IGF1R, and other HER2/3, gives rise to these compensatory mechanisms, permitting pathway reactivation and resistance to EGFR inhibitors (34). A prevalent mechanism of resistance, which may compromise the clinical efficacy of first-, second- and third-generation EGFR inhibitors alike, is amplification or overactivation of the oncogenic receptor tyrosine kinase MET (80,103).

MET is another proto-oncogenic RTK that may be activated by its ligand, the hepatocyte growth factor (HGF) (104). MET amplification is prevalent in cancers, including those of the lung,
and both MET protein overexpression, or overactivation as a result of HGF overexpression are documented drivers of resistance to targeted EGFR kinase inhibitors through reactivation of canonical EGFR-downstream signaling cascades (34,105). The mechanism of activation of downstream kinase cascades by MET is mostly conserved with the mechanisms observed in EGFR, with a notable exception being that the ERBB RTKs only contain binding sites for SH2 domains, requiring adaptor proteins such as Grb2 to interface with SH3 domain-containing scaffolding and kinase-associated proteins. In contrast to these receptors, the intracellular domain of MET contains SH3 domains, which can directly recruit SH3 domain-containing proteins such as Gab1 (104,106).

Reactivation through upregulation of MET expression or activation is a common bypass pathway in EGFR mutant lung cancers (18,34). MET amplification, MET overexpression, and MET overactivation by its ligand, HGF, have all been implicated in inducing MET-mediated drug resistance to EGFR-targeted therapies (34,105). As with the secondary EGFR T790M mutation, experimental evidence suggests that MET amplification may either be acquired as a result of EGFR inhibitor treatment, or occur de novo in pre-treatment tumors (107,108). In either case, MET amplification is a robust predictor of MET kinase-mediated resistance to EGFR inhibitors in EGFR-mutant NSCLC. Tumors with concurrent EGFR activating mutation and MET amplification are expected to be refractory to treatment with either EGFR or MET TKIs individually, but respond to combination treatment with concurrent EGFR and MET inhibitors (34).

As with the EGFR tyrosine kinase, MET kinase is targetable by small molecule kinase inhibitors (109). The first MET TKIs to be used clinically were originally developed as Alk kinase inhibitors to treat Alk fusion-positive NSCLCs, but subsequently found to also have inhibitory
efficacy against MET as well as ROS kinases (110). MET-specific inhibitors have also since been developed (110,111). These new MET inhibitors include the experimental MET savolitinib, as well as type II MET inhibitors including merestinib and glesatinib (111). In contrast to EGFR, MET kinase is not always found to harbor activating mutations in oncogenic contexts. Particularly in instances of MET-driven resistance to EGFR kinase inhibitors, the drug resistance is often perpetuated by amplification, overexpression, or hyperactivation of wild type MET kinase, necessitating the use of inhibitors that retain efficacy against the wild type receptor (103).

Outside of its role as a defined mediator of drug resistance to EGFR-targeted therapies, the role of MET as a targetable driver oncogene in NSCLC is poorly understood. While MET amplification may predict sensitivity to clinical MET inhibitors in some lung cancer contexts, it is not a predictive biomarker of drug sensitivity (112,113). As such, MET inhibitors are not approved for clinical use in thoracic malignancies exhibiting MET amplification. At present, first-line therapy with MET inhibitors in NSCLC is under clinical development for the treatment of activating MET mutations, including a subset of recently-characterized activating exon 14 splice variants (26,27).

1.5. Preclinical models of driver oncogenes and drug resistance in NSCLC

Drug sensitivity and resistance in NSCLC can be modeled using commercially available and novel primary cell line models derived from patients with oncogene-driven lung cancers. These models faithfully recapitulate cell-autonomous processes that influence cancer drug susceptibilities, and may even accurately predict clinical outcomes of treatment (111). Our lab has established and banked hundreds of patient-derived specimens in an effort to model responses and resistance to targetted therapies. Patient-derived models of NSCLC may both be
perpetuated in culture, or propagated in vivo, to gain insight into drug resistance development through both contexts.

In some cases, primary cell lines are established from patients who have progressed on targeted therapy, and may be studied to understand the mechanisms underlying drug resistance. Alternatively, resistance to targeted tyrosine kinase inhibitors can be modeled through long-term treatment of established or patient-derived cell lines with inhibitor (84,103,107,114,115). This is achieved through dose-escalation drug treatment to establish resistant cells and characterize the emergent properties giving rise to drug resistance. These models faithfully recapitulate cell-autonomous drug resistance mechanisms that emerge in patients (74,84,114). The primary cell line models, both commercially available and established in the lab, that we have used to study sensitivity and resistance to targeted therapies are listed in Table 1-3.

### Table 1-3: Non-Small Cell Lung Cancer Cell Line Models

<table>
<thead>
<tr>
<th>Model</th>
<th>Source</th>
<th>Mutational Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC9</td>
<td>Established</td>
<td>EGFR Del19</td>
</tr>
<tr>
<td>H3255</td>
<td>Established</td>
<td>EGFR L858R</td>
</tr>
<tr>
<td>HCC827</td>
<td>Established</td>
<td>EGFR Del19</td>
</tr>
<tr>
<td>HCC827GR6</td>
<td>Established</td>
<td>EGFR Del19, MET Amplification</td>
</tr>
<tr>
<td>EBC-1</td>
<td>Established</td>
<td>MET Amplification</td>
</tr>
<tr>
<td>DFC181</td>
<td>Patient-derived primary</td>
<td>EGFR Del19, MET Amplification</td>
</tr>
<tr>
<td>DFCI110</td>
<td>Patient-derived primary</td>
<td>EGFR Del19, MET Amplification</td>
</tr>
<tr>
<td>DFCI161</td>
<td>Patient-derived primary</td>
<td>EGFR L858R, MET Copy Number Gain</td>
</tr>
<tr>
<td>DFCI202</td>
<td>Patient-derived primary</td>
<td>EGFR L858R, MET Amplification</td>
</tr>
<tr>
<td>DFCI307</td>
<td>Patient-derived primary</td>
<td>EGFR Del19, MET Amplification</td>
</tr>
</tbody>
</table>
In addition to lung cancer cell lines, murine Ba/F3 pro-B cells can be used to test the transforming ability of oncogenes (116). Parental Ba/F3 cells require IL-3 supplementation for survival. Introduction of an ectopic mutant oncogene can render the cells IL-3 independent, instead making them dependent on the oncogene expressed (73,116). This oncogene dependency predisposes Ba/F3 cells to be sensitive to inhibitors targeting the overexpressed oncogene, making them a versatile and tractable platform for screening new targeted inhibitors. Ba/F3 cells may also be utilized to model the emergence of secondary or tertiary drug resistance mutations. To this end, following IL-3 withdrawal, the newly oncogene-driven Ba/F3 cells are subjected to an accelerated mutagenesis assay in the presence of targeted inhibitors to identify mutations that can bypass drug activity (117,118). The emergence of the C797S tertiary resistance mutation was initially predicted through a Ba/F3 mutagenesis assay prior to being observed in the clinic (117). Following overexpression of mutant EGFR in Ba/F3 cells and IL-3 withdrawal, the cells were treated with the mutagen N-ethyl-N-nitrosourea (ENU) concurrently with the EGFR inhibitor osimertinib. This ENU mutagenesis assay was able to reliably predict not only the emergence of C797S, but also of several other clinical tertiary EGFR mutations that conferred resistance to mutant-selective EGFR inhibitors (92,117).
Table 1-4: Preclinical models of sensitivity and resistance to targeted therapy in NSCLC

<table>
<thead>
<tr>
<th>Model</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba/F3 models</td>
<td>• Rapid screening</td>
<td>• Mouse pro-B cell line: non-human, non-cancer model</td>
</tr>
<tr>
<td></td>
<td>• De novo prediction of oncogene reactivating mutations through ENU accelerated mutagenesis</td>
<td>• May exhibit artificial drug sensitivity due to the absence of concurrent mutations or oncogenic interacting factors present in cancer</td>
</tr>
<tr>
<td></td>
<td>• Clean model to study drug sensitivity in the context of an individual oncogene</td>
<td></td>
</tr>
<tr>
<td>Cancer cell lines (previously established and new patient-derived models)</td>
<td>• Tractable models for studying cancer cell-autonomous processes, drug responses, and mechanisms of drug resistance</td>
<td>• Limited by availability and ability to establish viable models</td>
</tr>
<tr>
<td></td>
<td>• Patient-derived cancer model, containing mutations and/or processes of interest in a clinically relevant context</td>
<td>• Heterogeneity among models</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Resistance modeling takes longer than ENU mutagenesis studies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Inability to model cancer cell non-autonomous resistance mechanisms</td>
</tr>
<tr>
<td>CRISPR-Cas9-modified cell lines</td>
<td>• Introduction of point mutations, fusions, deletions, etc. to establish a desired genetic background for screening and drug discovery purposes</td>
<td>• Limited by CRISPR efficiency—although this can be greatly increased by drug selection if studying resistance mutations</td>
</tr>
<tr>
<td></td>
<td>• Creation of isogenic models harboring distinct mutational profiles</td>
<td>• Some lesions, such as amplifications, are difficult to introduce. CRISPR-A mediated expression upregulation would be more straightforward in these contexts</td>
</tr>
<tr>
<td>Patient-derived xenograft models (PDX)</td>
<td>• If derived directly from patient without intermediate cell culture, may more faithfully recapitulate tumor biology</td>
<td>• Subcutaneous implantation may not accurately recapitulate endogenous tumor microenvironment</td>
</tr>
<tr>
<td></td>
<td>• Assessing in vivo kinetics of drug treatment and resistance development while using patient-derived tumors with correspondingly variable genomic backgrounds</td>
<td></td>
</tr>
</tbody>
</table>
A newer model to study resistance to targeted therapy and novel disease mutations is the direct genomic modification of lung cancer cell lines using CRISPR/Cas9-mediated gene editing (119). These techniques can be useful to generate secondary/tertiary drug resistance mutations rather than time-consuming drug selection that allows resistance to develop naturally. Genome-edited lines also provide for more representative isogenic controls and a stronger tool to compare cell lines in drug sensitive/resistant states. Each of these tools is useful toward characterizing features of drug resistance and correlating with preclinical and clinical outcomes. A comparison of the strengths and weaknesses of each model is shown in Table 1-4. Herein, we leverage each of these models toward understanding resistance and sensitivity to targeted therapies in EGFR mutant lung cancer models.
Chapter 2: Modeling point mutations implicated in drug resistance using CRISPR/Cas9-mediated genome engineering
Attributions

Contributors to this work include Pınar Ö Eser, Kari J. Kurppa, Ciric C. To, Maggie Wilkens, Praful Gokhale, Dalia Ercan, Yanan Kuang, Julianna Supplee, Cloud Pawleewitz, and Pasi A. Jänne. P.Ö.E. and P.A.J. conceived the studies. K.J.K. optimized the CRISPR system used for the lab. All authors helped design and carry out the experiments, and P.Ö.E., K.J.K., M.W., Y.K, and J.S. analyzed and interpreted the results. P.G. and C.P. supervised in vivo studies and molecular work, respectively, and P.A.J. supervised the project. P.Ö.E. wrote the text with feedback from P.A.J.

2.1. Introduction

The preclinical development and validation of novel inhibitors for the treatment EGFR driven lung cancer relies, in part, on cell line and tumor models harboring sensitizing EGFR mutations. The irreversible EGFR inhibitor osimertinib was approved in 2016 for second-line use in T790M-positive erlotinib resistant lung cancer, and recently approved as a first-line therapy in EGFR-mutant NSCLC due to improved progression-free survival compared to erlotinib (21,60,120). While the clinical use of osimertinib represents a relatively recent advancement in the clinical treatment of NSCLC, results from clinical studies show that a range of 20-40% of patients develop osimertinib resistance through the secondary EGFR mutation C797S, while up to 20% develop resistance mediated by other rarer secondary mutations (70-72). Fortunately for patients who receive first-line osimertinib treatment, and whose tumors progress through C797S, the activating mutant/C797S genotype retains sensitivity to reversible EGFR inhibitors such as gefitinib and erlotinib. Unfortunately, however, the clinical efficacy of these latter inhibitors is also limited by the drug resistance, predominantly through T790M acquired in cis with the EGFR activating mutation and C797S. Thus, regardless of whether a patient receives first-line erlotinib
followed by osimertinib, or first-line osimertinib treatment followed by erlotinib, triple mutant
*EGFR* (L858R/T790M/C797S or del19/t790M/C797S) is still expected to emerge following
longitudinal treatment with these two classes of EGFR inhibitor, and be the untargetable
mutational endpoint in a subset of patients (*Figure 1-2*). With a lack of available EGFR inhibitors
effective against this genotype, patients whose tumors harbor activating
mutation/T790M/C979S *EGFR* continue to be relegated to chemotherapy, experiencing only
modest benefits in progression-free and overall survival.

Because triple mutant *EGFR* remains untreatable by targeted kinase inhibitors, there is a
need to establish NSCLC models harboring this genotype in order to screen and develop novel
next-generation inhibitors that can be used to target drug resistant tumors. There are presently
no patient-derived cell line or xenograft models harboring the C797S resistance mutation.
Although activating mutant/T790M/C797S EGFR has been ectopically overexpressed in both lung
cancer and non-cancer contexts, we wished to introduce it into lung cancer lines at
stoichiometrically relevant levels, which we anticipate will more accurately replicate the biology
of the mutation and specific targeted drug vulnerabilities in the context of lung cancer.

To this end, CRISPR-Cas9 genome engineering was harnessed to introduce the drug
resistance mutations T790M and C797S, both individually and in *cis* into EGFR inhibitor-naïve cell
lines harboring activating EGFR mutations (*Figure 2-1*). CRISPR-Cas9 genome editing (121,122)
provides a tractable tool to study drug resistance, expediting the generation of drug-resistant
cells while diminishing the likelihood of accumulating coding passenger mutations associated
with long-term dose-escalating drug treatment approaches to drug resistant cell line selection
(114,115). *EGFR* T790M and C797S were introduced into both PC-9 cells, which harbor the
activating five amino acid exon 19 $EGFR$ deletion (del 2235-2249), and H3255 cells, which have the point mutation resulting in the L858R substitution (c.2573 T>G) (123,124). Interestingly, both of these cell line models develop T790M-mediated gefitinib resistance following drug exposure in culture, although neither has been shown to develop spontaneous C797S-mediated osimertinib resistance (84,124).

**Figure 2-1: CRISPR Study Design**
Experimental workflow of CRISPR studies: two different cell lines, PC9 and H3255, will be used for introduction of drug resistance mutations into each of the two most prevalent $EGFR$ driver mutation backgrounds: exon 19 deletion and L858R, respectively. Following introduction of T790M to confer resistance to first-generation EGFR inhibitors, including gefitinib, and T790M/C797S to induce resistance to covalent mutant-selective EGFR inhibitors such as osimertinib (as well as C797S alone as a control—although C797S in the absence of T790M, or in the absence of administration of covalent second- or third-generation EGFR inhibitor has yet to be observed), successfully recombined CRISPR clones will be selected through high-dose drug treatment with gefitinib (for T790M) or osimertinib (for T790M, C797S)
2.2. Introducing EGFR inhibitor resistance mutations using CRISPR-Cas9 genome engineering

An advantage of using CRISPR-Cas9 genome engineering to introduce resistance mutations into EGFR mutant NSCLC was our ability to utilize tyrosine kinase inhibitors in the selection of correctly recombined clones. Without this drug selection, our screening process to identify successfully recombined clones would have been appreciably more complex, not only because of the low rate of homologous recombination (125), but also because the T790M and C797S mutations must recombine in cis with the activating EGFR mutation in order to confer drug resistance (126). Our efforts were also aided by the proximity of the T790M and C797S mutations to each other in the same exon, allowing us to use a single donor template to introduce both mutations. We set out to create cell line models of the mutational profiles listed in Table 2-1.

Table 2-1: CRISPR Cell Line Creation

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Activating Mutation</th>
<th>Mutation to Introduce</th>
<th>Drug Selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC9</td>
<td>EGFR Del 19</td>
<td>T790M</td>
<td>1 µM Gefitinib</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T790I</td>
<td>1 µM Gefitinib</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C797S</td>
<td>1 µM Osimertinib</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T790M/C797S</td>
<td>1 µM Osimertinib</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T790I/C797S</td>
<td>1 µM Osimertinib</td>
</tr>
<tr>
<td>H3255</td>
<td>EGFR L858R</td>
<td>T790M</td>
<td>1 µM Gefitinib</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C797S</td>
<td>1 µM Osimertinib</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T790M/C797S</td>
<td>1 µM Osimertinib</td>
</tr>
</tbody>
</table>

Highlighted in blue: cloning and validation ongoing; highlighted in red: pending development.

Following optimization of CRISPR constructs and conditions, PC9 and H3255 cells were electroporated with Cas9 protein, crRNA guide, and a donor template (T790M, C797S, or T790M/C797S) (Supplemental Figure I-1). Successfully recombined cells were selected with corresponding EGFR kinase inhibitors, as described in Table 2-1. Gefitinib and crizotinib selection were maintained continuously on the corresponding cells, to purify the successfully recombined
drug resistant cells in each population. As drug resistant clusters began to emerge from the CRISPR edited PC9 cells, five clones were picked and expanded from each construct, alongside the parental bulk population. Once lines were established, the resistance profile of each bulk population and subclone was verified by MTS assay compared to parental PC9 cells (Figure 2-2). Additionally, CRISPR sequencing was used to validate the presence and prevalence of the resistance mutation-harboring donor template in the engineered post-selection bulk populations.

Using MTS assays, we evaluated the sensitivity of our CRISPR edited PC9 cells to three generations of EGFR inhibitor to confirm that targeted mutations resulted in expected drug sensitivity profiles. While the gatekeeper mutation EGFR T790M, when found in cis with activating EGFR mutations (exon 19 deletion in PC9 cells, L858R in H3255 cells) confers resistance to first-generation EGFR inhibitors such as gefitinib and erlotinib, T790M-mutant cells retain some sensitivity to irreversible second-generation EGFR inhibitors such as afatinib and dacomitinib, which are structurally analogous to first-generation EGFR inhibitors but bind the receptor covalently at cysteine 797. T790M-mutant EGFR also retains sensitivity to irreversible third-generation inhibitors, such as osimertinib and rocilitinib, which are not only covalent, but also structurally distinct, comprised of a pyrimidine scaffold in lieu of the quinazoline scaffold found in first- and second-generation EGFR inhibitors (127). Of note, while second-generation EGFR inhibitors do not require covalent binding for their activity, receptor binding at cysteine 797 is critical for the activity of third-generation pyrimidine-based inhibitors (126). These expected drug sensitivity differences were observed in CRISPR engineered PC9 subclones and bulk populations alike (Figure 2-2a, b).
Figure 2-2: Validation of drug resistance and mutational profiles of CRISPR-edited PC9 cells

(a) Drug sensitivity profiles of CRISPR-edited PC9 bulk populations reveal that cells receiving the T790M donor exhibited gefitinib resistance but relative sensitivity to afatinib and osimertinib. By contrast, C797S bulk populations showed osimertinib resistance but gefitinib and afatinib sensitivity. As anticipated, bulk populations engineered to harbor T790M/C797S mutations in cis showed resistance to first-, second-, and third generation EGFR inhibitors. (b) Single-cell clones of each mutational profile showed identical drug sensitivities to its corresponding bulk population. (c) CRISPR sequencing of bulk CRISPR populations confirmed genomic integration of mutant EGFR alleles following Cas9+donor template nucleofection followed by drug selection.

To profile integration of the donor templates following Cas9-mediated cleavage, we sequenced bulk CRISPR-edited, post-selection PC9 populations to distinguish parental EGFR and
recombined alleles from off-target Cas9- and drug-mediated events (Figure 2-2c). Each donor that was introduced into the cells harbored not only the drug resistance mutation of interest, but also a signature of silent mutations (Supplemental Figure I-1), with the dual purposes of protecting it from cleavage, and permitting differentiation of the resistance mutations that we introduced from mutations that developed spontaneously over the course of drug treatment. Reassuringly, sequencing the bulk populations revealed donor recombination rates of 21%, 59%, and 28%, for T790M, C797S, and T790M/C797S donors, respectively (Supplemental Table I-1). Mutant EGFR alleles are often amplified in EGFR-driven NSCLC, making these population-based allele ratios difficult to interpret. Sequencing individual subclones in the future will lend clarity to resolving on- and off-target recombination and mutation events in these models.

In addition to the PC9-based CRISPR models, we also developed a panel of drug resistant cells with the H3255 background. Our primary objective in creating these models was to have both of the predominant EGFR activating mutations, EGFR Del19 and L858R point mutation, represented in future screening efforts. A major difference between these two activating mutations is their susceptibility to allosteric inhibitors, currently under development as a proposed next-line therapy in EGFR mutant NSCLC (98). While able to inhibit T790M and or C797S mutant EGFR, present allosteric inhibitors cannot bind EGFR harboring the exon 19 deletion, and only show efficacy against the EGFR L858R activating mutation (98).

Although slow-growing, H3255 is among the few established EGFR L858R mutant NSCLC lines. Development of H3255-based CRISPR models of triple mutant EGFR holds promise for prescreening new generations and scaffolds of allosteric EGFR inhibitors, among others. Using the same methodology used to generate PC9-based CRISPR models, we nucleofected H3255
parental cells with Cas9 protein alongside targeting crRNAs and donor templates encoding drug resistance mutations. While we were eventually able to recover drug resistant bulk populations of CRISPR-edited H3255 cells, these models remain too sparse at present for meaningful analysis of drug sensitivities. CRISPR-edited H3255 cells showed high attrition rates following initiation of drug selection, and took significantly longer than PC9 cells to grow out under selection following nucleofection (PC9 cells grew out in under one month after nucleofection, versus over four months for H3255 cells). Nevertheless, we eventually observed H3255 colonies begin to emerge, while control crRNA nucleofected cells ultimately succumbed to drug treatment (Figure 2-3a).

Surviving colonies of CRISPR-edited H3255 cells were suspect, however, because of their lengthy recovery time, and significant phenotypic heterogeneity (data not shown). Whereas we had confidence in our engineered PC9 colonies, which recovered and expanded quickly and showed expected drug resistance profiles for their respective mutations, the lengthy recovery time of the H3255 cells gave rise to concerns over the of the emergence of spontaneous and rogue mechanisms of resistance during drug selection. Comparison of pre-selection to post-selection sequencing results revealed significant enrichment of detectable donor template for each mutational profile: T790M donor reads were enriched from 0.22% to nearly 4%; C797S reads increased from 0.27% to 7.2%; and T790M/C797S reads went from undetectable to just under 2% (Figure 2-3b). In spite of this enrichment of CRISPR-recombined cells over three months, the allele frequencies of targeted mutations of interest remained very low in the H3255 cells, and were accompanied by a high burden of off-target point mutations and indels.
Figure 2-3: Quantification of mutant allele enrichment in CRISPR-edited H3255 cells

(a) Upper panels: Emergence of viable colonies of CRISPR-edited H3255 following weeks of drug selection. From left to right: nucleofected with T790M donor/selected with gefitinib; C797S donor/selected with osimertinib; T790M/C797S donor/selected with osimertinib. Lower panels: Negative controls succumb to drug treatment: identical conditions, nucleofected with a non-targeting control crRNA. (b) The prevalence of the donor allele introduced through homologous recombination, shown in red, was enriched in the bulk population over time. Osimertinib-selected CRISPR edited cells, but not untargeted control counterparts, additionally exhibited spontaneous development of C797S without corresponding integration of the donor strand. Accumulation of spontaneous mutations at other sites within exon 20 was also observed.
We considered the possibility that relative allele frequencies of EGFR in the PC9 background as compared to H3255 cells might explain the lower enrichment of successful gene targeting events in H3255 cells. PC9 cells harbor ~10 copies of EGFR (predominantly del19) while H3255 cells have levels of amplification nearing 25 copies (84). One copy of T790 or C797S is sufficient to confer drug resistance. Consequently, at a population level, one copy per cell would be equivalent to 10% allele enrichment in PC9 cells, as compared to approximately 4% allele enrichment in H3255 cells. Based on our bulk population CRISPR sequencing results, we inferred that we may have attained that level in the H3255 cells, even with the comparatively low recombined allele frequencies that were observed. Nonetheless, the high attrition rates of the post-CRISPR edited cells during selection, along with the prevalence of non-specific CRISPR- and drug selection-associated off-target events warrants subcloning or re-establishment of the H3255 panel before drug screening efforts with the cells may be undertaken.

Among these “off-target” events were the spontaneous emergence of C797S, as well as a few other notable mutations, over the course of drug selection. We were able to discriminate successfully recombined drug resistance mutations from de novo acquired mutations based on the silent mutations incorporated into the donor oligonucleotides (Supplemental Figure 1-2). In fact, in the case of H3255 cells, the prevalence of “spontaneously” acquired C797S—that is, C797 in the endogenous genome that mutated, rather than recombined from the donor template—surpassed the prevalence of donor oligo C797S that successfully integrated for both T790M- and T790M/C797S-recombined populations (Figure 2-3b). Importantly, because T790M does not affect osimertinib sensitivity, the spontaneously-emerging C797S in the T790M/C797S population was not accompanied by the gatekeeper mutation.
**Figure 2-4: Prevalence of spontaneous mutations in CRISPR edited H3255 cells**

The identity and frequency of spontaneous point mutations that emerged over the course of gefitinib and osimertinib selection following CRISPR editing of H3255 cells to introduce point mutations conferring drug resistance. Analysis of sequencing amplicons reveals that the L798 silent mutation, which coincides with the CRISPR PAM sequence, which is essential for Cas9-mediated DNA cleavage, is the most prevalent event, occurring spontaneously in all surviving cells. It was also shown that C797S emerges in the population independently of donor construct recombination (red) more frequently than as a result of Cas9-associated recombination (light gray).
Notably, although C797S emerged independently of the donor template at relatively high frequency in osimertinib-selected H3255 cells, none of the H3255 cells nucleofected with non-coding control crRNA developed the spontaneous mutation. This indicates that the Cas9-mediated perturbation was somehow essential, even to facilitate the spontaneous (non-homologous recombination-mediated) development of C797S. While the C797S mutation developed spontaneously in both PC9 and H3255 cells in response to osimertinib selection, spontaneous T790M emergence was not detected in either model following gefitinib selection (Figure 2-4 and Supplemental Figure I-2). This discrepancy may attest to osimertinib as a stronger selection, perhaps as a result of its covalent and mutant-specific EGFR inhibition. Interestingly, C797 and V802 mutations, both associated with clinical osimertinib resistance, developed spontaneously in at low frequency in H3255 cells selected with gefitinib. In addition to the aforementioned mutations, a silent substitution at L798 was detected across all models in both cell lines (Figures 2-4 and Supplemental Figure I-2). This is the CRISPR-associated PAM sequence that is essential for cleavage, and is therefore associated with development of Cas9 resistance rather than crizotinib resistance (Supplemental Figure I-1).

2.3. Using a CRISPR-engineered model of EGFR del19/T790M/C797S to predict assess the efficacy of experimental therapeutic regimens against triple mutant EGFR

Studies are ongoing to identify next-line treatments for triple EGFR-mutant (activating/T790M/C797S) NSCLC, including targeting alternative sites of the receptor with both allosteric inhibitors and antibody-based therapies. However, these drug development efforts require a reliable preclinical platform to test drug efficacy in cell line and tumor models of lung cancer. Having established and validated our CRISPR-edited PC9-based models of EGFR...
T790M/C797S, we sought to pilot their use as a predictive platform in the development of a xenograft-based model of drug sensitivity.

Allosteric inhibitors are not effective against \textit{EGFR} del19, so we decided to explore an antibody-based combination regimen to assess its ability to target \textit{EGFR} del19/T790M/C797S. We used our CRISPR-derived PC9 cells to study the efficacy of combining osimertinib with the monoclonal EGFR inhibitory antibody necitumumab. Our study was designed to mirror an ongoing clinical trial (NCT02496664) to test the efficacy of combined osimertinib + necitumumab therapy on \textit{EGFR} mutant NSCLC progressed on standard of care therapies (128). Out of the many ongoing efforts to find strategies to overcome osimertinib resistance, we modelled this one in our triple mutant PC9 cells because there were anecdotal reports of patient benefit. There is also precedent for the efficacy of concurrent antibody plus small molecule TKI therapy in \textit{EGFR} mutant NSCLC. In the pre-osimertinib era, a combination of the EGFR inhibitor afatinib and the EGFR inhibitory antibody cetuximab showed both preclinical and clinical efficacy in targeting EGFR T790M mutant lung cancer (129,130). The combination afatinib + cetuximab treatment approach in these reports worked by promoting EGFR degradation, and we were interested in determining whether an analogous approach would be effective in C797S-positive triple mutant NSCLC.

To this end, the PC9 T790M/C797S clone #3 was expanded and implanted subcutaneously into mice. Once the tumors engrafted, the mice were treated with osimertinib, the monoclonal \textit{EGFR} inhibitory antibody necitumumab, or an osimertinib + necitumumab combination regimen to assess the efficacy of antibody therapies in tumors harboring a C797S mutation. Interestingly, our mouse xenograft-based clinical trial revealed comparable efficacy and tolerability for single-agent necitumumab and combination necitumumab + osimertinib treatment regimens (Figure 2-
Although neither regimen induced tumor shrinkage, both arms were associated with some anti-tumor activity manifested as significantly reduced tumor growth rates. Unexpectedly, our aggregate results also revealed some slowed growth associated with single-agent osimertinib treatment, which was not anticipated since the tumors harbored the C797S osimertinib resistance mutation (Figure 2-5a). Although we speculated that these effects may be attributable to the presence of non-CRISPR modified mutant EGFR alleles in the tumor, subsequent western blot analysis supported an EGFR-independent mechanism underlying this apparent vulnerability, because there were no discernible differences in EGFR phosphorylation between vehicle treated and osimertinib treated mouse tumors (Figure 2-6). Finally, there were no remarkable differences in the tolerability of any of the drugs, as assessed by a lack of dramatic fluctuations in the weight of the animals (data not shown).
Figure 2-5: In vivo assessment of the efficacy of the EGFR-inhibitory antibody necitumumab against PC9 cells harboring T790M and C797S drug resistance mutations

(a) Aggregate tumor growth following treatment with single agent versus combination osimertinib and necitumumab (b) Body weight fluctuations over the course of the study as a metric for drug tolerability (c) Intra-individual variability among the mice in each treatment group in tumor drug response.
Analyzing the growth curve data, we observed a high degree of inter-individual variability within each treatment cohort. This prompted us to stratify our analysis by individual mice \((\text{Figure 2-5c})\). When the aggregate response curves were broken down, there was striking variability in the inter-individual variability, with some mouse tumors exhibiting “extraordinary responses” to each arm of therapy, while others displayed resiliency to drug treatment. In an effort to tease apart the mechanisms underlying these differences, the tumors of mice in each treatment group were lysed and analyzed by western blot. Examination revealed relative homogeneity within each treatment condition. Levels of total and phosphorylated EGFR were consistent across vehicle and osimertinib-treated conditions, and diminished by necitumumab treatment. This observation was expected because necitumumab, among other antibody-based EGFR inhibitors, functions in part by inducing receptor degradation \((\text{Figure 2-6})\) \((131)\). Akt phosphorylation and activation were also downregulated, secondary to EGFR degradation. Unexpectedly, while total Akt and total ERK1/2 levels were unaffected by necitumumab treatment, the downstream target ribosomal protein S6 was also downregulated by necitumumab treatment, presumably through degradation.

Our results not only reveal no added benefit of combination treatment of osimertinib with necitumumab, but also indicate a degree of antagonism, resulting in decreased necitumumab-mediated phospho- and total EGFR downregulation \((\text{Figure 2-6})\). This antagonistic effect is also observed in downstream ribosomal protein S6, which also shows resurgence in both total and phospho- protein levels when treated with concurrent osimertinib and necitumumab. While the mechanism underlying this antagonism remains unclear, it does not appear to affect tumor growth kinetics \((\text{Figure 2-5a})\), and was therefore not interrogated further.
Figure 2-6: Heterogeneity in the drug response of PC9 T790M/C797S xenografts to osimertinib and necitumumab treatment

Correlation between overall tumor growth in each mouse and tumor signaling. Four tumors for each drug treatment condition were harvested four hours after the final dose of drug, and the remaining four were harvested at 24 hours following treatment. Tumors that exhibited no growth over the four-week study are indicated in red; all of the remaining tumors grew, though at variable rates based on their drug tolerance. Below each fold growth plot is the western blot analysis of each corresponding tumor.
Ultimately, we were unable to identify the basis of extraordinary drug responses in this mouse cohort. When individual changes in tumor growth were correlated with western blot pathway analysis, there was no apparent correlation between extent of EGFR or downstream pathway inhibition and tumor size. While tumors harvested 24 hours after drug treatment showed strong correspondence between most pronounced tumor growth inhibition and highest ratio of cleaved to total PARP, this trend was not conserved in the tumors harvested at 4 hours following the last dose of drug. This indicated that PARP cleavage was either a later apoptotic response, or no had predictive value in this model. Phosphorylation of canonical EGFR-downstream pathway components and upregulation of the pro-apoptotic protein Bim also showed no correlation with tumor drug response, so we were unable to pinpoint a mechanistic basis of the interindividual variability we observed.

However, based on analysis of the aggregate western blot data, we were able to corroborate that necitumumab treatment degraded EGFR. As observed in the prior studies on afatinib + cetuximab, EGFR degradation correlated with drug efficacy (129,130). This observation has inspired renewed efforts to identify strategies aimed at EGFR degradation in osimertinib resistant models of EGFR-mutant NSCLC.

2.4. Characterizing the development and biology of the predicted drug resistance mutation T790I in cell line models of NSCLC

As a final application of CRISPR modelling of NSCLC drug sensitivity mutations, we are interested in assessing the development of T790I as an alternative mutation to T790M to confer resistance to first-generation EGFR inhibitors. This idea emerged from the existence of a minor allele encoding ACA (instead of the major allele ACG) at threonine 790, encountered in less than
0.01% of the population (132). In patients harboring the predominant allele at codon 790, the transition ACG>ATG results in the T790M gatekeeper mutation. In patients who instead harbor the minor threonine allele at this codon, the equivalent transition would result in the mutation ACA>ATA, or an alternative gatekeeper mutation T790I (Table 2-2). The oncogenic activity of T790I has previously been confirmed by Azam et. al. in 2008 (73), but to our knowledge no further validation has been done of the emergence of a T790I gatekeeper mutation in NSCLC, or its implications for resistance to first generation EGFR inhibitors. While EGFR T790I has not been observed in erlotinib- or gefitinib-refractory lung cancers in the clinic, this may be attributed to the scarcity of the corresponding parental ACA SNP at codon 790 (132). Having applied CRISPR-Cas9 genome editing to introduce a panel of resistance mutations into PC9 cells, we intend to apply this approach for introducing and interrogating the biology of a T790I mutation in cis with EGFR activating mutations, as well as in cis with C797S, in an EGFR-driven NSCLC context (Table 2-1).

<table>
<thead>
<tr>
<th></th>
<th>c.2368</th>
<th>c.2369</th>
<th>c.2370</th>
<th>Amino Acid</th>
</tr>
</thead>
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<td>WT Codon</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>Thr</td>
</tr>
<tr>
<td>T790M</td>
<td>A</td>
<td>T</td>
<td>G</td>
<td>Met</td>
</tr>
<tr>
<td>Minor Allele Codon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T790M-analagous Base Transition in Minor Allele Codon</td>
<td>A</td>
<td>T</td>
<td>A</td>
<td>Ile</td>
</tr>
</tbody>
</table>

Prior to developing CRISPR models and initiating this characterization, however, we began by verifying the drug resistance of the T790I mutation in cis with the del19 EGFR activating mutation in Ba/F3 murine leukemia cells. Oncogene overexpression in Ba/F3 cells renders the cells dependent on that oncogene, and provides a simple platform to predict drug vulnerabilities of the transduced oncogene. We found that the T790I mutation conferred resistance to gefitinib,
as expected—a feature which will be used to select successfully recombined cells in future CRISPR studies. Interestingly, EGFR del19/T790I was significantly more susceptible to the irreversible inhibitor afatinib when compared to EGFR del19/T790M in a Ba/F3 context (Figure 2-7a). It did, however, still confer afatinib resistance. Interestingly, we found that the del19/T790I mutation was very sensitive to osimertinib, with preliminary results suggesting it was even more susceptible than the del19/T790M model (Figure 2-7a).

Figure 2-7: Preliminary characterization of EGFR T790I in cis with activating EGFR mutation as a drug resistance mechanism

(a) Like the established EGFR T790M gatekeeper mutation, the T790I mutation confers resistance to gefitinib, but retains osimertinib sensitivity when present in cis with the activating EGFR exon 19 deletion in a Ba/F3 model of oncogene dependency. Although T790I also confers relative afatinib tolerance compared to the activating mutation alone, it remains somewhat susceptible to inhibition by afatinib. (b) Both T790M and T790I mutations preserve phosphorylation of EGFR and downstream kinases in the presence of 1 µM gefitinib, but lose activation of both EGFR and downstream signaling following treatment with 1 µM of mutant-selective inhibitor osimertinib.

Finally, we verified that inhibition of EGFR and downstream factors in this T790I model were consistent with the molecular biology observed in T790M-harboaring Ba/F3 cells (Figure 2-
7b). Of note, the baseline EGFR phosphorylation appeared to be lower in del19/T790I Ba/F3 cells compared to their del19 and del19/T790M counterparts. This was consistent with a qualitative observation of slightly slower growth of Ba/F3 cells harboring this mutational profile (data not shown).

2.5. Discussion of ongoing and future directions

We will continue to study the biology and drug resistance properties of the T790I gatekeeper mutation, interrogating whether the mutation develops in response to drug selection and using CRISPR-Cas9 genome editing to introduce the mutation into a lung cancer background. To this end, we will first introduce the silent mutation at c.2370 into *EGFR* del19 and *EGFR* L858R expression vectors through site-directed mutagenesis to convert wild type T790 into the ACA allele of T790. This construct will then be introduced into parental Ba/F3 cells, and be subjected to ENU mutagenesis in the presence of gefitinib to assess whether the T790I allele emerges, with what frequency, and whether it has distinct biological features when it is in *cis* with the del19 activating mutation versus L858R. Because we have demonstrated that the T790I mutation is able to confer resistance to gefitinib, we anticipate that the tendency of cells to mutate the second position to develop resistance of codon 790 will prevail, and the Ba/F3 cells will express T790I as a novel drug resistance mutation. On the other hand, if T790I is a weaker resistance mutation than T790M—as shown in the preliminary BaF3 results—it is also possible that the activating mutant EGFR allele may mutate both the second and third residues of the altered T790 codon to develop T790M following drug treatment, which would indicate that T790M is indispensable for drug resistance. If we are able to recover T790I through an accelerated mutagenesis assay in Ba/F3 cells, we will next establish and characterize T790I and T790I/C797S
PC9 models. If the L858R activating mutation is found to influence the biological activity of the T790I mutation in Ba/F3 cells, H3255 models of T790I and T790I/C797S will also be established and characterized in addition to PC9-based models.

Having already established PC9-based models of drug resistance mediated by EGFR T790M and C797S mutations, we hope to use these models in future drug screening efforts to identify novel compounds and combination regimens that show efficacy against clinically untargetable triple mutant EGFR. One approach we hope to pursue is the investigation of HER3 as a potential therapeutic target in EGFR-driven NSCLC. HER3 is phosphorylated by EGFR, and is an effector of many of the downstream processes crucial to cancer cell survival. Thus, HER3 may represent a viable therapeutic target in cancers that accrue secondary and tertiary EGFR mutations. We found HER3 to be highly expressed in osimertinib resistant models, including the PC9 T790M/C797S clone #3 tumors (data not shown), and plan to next use this model to test the efficacy of a HER3-directed antibody-drug conjugate under clinical investigation, U3-1402, on our cells (NCT03260491) (128). Finally, to establish a platform for screening novel allosteric EGFR inhibitors on C797S-positive lung cancer cells, the H3255 CRISPR panel will be retargeted and expanded.
Chapter 3: Identification and characterization of MET kinase dependency in EGFR-mutant, MET-overexpressing NSCLC
Attributions:

Contributors to this work include Pınar Ö Eser, Raymond M. Paranal, Atsuko Ogino, Pavlos Missios, Jenny Choi, Mika Lin, Michael Poitras, Praful Gokhale, Steven Wang, Man Xu, Sangeetha Palakurthi, Masahiko Yanagita, George Q. Daley, and Pasi A. Jänne. P.Ö.E. and P.A.J. conceived the studies. G.Q.D. provided feedback on experimental design and data presentation. All authors helped design and carry out experiments, and P.Ö.E., R.M.P., and M.P. analyzed and interpreted the results. P.G. and S.P. supervised in vivo studies and P.A.J. supervised the project. P.Ö.E. wrote the text with feedback from P.A.J.

3.1. Introduction

Amplification of the receptor tyrosine kinase MET is a conserved mechanism of resistance to EGFR inhibitors, observed in 5-20% of patients (depending on the cohort studied) progressed on reversible EGFR TKI (68,103,133). In contrast to point mutations associated with EGFR inhibitor resistance, MET amplification and overexpression may confer resistance to first, second, and third-generation EGFR inhibitors alike (70,134). EGFR mutant and MET amplified NSCLC becomes co-dependent on the activation of both EGFR and MET kinases. Clinically, tumors harboring concomitant EGFR mutation and MET amplification do not respond to single agent EGFR or MET inhibitors, but require treatment with a combination of EGFR and MET TKI (107,133). Small molecule MET kinase inhibitors in clinical use include crizotinib (Xalkori), the experimental drug savolitinib, and the broad-spectrum type II kinase inhibitor cabozatinib (Cabometyx) (109,111).
HCC827GR6 is a cell line model of gefitinib resistance mediated by MET amplification, which was developed through long-term dose escalation gefitinib treatment of the parental HCC827 cell line (103). As with patients harboring EGFR mutant tumors that become drug resistant through MET amplification, HCC827GR6 cells are resistant to treatment with single agent gefitinib or crizotinib, but sensitive to combination TKI treatment (103). We have recently established three new patient-derived cell line and xenograft models of lung adenocarcinoma: DFCI81, DFCI161, and DFCI307. All three of these models exhibit concurrent EGFR activating mutations and MET copy number gain—a genomic profile that predicts codependency on concurrent EGFR and MET activation. We generated both cell line and xenograft tumor models from the DFCI81 and DFCI161 specimens, while only a xenograft model could be generated from DFCI307 (Table 3-1).

Table 3-1: Patient-derived cell lines and xenograft models being characterized

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Activating EGFR Mutation</th>
<th>Source</th>
<th>EGFR Inhibitor Sensitivity</th>
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<tr>
<td>DFCI81</td>
<td><em>EGFR</em> del19</td>
<td>Cell line derived from post-erlotinib pleural effusion. MET amplified.</td>
<td>Resistant</td>
</tr>
<tr>
<td>DFCI161</td>
<td><em>EGFR</em> L858R</td>
<td>Cell line and xenograft models derived from post-erlotinib pleural effusion. MET overexpressing.</td>
<td>Resistant</td>
</tr>
<tr>
<td>DFCI307</td>
<td><em>EGFR</em> del19</td>
<td>Xenograft model derived from post-osimertinib tumor. MET amplified.</td>
<td>Resistant</td>
</tr>
</tbody>
</table>
All three patient-derived models were established from the tumors of patients who had progressed on EGFR-targeted therapy (Figure 3-1a). Clinical targeted OncoPANEL sequencing analysis (135) done at the time of specimen collection revealed the activating exon 19 ELREA deletion is present in DFCI81 (96% of reads) and DFCI307, while DFCI161 harbors the activating exon 21 L8585R point mutation (86% of reads). The high allele frequencies of mutant EGFR in the DFCI81 and DFCI161 models provides evidence of amplification of the mutant allele, a common feature of EGFR-driven lung cancers (136). Western blot analysis with mutant-specific antibodies revealed that the activating EGFR mutations are expressed in each model, while FISH analysis corroborated MET amplification in the DFCI81 and DFCI307 models, and revealed chromosome 7 copy number gain in DFCI161 cells (Figure 3-1b). Genomic MET amplification is defined, somewhat arbitrarily, by the ratio of centromere 7 to MET copy number. Generally, a MET:centromere 7 ratio of ≥5:1 is considered amplified, while any smaller ratio is not amplified (28). However, polysomy 7, which would result in increased MET copy number may result in MET copy number gain without being defined, strictly speaking, as amplification. Even in the absence of genomic MET amplification, the DFCI161 cell line exhibits polysomy 7 and overexpresses MET protein at levels comparable to those observed in MET amplified cell lines.
Figure 3-1: Three new patient-derived models of *EGFR* mutant MET-amplified NSCLC

(a) Clinical histories of patients from whom DFCI81, DFCI161, and DFCI307 specimens were obtained. DFCI81 and DFCI161 models were derived from pleural effusions, while the DFCI307 xenograft model was generated from a biopsy. Each specimen was collected following patient progression on an EGFR-targeted therapy. (b) FISH analysis of the DFCI81 cell line and DFCI307 xenograft model reveals MET amplification, while the DFCI161 cell line exhibits MET copy number gain in the form of polysomy of chromosome 7.

3.2. Identification of a switch to MET dependency in a subset of *EGFR* mutant, *MET* amplified NSCLC

Taken together, the DFCI81, DFCI161, and DFCI307 genomic profiles (EGFR mutant, MET upregulated) and patient treatment histories (each patient received at least one regimen of EGFR-targeted therapy) implied a classic paradigm of MET-mediated resistance to EGFR inhibitors. We would anticipate tumors from these patients to be resistant to perturbation of
either EGFR or MET receptor activity individually but respond to combinatorial inhibition of both receptors. Unexpectedly, however, our analysis of tumor-derived cell line and xenograft models revealed a novel paradigm. Instead of exhibiting classic EGFR/MET-codependency, all three patient-derived specimens showed a switch away from EGFR signaling to complete MET oncogene dependency (Figure 3-2).

We identified this drug sensitivity profile by treating the cell line models of DFCI81 and DFCI161 with dose ranges of EGFR and MET kinase inhibitors and quantifying cell viability by MTS assay at the end of 96 hours. A panel of EGFR and MET inhibitors was tested on the cell lines to ensure that the drug sensitivities observed were not a result of any off-target activity, and IC_{50} values were extrapolated from the corresponding dose curves. The DFCI81 and DFCI161 cell lines exhibited resistance to the EGFR inhibitors gefitinib, afatinib, and osimertinib, and sensitivity to the MET inhibitors crizotinib, savolitinib, and merestinib (Figure 3-2a). Single agent treatment with the MET inhibitor crizotinib was also sufficient to induce apoptosis, as quantified by caspase 3/7 activation, in both DFCI81 and DFCI161 cell lines (Supplemental Figure II-1). The EGFR/MET-codependent cell line HCC827GR6 and its EGFR-dependent parental counterpart HCC827 were used throughout these studies as controls to contextualize our observations in the patient-derived models.
Figure 3-2: Patient-derived cell line and xenograft models exhibit sensitivity to MET inhibitors

(a) Patient-derived DFCI81 and DFCI161 cells were treated with a panel of EGFR and MET inhibitors to assess their drug dependencies. The primary cell lines were treated alongside the EGFR/MET codependent cell line HCC827GR6, and EGFR-dependent parental HCC827 cells as. DFCI81, DFCI161, and HCC827GR6 cells all exhibited resistance to the three EGFR inhibitors tested: gefitinib, afatinib, and osimertinib. HCC827 parental cells, on the other hand, were sensitive to all three inhibitors. The codependent HCC827GR6 cells were sensitized by adding crizotinib to the gefitinib treatment (signified by G+C) induced sensitivity in this model. When treated with single agent MET inhibitors crizotinib, savolitinib, and merestinib, both HCC827 and HCC827GR5 were drug resistant. On the other hand, DFCI81 cells and DFCI161 cells exhibited exquisite sensitivity to each of the three inhibitors, revealing MET oncogene dependency. (b) The sensitivity of DFCI81 and DFCI161 to single agent MET inhibitors was corroborated through patient-derived xenograft studies. Each cell line, alongside another patient-derived model, DFCI307, was engrafted into mice and treated with drug to monitor tumor drug vulnerabilities. As predicted from the cell culture models, all three cell lines exhibited resistance to the EGFR inhibitors and sensitivity to treatment with single-agent MET inhibitors.

To assess tumor xenograft drug sensitivity, patient-derived cells were implanted subcutaneously into immunocompromised mice, allowed to grow until all tumors reached
uniform size, then initiated on EGFR or MET inhibitor treatment alongside vehicle control. Tumors were measured routinely with calipers, and time course measurements were plotted (Figure 3-2b). The DFCI81 and DFCI161 patient-derived xenografts corroborated the trends of EGFR inhibitor resistance and MET inhibitor sensitivity that we had observed in the tissue culture models. Further, the xenograft model of DFCI307 also exhibited this drug sensitivity profile. Taken together, these observations support a complete switch in oncogene dependency from EGFR to MET, a previously uncharacterized mechanism of drug resistance in EGFR-driven lung cancers. To corroborate that this dependency was unique to our patient-derived models, we conducted a small screen to assess the drug sensitivities of a panel of lung cancer cell lines, affirming that no other EGFR mutant, MET amplified models exhibited this switch to MET dependency (Supplemental Figure II-2).

Having shown that the DFCI81 and DFCI161 cell lines, and DFCI81, DFCI161, and DFCI307 patient-derived xenograft models, were resistant to EGFR inhibitors and highly sensitive to MET inhibitors, we next examined the effects of MET inhibition on downstream signaling pathways. The phosphorylation status of canonical EGFR-downstream kinases ERK1/2, Akt, and S6 ribosomal protein was examined to corroborate a shift in pathway dependency (Figure 3-3). Characterization of downstream pathways in DFCI81 and DFCI161 cells alongside HCC827 and HCC827GR6 controls revealed distinct receptor dependencies among the cell lines. In the gefitinib sensitive HCC827 cells, activation of the downstream kinases ERK1/2, and Akt relied on EGFR activation, and was inhibited by single agent gefitinib treatment. By contrast, in the EGFR and MET co-dependent HCC827GR6 cell line, the downstream kinases could be activated by both EGFR and MET, and simultaneous inhibition of both receptors was required to ablate the
downstream signaling. Finally, in the case of the \textit{EGFR} mutant, MET addicted DFCI81 and DFCI161 cells, gefitinib had no effect on signaling, but single agent crizotinib was sufficient to inhibit the phosphorylation, and thereby activation, of downstream kinases (\textbf{Figure 3-3}). DFCI307 xenograft lysates from mice treated with the EGFR inhibitor osimertinib and the MET inhibitor savolitinib revealed an analogous MET dependency of downstream signaling (\textbf{Supplemental Figure II-3}). Notably, although EGFR phosphorylation is reduced in all three patient-derived models following treatment with EGFR inhibitor, combination treatment with EGFR and MET inhibitors is required to completely ablate EGFR activation, indicating that MET may also participate in EGFR phosphorylation these contexts (\textbf{Figure 3-3, Supplemental Figure II-3}).

Having validated that these three patient primary models exhibited exquisite sensitivity to single-agent MET inhibitors, and MET-dependent activation of downstream signaling, we sought to understand the mechanistic basis of this vulnerability. We were interested in interrogating this MET dependency for several reasons. To our knowledge, this kind of oncogenic switch—whereby a cancer harboring an activating mutation in a driver oncogene mutation exhibits complete dependency on a different oncogenic driver—has not been previously characterized in lung cancer. While the presence and amplification of activating EGFR mutations in our models may be a relic, it is serves as a strong indicator that at some point along tumor progression, each of these models was dependent on EGFR. Although refractory to EGFR inhibitor treatment, each cell line and tumor model also shows clear evidence of dependency on canonical EGFR-downstream signaling. Consequently, we cannot discount that this switch may have occurred as a mechanism of either \textit{de novo} or acquired resistance to EGFR inhibitor treatment.
To evaluate whether the MET dependency observed in DFC81 and DFCI161 models coincided with control of downstream kinase signaling, we treated the patient-derived MET-dependent cell lines with EGFR and MET kinase inhibitors, individually and in combination, and compared the signaling pathways with HCC827 and HCC827GR6 cells treated with the same inhibitors and run on the same gel. We examined total and phosphorylated levels of EGFR and MET to ensure the inhibitors were engaging their targets. We next examined the downstream dependencies of EGFR and MET in each model. To this end, we assessed phosphor and total levels of the adaptor proteins HER3 and Gab1, and the downstream kinases Akt, ERK1/2, and S6 ribosomal protein. Finally, we looked at Bim level to gauge induction of apoptosis secondary to kinase inhibition.

Figure 3-3: Patient-derived cell line and xenograft models exhibit MET-dependent modulation of downstream kinase signaling

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To evaluate whether the MET dependency observed in DFC81 and DFCI161 models coincided with control of downstream kinase signaling, we treated the patient-derived MET-dependent cell lines with EGFR and MET kinase inhibitors, individually and in combination, and compared the signaling pathways with HCC827 and HCC827GR6 cells treated with the same inhibitors and run on the same gel. We examined total and phosphorylated levels of EGFR and MET to ensure the inhibitors were engaging their targets. We next examined the downstream dependencies of EGFR and MET in each model. To this end, we assessed phosphor and total levels of the adaptor proteins HER3 and Gab1, and the downstream kinases Akt, ERK1/2, and S6 ribosomal protein. Finally, we looked at Bim level to gauge induction of apoptosis secondary to kinase inhibition.
Figure 3-3 (Continued): As expected, we observed that EGFR activation governed phosphorylation of adaptor and downstream kinases and induction of apoptosis in parental HCC827 cells. In the EGFR/MET codependent HCC827GR6 cells, combination treatment with gefitinib and crizotinib was required to fully inhibit phosphorylation of HER3, Akt, and ERK1/2, and upregulate Bim expression. My contrast, in the MET-dependent models DFCI81 and DFCI161, the activation of all downstream adaptors and kinases, and the induction of apoptosis were intimately linked to MET inhibition. In these cellular contexts treatment with single-agent crizotinib was sufficient to inhibit all downstream signaling pathways and induce apoptosis.

Understanding the basis of MET oncogene dependency in these cells may also have implications for identifying the link between MET amplification and sensitivity to targeted MET inhibitors, which remains poorly understood. In some models of NSCLC, such as the cell line EBC-1, MET amplification corresponds MET dependency, and MET inhibitor sensitivity (137). On the other hand, many MET-amplified tumors are refractory to MET inhibition, with no discernible dependency on MET signaling (113,138). Understanding the biology underlying MET dependency in the DFCI81, DFCI161, and DFCI307 cell lines, which harbor either MET amplification or MET protein upregulation associated with genomic MET gain, may illuminate the basis of amplification-associated MET dependency in models without concomitant EGFR mutation.

The switch to MET dependency that we observe in our three models may be a prevalent phenomenon in EGFR mutant lung cancer. There is no way of knowing how frequently a switch to MET dependency occurs in clinical lung cancers harboring concomitant EGFR mutation and MET amplification, because this subtype has not previously been characterized. As a result, all patients with this genomic profile are administered combination EGFR and MET inhibitor treatment—although it now appears a subset of them could stand to benefit from single-agent MET inhibition, which would not only be better tolerated, but may permit additional next-line treatment options when the tumors become refractory. Recently, two separate case reports
were published documenting patients whose tumors harbored *EGFR* activating mutations, and became refractory to therapy through *MET* amplification (139,140). In each case, the patient was unable to tolerate continued EGFR-targeted therapy and relegated to single agent MET inhibitor, simply because their clinicians deviated from standard clinical protocols in an effort to keep the patients on targeted therapy. Remarkably, in each case, the patient’s tumor responded to MET inhibitor monotherapy, setting a clinical precedent for our preclinical observations.

It will be important for us to characterize the biology underlying this switch to MET dependency in *EGFR* mutant NSCLC, and identify features that may serve as clinical predictors of this novel therapeutic vulnerability. Because of a lack of longitudinal patient models available, we have relied heavily on comparing the patient-derived MET kinase dependent models to the HCC827GR6 line, a model of EGFR/MET co-dependency. Through these comparisons, we tested perturbations that may induce EGFR/MET-codependency in our MET-dependent models, in an effort to uncover the underlying basis of a shift away from EGFR codependency in these patient-derived models.

### 3.3. Exogenous ERBB ligand treatment induces crizotinib resistance in MET-dependent cell line models

In addition to the activation of downstream kinases ERK1/2 and Akt, we noted that phosphorylation of HER3 was sensitive to single agent crizotinib treatment in DFCI81 and DFCI161 cell line models (Figure 3-3). While HER3 phosphorylation was MET-dependent, HER2 phosphorylation in DFCI161 cells was shown to be EGFR dependent, and HER4 protein could not be detected, leading us to focus on HER3 phosphorylation dynamics over the other ERBB family members (data not shown). In the case of DFCI307, while HER3 phosphorylation was not
dramatically changed in the absence versus presence of the MET inhibitor savolitinib, we noted a significant compensatory upregulation of total HER3, signifying a dramatic reduction in HER3 phosphorylation ratio compared to vehicle and osimertinib treated cells (Supplemental Figure II-3). These observations of MET-mediated HER3 phosphorylation contrasted with HER3 regulation in HCC827 and HCC827GR6 cells, in which HER3 phosphorylation is disrupted by treatment with EGFR inhibitor or combination treatment with EGFR and MET inhibitors, respectively (Figure 3-3).

Engelman et. al. previously demonstrated a hallmark of EGFR/MET codependency is concurrent regulation of HER3 phosphorylation by the EGFR and MET receptors (103). In parental HCC827 cells, they showed that the basis of gefitinib sensitivity was that EGFR was the exclusive kinase that dimerized with and activated HER3. Conversely, in MET-amplified HCC827GR6 cells, they demonstrated that HER3 may equally dimerize with and be activated by both EGFR and MET kinases, resulting in the EGFR/MET codependency observed in these cells (103).

Intriguingly, while EGFR is both expressed and phosphorylated in our MET-dependent models (albeit at lower levels compared to their HCC827 counterparts), it does not appear to have any bearing on the phosphorylation of HER3 in this context. This observation is unusual, not only in light of the published data, but also because EGFR is known to freely dimerize with HER3 across different models, both physiological and pathological (39,51,103). To investigate this dichotomy, we sought to determine whether we could rescue EGFR/MET codependency in the DFCI81 and DFCI161 models by artificially inducing EGFR-HER3 dimerization through treatment with ERBB family activating ligands. We predicted the MET dependency observed in our models may result from preferential formation of MET-HER3 dimers over EGFR-HER3 dimers (Figure 3-
4), and that this dependency may be modulated through treatment with exogenous ERBB family ligands to artificially induce EGFR-HER3 dimerization.

Figure 3-4: Hypothesized basis of MET oncogene dependency versus EGFR-MET codependency may rely on differential dimerization with and activation of HER3

Based on our observations of context-dependent HER3 phosphorylation by EGFR versus MET, we hypothesize that HER3 preferentially dimerizes with EGFR in the EGFR-dependent HCC827 cells, and with MET in the MET-dependent DFCI161 and DFCI81 cells. By contrast, we expect that HER3 interacts with both oncogenes in the EGFR and MET co-dependent HCC827GR6 model. We predict the dominance of EGFR in the HCC827 context, and of MET in the DFCI161 and DFCI81 contexts may be determined by differences in growth factor and kinase expression, or other variables that offset the balance in favor of cell line EGFR versus MET dependence.
EGFR is expressed and phosphorylated in the MET-dependent cell lines, yet this level of expression and activation appears uncoupled from activation of downstream signaling. To interrogate whether enhancing EGFR activation through treatment with exogenous ERBB ligands could induce EGFR/MET codependency—and thereby crizotinib resistance—in our MET-dependent models, DFCI81 and DFCI161 cells were treated with kinase inhibitors in the presence recombinant ERBB ligands including EGF, EPR, and NRG1 (Table 1-1), alongside vehicle BSA and the MET ligand HGF as controls. Our experimental rationale was reaffirmed when we observed significantly reduced transcript expression of the ERBB ligands epiregulin (EPR) and neuregulin-1 (NRG) in the DFCI81 and DFCI161 cells compared to HCC827GR6 cells (Supplemental Figure II-4a).

MTS assay quantification of drug sensitivity revealed that NRG1 co-treatment induced crizotinib resistance in both DFCI81 and DFCI161 cell lines (Figure 3-5). Stimulation with EGF and EPR additionally induced crizotinib resistance in DFCI161, but not DFCI81, cells (Figure 3-5a, c). In all cases, gefitinib resistance was unaffected by concurrent ligand treatment. We also tested control cell lines to ensure the induction of drug resistance by growth factors was not universal, non-specific phenomena. Reassuringly, sensitivity of parental HCC827 cells to gefitinib, of HCC827GR6 cells to combination treatment with gefitinib and crizotinib, and of EBC-1 cells to crizotinib treatment were unaffected by growth factor treatment (data not shown).

Examination of downstream signaling cascades in DFCI81 and DFCI161 cells revealed that the growth factors which induced crizotinib resistance promoted sustained phosphorylation of the downstream kinases ERK1/2 and Akt, even in the presence of crizotinib when MET activation was ablated (Figure 3-5b, d). When DFCI81 and DFCI161 cells were treated with inhibitors in the
presence of ligands that conferred crizotinib resistance (NRG1 only in the case of DFCI81; EGF, EPR, and NRG1 for DFCI161), the cells’ downstream signaling was unaffected by treatment with single-agent crizotinib. However, when EGFR inhibitor and MET inhibitor were combined, downstream kinase cascades were once again ablated (Figure 3-5b, d). This phenotype resembled the downstream signaling dependencies of conventional EGFR/MET codependent models such as HCC827GR6, and we postulated that treatment with recombinant growth factors was activating EGFR signaling to induce crizotinib resistance in this cell line.

Based on the receptor profiles of the ligands that induced resistance in DFCI81 and DFCI161 cells, we hypothesized that the ligands were promoting EGFR-HER3 dimerization to induce EGFR/MET codependency in these MET-dependent backgrounds. To test this hypothesis, we treated DFCI81 and DFCI161 cells with a combination of EGFR and MET inhibitors (1 μM crizotinib + dose-escalation gefitinib) in the presence of ligand. In DFCI161 cells, the combination treatment resensitized the cells, and it was thus confirmed that the crizotinib resistance induced by all three ligands—EGF, EPR, and NRG1—was mediated by EGFR activation. Conversely, DFCI81 cells treated with a combination of EGFR and MET inhibitors in the presence of NRG1 remained viable, indicating that NRG1 may be inducing HER3 dimerization with and activation by another kinase in the context of DFCI81 cells (Supplementary Figure II-5). Though reproducible, this observation was surprising because combination treatment with gefitinib and crizotinib were sufficient to inhibit phosphorylation and activation of downstream ERK1/2 and Akt, even in the presence of NRG1 ligand (Figure 3-5b).
Figure 3-5: EGFR activation through treatment with exogenous ERBB family ligands is sufficient to induce MET inhibitor resistance through downstream pathway reactivation

(a) Treatment of DFCI81 cells with the ERBB ligands EGF, EPR, and NRG1, alongside controls BSA and the MET ligand HGF, revealed NRG1 alone induced crizotinib resistance, and (b) permitted sustained HER3 activation and propagation of downstream signaling in the presence of crizotinib. (c) All three ERBB ligands—EGF, EPR, and NRG1, induced crizotinib resistance when added acutely to the cells prior to TKI treatment (d) While all three ligands induce crizotinib resistance through bypass activation of MET downstream signaling in the presence of crizotinib, of the three ligands, only NRG1 appears to activate HER3.
Figure 3-5 (Continued)

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Crizotinib

Gefitinib

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HER3

pEGFR

EGFR L858R

pMET

MET

pAkt

Akt

pERK1/2

ERK1/2

pS235/6 S6

pS240/4 S6

S6

HSP90
Interested in the biology underlying the observed discrepancy between the ligand-induced crizotinib resistance profiles between the DFCI81 and DFCI161 cells, we postulated that the basis for this inconsistency may lie in the relative EGFR expression levels of the two cell lines. There is a clear deficiency in total EGFR protein expression of the MET-dependent models compared to the HCC827 parental and GR6 cells (Figure 3-3). While both MET-dependent models expressed significantly reduced total EGFR protein compared to controls, DFCI161 cells still expressed considerably more EGFR than DFCI81 cells. This observation was corroborated by quantitative RT-PCR to compare transcript levels. Indeed, there was a tiered difference between EGFR expression in DFCI81 and DFCI161 cells, although both models expressed significantly less EGFR transcript than HCC827GR6 cells (Supplemental Figure II-4b). On the other hand, levels of HER3 and MET expression were comparable across the three cell lines.

Our recombinant ligand-mediated drug resistance studies highlighted differences in the repertoire of ligands that induced crizotinib resistance. DFCI161 cells were more readily induced to EGFR/MET codependency through treatment with EGFR activating growth factors. Further, each growth factor that conferred crizotinib resistance did so in an EGFR-dependent manner. Conversely, DFCI81 cells were unaffected by EGFR-targeting growth factors, and only became crizotinib resistant when treated in the presence of the HER3-activating ligand NRG1. Furthermore, NRG1 did not appear to induce resistance entirely through EGFR signaling. These observations, coupled with our validation of the disparate levels of EGFR expression led us to postulate that differences in EGFR expression could account for the different ligand responses observed in these cells.
Indeed, when we overexpressed wild type EGFR in DFCI81 cells, we observed a ligand-mediated codependency profile that phenocopied the DFCI161 cells. The DFCI81/WT EGFR cells not only became responsive to EGF as a mediator of crizotinib resistance, they also responded to NRG1 in a more EGFR-dependent manner. Whereas before, the NRG1-mediated crizotinib resistance in DFCI81 cells was largely independent of EGFR activation, overexpression of WT EGFR in these cells sensitized them to combination treatment with crizotinib and gefitinib in the presence of NRG1 ligand (Supplemental Figure II-6). Taken together, these findings demonstrate the potential plasticity of MET-dependent cells to become EGFR/MET co-dependent, and vice versa. Irrespective of the mutant EGFR expressed by these cells, upregulating and/or activating wild type EGFR was sufficient to induce a classic EGFR/MET codependency phenotype in these MET-dependent cell lines.

Even at baseline, however, the HER3-activating ligand NRG1 was the common denominator that induced crizotinib resistance in both of our MET-dependent models. Although ligands function primarily by changing receptor confirmation to induce dimerization, co-immunoprecipitation studies did not reveal a significant change to HER3-EGFR or HER3-MET dimers in the DFC161 and DFCI81 cell lines (Supplemental Figure II-7). Even so, our recombinant NRG1 findings indicated a potential role for HER3 in the determination of oncogene dependency. We next sought to characterize HER3 dimerization with MET versus EGFR at baseline as a surrogate marker that could permit de novo prediction of the oncogene dependencies of EGFR-mutant, MET amplified NSCLC models.
3.4. Assessing the predictive potential of activated EGFR-HER3 versus MET-HER3 dimers as an indicator of single oncogene MET dependency in EGFR-mutant, MET-amplified models of NSCLC

Given that HER3 has previously been shown to depend on EGFR for phosphorylation in EGFR-dependent models (such as the HCC827 parental cells) and rely equally on EGFR and MET kinases for activation in EGFR/MET-codependent cells (such as the HCC827GR6 model) (103), we postulated that HER3 may also be the principal effector of MET dependency in DFCI81 and DFCI161. HER3 phosphorylation appeared strongly predictive of oncogene dependency in all three models tested (Figure 3-3 and Supplemental Figure II-3). We therefore hypothesized that HER3 dimerization partners could be a predictive marker of oncogene dependency in EGFR mutant, MET amplified models (as illustrated in the simplified hypothetical model shown in Figure 3-4). This hypothesis was fortified by the observation that the HER3 ligand NRG1 is the only ligand that induces drug resistance in both DFCI81 and DFCI161 cell lines (Figure 3-5).

We anticipated that HER3 would dimerize predominantly with MET in DFCI81 and DFCI161 cells, preferentially with EGFR in the EGFR-dependent HCC827 cells, and agnostically with both MET and EGFR in the EGFR-MET codependent HCC827GR6 cells. To test this hypothesis, we first did a baseline immunoprecipitation, pulling down untreated, uncrosslinked DFCI81, DFCI161, HCC827, and HCC827GR6 lysates with HER3, EGFR, or MET antibodies and blotting for HER3, EGFR, and MET (Figure 3-6a). The results of this pulldown did not support our hypothesis of differential HER3 binding partner preference between cell lines with different oncogene dependencies. Instead, the only variations observed between the pulldowns from different cell lines appeared to result from lower EGFR expression in DFCI81 and DFCI161.
Figure 3-6: Assessment of the prevalence of activated EGFR-HER3 versus MET-HER3 dimers as a potential predictor of MET dependency

(a) DFCI81 and DFCI161 cells do not reveal differential dimerization patterns that distinguish them from HCC827 and HCC827GR6 cells when total proteins are immunoprecipitated and immunoblotted at baseline. (b) Pulldown for pHER3 appears to show an enrichment of MET pulldown in the MET-dependent DFCI81 and DFCI161 cells compared to control cell lines. (c) DFCI81 and DFCI161 cells, alongside HCC827 and HCC827GR6 control lines, were treated with DMSO (vehicle), gefitinib, crizotinib, or combination gefitinib + crizotinib, lysed, and immunoprecipitated with total HER3 and total MET. While the HER3 pulldown did not reveal any crizotinib-mediated changes in dimerization, the MET pulldown did show crizotinib-associated downregulation of both MET-HER3 dimers and MET-EGFR dimers. In the DFCI81 cells, crizotinib also appeared to ablate MET-p85 (PI3K regulatory subunit) dimers.
We next turned our attention to phosphorylated HER3 dimerization, reasoning that some of the EGFR-HER3 dimers detected in the MET-dependent cells and cells could be inactive. Interestingly, although pHER3 was not enriched in DFCI81 and DFCI161 pulldowns for total MET, we observed an increase in total MET protein that immunoprecipitated with pHER3 from DFCI81 and DFCI161 lysates versus HCC827 and HCC827GR6 (Figure 3-6b). Although promising, this result did not account for our expectation to find activated HER3-MET dimers in the EGFR/MET-codependent HCC827GR6 model. A final IP was conducted to assess whether treatments with gefitinib and/or crizotinib affected dimerization. In keeping with our postulate that dimerization may not be indicative of HER3 activation in all cases, crizotinib treatment did not ablate MET-HER3 dimers in DFCI81 and DFCI161 cells; nor did treatment with a combination of gefitinib + crizotinib diminish MET-HER3 dimers in HCC827GR6 cells (Figure 3-6c)—even though these drug treatments were shown to inhibit the HER3 phosphorylation in each of these respective models.

In the context of EGFR/MET codependent cancer models, it has been shown that HER3 primarily signals through PI3K to induce MET-mediated resistance to EGFR inhibitors (103). In HCC827GR6 cells, HER3 protein co-immunoprecipitated with p85 regulatory subunit of PI3K, and that disruption of the HER3-p85 correlated with drug sensitivity. In parental EGFR-dependent HCC827 cells, gefitinib treatment was sufficient to disrupt these dimers; in EGFR/MET-codependent HCC827GR6 cells, combination treatment with gefitinib and crizotinib was required to disrupt p85-HER3 dimers (103). We were interested to assess whether these observations could now be expanded the novel subset of EGFR mutant, MET dependent lung cancer we had identified. Our IP studies appear to support this in showing disruption of both HER3-p85 and MET-p85 dimers by single agent crizotinib treatment in DFCI81 cells, although results more
difficult to resolve in DFCI161 cells (Figure 3-6c, Supplemental Figure II-8). HER3 is an essential intermediary protein in the signal transduction from receptor tyrosine kinases to their downstream effectors, and as such we will continue to seek improved approaches to visualizing the stability of the interaction between the triad of MET, HER3, and PI3K in our MET-dependent models.

3.5. Ectopic overexpression of activating mutant EGFR induces resistance to single-agent crizotinib in MET-dependent cell lines

Although our findings of crizotinib-mediated physical disruption of HER3 signaling through PI3K were promising, we remained unable to account for the mechanism underlying this preferential interaction between MET and HER3. As we observed in Chapter 3.3, MET-dependent models expressed significantly reduced EGFR transcript and protein compared to EGFR-dependent and co-dependent models (Figure 3-3 and Supplemental Figure II-4b). Moreover, we observed that ectopically overexpressing wild type EGFR in DFCI81 cells was sufficient to shift them to ligand-mediated EGFR/MET codependency.

We next set out to examine whether EGFR:MET expression ratio could be a determinant of oncogene dependency. We hypothesized that HER3 binding partner preference, and thereby oncogene dependency, may be dictated by the relative expression or activity levels of the EGFR versus MET in different cell lines. Because DFCI81 cells express a lower baseline level of EGFR, we first tested our mutant EGFR expression stoichiometry hypothesis on them. Using a doxycycline-inducible expression vector, we infected DFCI81 cells with either EGFR Del19, or a vector control. Following puromycin selection, the infected cells were used to evaluate the effects of modulating mutant EGFR expression on drug resistance (Figure 3-7).
Figure 3-7: Ectopic overexpression of activating mutant EGFR is sufficient to induce EGFR/MET codependency in MET-dependent models of EGFR-mutant NSCLC

(a) Ectopic doxycycline-inducible overexpression of EGFR Del19 is sufficient to induce crizotinib resistance in DFCI81 cells to near micromolar ranges. Two-way ANOVA was used to test variance among groups (b) Crizotinib resistance observed in the context of dox-inducible EGFR Del19 overexpression is mediated by EGFR, and confers EGFR/MET co-dependency on lentivirus-infected DFCI81 cells in the presence of 2 μg/mL doxycycline (c) Western blot analysis of DFCI81 parental cells, vector controls, and EGFR Del19-expressing cells reveals that the presence of increased levels of EGFR del19 in the cells results in EGFR and MET jointly mediating phosphorylation of the HER3 adaptor protein, and thereby of the downstream kinases ERK1/2, Akt, and S6. As with HCC827GR6 cells, doxycycline-induced co-dependent DFCI81 cells also become refractory to EGFR and MET inhibitors individually, and require combination treatment to inhibit downstream signaling and induce apoptosis, as evidenced by Bim protein upregulation.
While overexpressing EGFR Del19 in a doxycycline-inducible manner in the DFCI81 model had no effect on the gefitinib resistance of the cells, it not only induced crizotinib resistance (Figure 3-7a), but also EGFR/MET codependency in the DFCI81 cells (Figure 3-7b). Furthermore, we were able to show dramatic dox-mediated upregulation of EGFR Del19 by western blot (Figure 3-7c). Examination of downstream kinase signaling cascades revealed that ectopic overexpression of activating mutant EGFR in the DFCI81 cells not only phenocopied the drug resistance profile of EGFR/MET co-dependent cells, it also induced co-dependent regulation of downstream kinases. As we had previously observed in the HCC827GR6 cells (Figure 3-3), phosphorylation of ERK1/2, Akt, and S6 were dependent on the combined activity of MET and EGFR in the DFCI81-Del19+DOX cells. Moreover, HER3 phosphorylation was also mediated by both receptors, and could only be inhibited by combination treatment with gefitinib + crizotinib (Figure 3-3). Finally, unlike the parental DFCI81 cells, or the no doxycycline control, EGFR-Del19 overexpressing Del19 cells required combination treatment with gefitinib and crizotinib to induce upregulation of Bim and apoptotic signaling.

Through ectopic expression of EGFR Del19 in the DFCI81 cells using a doxycycline-inducible system, we demonstrated that mutant EGFR overexpression is sufficient to confer resistance to single-agent MET inhibitors and induce EGFR/MET codependency through concurrent phosphorylation of HER3. In many ways, this paradigm is analogous to the process by which EGFR-dependent tumors develop EGFR/MET co-dependency through upregulation of MET. In the case tumors and cell lines that become MET dependent, it is possible that, having already upregulated MET to develop EGFR inhibitor resistance, at some point in their malignant progression tumors may downregulate EGFR to become completely MET oncogene dependent.
Having demonstrated that increasing levels of mutant EGFR expression through ectopic overexpression is sufficient to rescue crizotinib sensitivity and induce EGFR/MET codependency in DFCI81 cells, we became interested in the genomic mechanism underlying EGFR downregulation in MET-dependent models. As a preliminary evaluation of mechanisms governing the transcriptional regulation of EGFR expression, we carried out a small-scale screen of epigenetic modifiers to determine whether any of our candidates could induce crizotinib resistance (Supplemental Figure II-9). Out of six epigenetic modifiers evaluated, we found one hit that induced crizotinib resistance in both DFCI81 and DFCI161 cell lines, and additionally appeared to sensitize DFCI161 cells to gefitinib (Figure 3-8). This compound, 5-Aza-2'-deoxycytidine, or decitabine, is a DNA methyltransferase inhibitor, and commonly used in the treatment of leukemias (141,142).

Figure 3-8: Treatment with DNA methyltransferase inhibitor decitabine induces crizotinib resistance in DFCI81 and DFCI161 cell lines
(a) DFCI81 and (b) DFCI161 cells were treated with decitabine in combination with gefitinib or crizotinib. While both cell lines exhibited a dramatic increase in crizotinib resistance, DFCI161 cells additionally appeared to become sensitized to gefitinib. Cell lines were treated simultaneously with 1 μM and 10 μM doses of decitabine, and subjected to a two-way ANOVA to assess variance. Although both concentrations showed some efficacy, for simplicity only results from the higher decitabine dose are shown.
This observation is very preliminary, and pending validation. While the differences observed are striking, DNA methyltransferase act on a genome-wide scale, so there are many off-target perturbations that could also influence the shift to crizotinib resistance in the DFCI81 and DFCI161 cells. Reassuringly, however, the induction of drug resistance was restricted to decitabine treatment, and not induced by other chromatin modifiers tested. We have yet to ascertain if this induction of resistance coincided with upregulation of EGFR expression, which we have already shown if sufficient to induce crizotinib resistance. While we cannot account for the gefitinib sensitization corresponding to decitabine treatment in the DFCI161 cells, we were able to glean that the decitabine-induced crizotinib resistance of DFCI81 cells is reversible through concurrent gefitinib treatment (Supplemental Figure II-10). This finding may imply that decitabine can induce EGFR/MET-codependency through a mechanism analogous to that induced by ectopic EGFR overexpression. Efforts are underway to validate the observation of decitabine-mediated crizotinib resistance and elucidate the mechanistic basis of this drug sensitivity shift.

3.6. DFCI161 cells develop EGFR-mediated acquired resistance to crizotinib

We have demonstrated that by overexpressing and/or activating EGFR in MET-addicted cells we are able to induce EGFR/MET codependency in the model of NSCLC. Based on these observations, we postulated that MET-driven NSCLC emerged as a result of EGFR downregulation tumors that initially exhibited MET-mediated resistance to EGFR inhibitors. Having documented this intricate interplay between these two driver oncogenes, we next sought to assess the mechanisms through which MET-dependent models developed resistance to MET inhibitor treatment. Because mutant EGFR continues to be expressed in these cells, albeit at lower levels,
we inferred they may exhibit a tendency to harness the potential of this known oncogenic driver and develop EGFR-mediated resistance to crizotinib. We were most interested in modelling the development of crizotinib resistance towards predicting a clinical course for prospective patients who may in the future receive clinical benefit from this therapeutic course.

To establish drug resistant clones, we subjected DFCI81 and DFCI161 cells to a dose-escalation regimen of continuous crizotinib selection, beginning at 100 nM of drug and increasing step-wise until 1 μM. We established resistant clones from two separate passages of each cell line, and perpetuated both bulk populations and resistant clones of each cell line. As each drug resistant population was established, we continued propagating the cells under crizotinib selection. However, drug was washed out for a minimum of one week prior to the plating of each experiment to avoid the confounding effects of residual crizotinib on the cells.

Once established, drug resistant DFCI81 and DFCI161 populations and subclones were profiled by MTS assay, both to verify crizotinib resistance, and assess the involvement of EGFR in drug conferring resistance. MTS characterization of eight DFCI161 clones not only confirmed their crizotinib resistance, but also revealed that, in becoming crizotinib resistant, seven of the eight subclones had become sensitized to single agent gefitinib treatment (Figure 3-9a). The remaining subclone, which retained gefitinib resistance when it acquired crizotinib resistance appeared to be crizotinib resistant through EGFR, as combination treatment with gefitinib and crizotinib was sufficient to restore drug sensitivity (Figure 3-9b). MTS assay characterization of the DFCI161 bulk resistant populations revealed that these cells phenocopied clone A1, exhibiting resistance to single agent gefitinib and crizotinib, but sensitivity to combination treatment (Supplemental Figure II-11a). While more extensive validation of the signaling differences underlying these two
subsets of crizotinib resistance in DFCI161 cells is ongoing, preliminary western blot analysis revealed significant downregulation in levels of both total and phosphorylated MET in the gefitinib sensitive subclones B4 and B5. On the other hand, the drug resistant bulk populations A and B, which exhibited EGFR/MET codependency rather than a switch to EGFR inhibitor sensitivity—showed intact levels of total MET (Supplemental Figure II-11b). In keeping with our expression stoichiometry postulate, these results indicate a strong correlation between increasing the EGFR: MET expression ratio, and reversing MET dependency.

Figure 3-9: DFCI161 cells develop crizotinib resistance through activation of EGFR signaling

(a) Crizotinib resistant clones of DFCI161 cells were plated for MTS assay to quantify drug response following one week of drug washout. All clones exhibited EGFR-mediated resistance to crizotinib treatment, with seven of the eight clones profiled exhibiting a switch to complete EGFR dependence, as evident in their gefitinib sensitivity (b) The remaining crizotinib resistant clone, CR A1, retained gefitinib resistance but showed sensitivity to combination treatment with gefitinib and crizotinib (G+C).

In contrast to DFCI161CR-Bulk A cells, both DFCI81CR-Bulk A and DFCI81-Bulk B cells retain crizotinib resistance, as well as resistance to gefitinib and gefitinib + crizotinib combination treatment (Supplemental Figure II-12a). Preliminary western blot analysis of DFCI81CR-Bulk A did not reveal any features distinguishing it from the parental DFCI81 cells. By contrast, DFCI81CR-Bulk B showed reactivation of downstream pAkt, pERK1/2, and pS6 signaling following
crizotinib treatment, indicating potential involvement of another upstream kinase to activate bypass signaling in the absence of MET activation (Supplemental Figure II-12b). A phospho-RTK array was developed to compare parental DFCI81 cells to DFCI81CR-Bulk B cells in an effort to identify candidate receptor tyrosine kinases that could be activating bypass signaling in the presence of crizotinib (Supplemental Figure II-12c). One plausible candidate that was phosphorylated in DFCI81CR-Bulk B + crizotinib, but not in crizotinib-treated parental cells was Axl kinase. An MTS assay investigating the effects of dual inhibition of MET and Axl in DFCI81CR-Bulk B cells is currently underway. Notably, drug resistance in DFCI81CR cells appears transient, with cells reverting to crizotinib sensitivity following two weeks of crizotinib withdrawal. By contrast, DFCI161CR cells retain comparably robust crizotinib resistance past one month of drug withdrawal (data not shown).

3.7. Discussion

In this Chapter, we sought to identify and characterize a novel clinical subset of EGFR-mutant NSCLC that progressed through a driver oncogene switch to MET dependency. All three models studied exhibited dramatic and robust MET dependency. The DFCI81 model exhibited such profound MET dependency that a xenograft model responded to drug for over 60 days, only growing back upon crizotinib withdrawal (data not shown). Unfortunately, due to the scarcity of specimen available, it was not possible to develop a DFCI307 patient-derived cell line, making this model less tractable for ex vivo mechanistic studies. However, both in vivo and patient data underscore the MET dependency of this model. The patient donor of DFCI307 participated in the Tatton trial, in which patients who progressed on first-line erlotinib treatment were administered a combination of osimertinib and savolitinib. While this patient responded to the combination
treatment for several months, her tumor ultimately developed resistance mediated by an acquired MET D1228V mutation (111). The development of savolitinib resistance through a MET mutation is a strong indicator that the tumor retained dependency on MET signaling throughout the course of combined osimertinib + savolitinib therapy. There is also evidence that the tumor retained MET dependency following development of savolitinib resistance, as the tumor continued to exhibit sensitivity to cabozatinib, a structurally distinct type II MET kinase inhibitor (111). Of the three models, DFCI161 appeared the most prone to reversion to EGFR codependency—both in its response to ligand treatment, and through its progression to crizotinib resistance following prolonged drug selection. Even so, DFCI161 was profoundly sensitive to crizotinib in both cell line and in vivo models, exhibiting nanomolar IC\textsubscript{50} values in toxicity curves.

An ability to predict MET dependency \textit{de novo} in the clinic would permit treatment of this subset of patients with MET inhibitor monotherapy rather than the less tolerable combination EGFR + MET kinase inhibitor regimen, which is the current standard of care for all patients harboring concurrent \textit{EGFR} mutation and genomic \textit{MET} amplification. This could provide a tangible clinical benefit, as the maximum tolerated dose of crizotinib nearly doubles when administered alone versus in combination with erlotinib (143,144). The observation that DFCI161 cells developed EGFR-mediated crizotinib resistance, with a majority of clones reverting to single-agent gefitinib sensitivity, underscores the clinical potential of identifying this vulnerability: instead of receiving combination EGFR and MET inhibitor therapy, eligible patients could instead benefit from a course of single agent crizotinib, and still have the option of combination
therapy—or even sequential EGFR inhibitor therapy—upon progression. If implemented, this clinical course could potentially prolong both progression-free and overall survival.

Herein, we have elucidated some mechanistic underpinnings of this subset of EGFR mutant, MET-dependent NSCLC. We have demonstrated that downregulated EGFR expression level (particularly relative to MET expression level) and predominantly MET-mediated HER3 activation are two prominent features of MET-dependent lung cancer models. Furthermore, we have shown these features may play a causative role in the evolution of MET dependency, since reversing them is sufficient to induce EGFR/MET codependency in our patient-derived models. Unfortunately, however, our sample size for these studies was too small to pinpoint reliable and diagnostic metrics that would allow pre-treatment classification of this subset of NSCLC.

There is reason to suspect that the observed switch to MET dependency in tumors harboring concomitant EGFR activating mutation and MET amplification may be prevalent in the clinic. In under a decade, our lab alone has banked three models exhibiting this oncogenic vulnerability. MET amplification is observed in 5-20% of cases of resistance to targeted EGFR inhibitors (to which only patients with EGFR mutant tumors—accounting for less than 15% of NSCLC cases in Caucasian patients—respond) (145). Given the relative scarcity of EGFR mutant, MET amplified tumors in the population, our lab has only banked specimens from ten different patients over the course of a decade (Table 3-2). Of the concurrent EGFR mutant/MET upregulated specimens we have identified: three have switched to single agent MET dependency, two have retained the expected phenotype of EGFR/MET codependency, and one could not be recovered and banked. The remaining four represented scarce samples that could only be
expanded *in vivo*. Without corresponding cell lines, the oncogene dependency of these specimens remains to be characterized (Table 3-2).

Table 3-2: Prevalence of MET dependency in patient-derived models

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EGFR Status</th>
<th>MET Status</th>
<th>Models Established</th>
<th>Oncogene Dependency</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFCI81</td>
<td>Del19</td>
<td>Amplification</td>
<td>Cell Line, PDX</td>
<td>MET Dependent</td>
</tr>
<tr>
<td>DFCI110</td>
<td>Del19</td>
<td>Amplification</td>
<td>Cell Line</td>
<td>EGFR/MET Codependent</td>
</tr>
<tr>
<td>DFCI161</td>
<td>L858R</td>
<td>Copy number gain</td>
<td>Cell Line, PDX</td>
<td>MET Dependent</td>
</tr>
<tr>
<td>DFCI191</td>
<td>L858R</td>
<td>Amplification</td>
<td>Insufficient sample</td>
<td>Undetermined</td>
</tr>
<tr>
<td>DFCI202</td>
<td>L858R</td>
<td>Amplification</td>
<td>Cell Line</td>
<td>EGFR/MET Codependent</td>
</tr>
<tr>
<td>DFCI307</td>
<td>Del19</td>
<td>Amplification</td>
<td>PDX</td>
<td>MET Dependent</td>
</tr>
<tr>
<td>DFCI357</td>
<td>Del19</td>
<td>Amplification</td>
<td>PDX</td>
<td>Undetermined</td>
</tr>
<tr>
<td>DFCI363</td>
<td>Del19</td>
<td>Amplification</td>
<td>PDX</td>
<td>Undetermined</td>
</tr>
<tr>
<td>DFCI431</td>
<td>L858R</td>
<td>Amplification</td>
<td>PDX</td>
<td>Undetermined</td>
</tr>
<tr>
<td>DFCI544</td>
<td>Del19/T790M</td>
<td>Amplification</td>
<td>PDX</td>
<td>Undetermined</td>
</tr>
</tbody>
</table>

In addition to the models that we have established, two recent clinical case publications also reported on patients who progressed on EGFR-targeted therapies and exhibited sensitivity to crizotinib monotherapy (139,140). Both patients described in these cases had EGFR mutant NSCLC, showed initial responses to EGFR inhibitor treatment, and had evidence of MET amplification in their post-progression biopsies. Although the standard of care for this clinical scenario is normally combination treatment with EGFR and MET inhibitors, in both cases the clinicians opted to treat the patients with single-agent crizotinib. The rationale given for this decision was poor tolerance of EGFR targeted therapy in one case, and patient progression on both erlotinib and osimertinib treatment in the latter case. In both cases, the patients showed clinical response to treatment and clear evidence of tumor regression no on therapy. While crizotinib was poorly tolerated and needed to be discontinued on one case, the other patient’s tumor shrank dramatically in response to crizotinib, and an ongoing progression-free survival of four months was reported at the time of publication (139,140).
It is presently impossible to ascertain the prevalence of MET dependency in EGFR mutant, MET amplified lung cancer. Current genome-based predictions of drug sensitivity fail to pinpoint these cases, as tumors harboring concurrent EGFR mutation and MET amplification are presumed to be co-dependent on activity of both oncogenes. We hope that illuminating some of the mechanistic underpinnings of this subset of EGFR mutant NSCLC may eventually advance the clinical diagnosis and treatment of these lung cancers.
Chapter 4: Identification of a steroid-induced switch in drug sensitivity in EGFR-mutant MET-overexpressing NSCLC models
Attributions

Contributors to this work include Pınar Ö Eser, Raymond M. Paranal, Jenny Choi, Steven Wang, Man Xu, Sangeetha Palakurthi, Atsuko Ogino, Chiara Ambrogio, Magda Bahcall and Pasi A. Jänne. P.Ö.E. and P.A.J. conceived the studies. All authors helped design and carry out the experiments, and P.Ö.E., R.M.P., J.C., and S.W. analyzed and interpreted the results. S.P. supervised in vivo studies and P.A.J. supervised the project. P.Ö.E. wrote the text with feedback from P.A.J.

4.1. Introduction

Glucocorticoids are steroid hormones that are widely prescribed in clinical care to mitigate inflammatory pathologies. The endogenous glucocorticoid cortisol is synthesized and secreted by the adrenal gland (146). Cortisol is predominantly released in response to stress and has both immune and metabolic functions, dampening inflammatory signaling and affecting glucose metabolism. Synthetic glucocorticoids, including hydrocortisone and dexamethasone are synthetic analogs of endogenous cortisol, and are commonly prescribed to mitigate chronic inflammation (146). Lung cancer patients frequently experience inflammation, both as a result of malignant progression, and secondary to antineoplastic treatment, and are frequently prescribed glucocorticoids as part of disease management (147). Like its endogenous corticosteroid counterparts, dexamethasone acts through engagement and activation of the glucocorticoid receptor (GR), a transcriptional factor that simultaneously functions in transrepression of inflammatory genes, and transactivation of metabolic signaling pathways (148,149).
Chronic use of glucocorticoids is associated with weight gain, osteoporosis, and diabetes, among other adverse events (150,151). Even with these side effects, the use of glucocorticoids in NSCLC palliative care is pervasive, since the side effects of sub-chronic steroid treatment are tolerated significantly better than the symptoms of cancer- and chemotherapy-associated inflammation that they are prescribed to combat. Notably, crosstalk has been observed between GR activation and EGFR signaling (152). Activation of GR by endogenous cortisol was shown to dampen EGFR-downstream signaling. Because cortisol secretion follows a circadian pattern, EGFR downregulation was associated with diurnal GR activation. Based on these observations, the authors recommended that EGFR inhibitors should be administered in at night—when cortisol levels are low and EGFR signaling is thereby expected to be most active—to maximize their efficacy (152,153). While there is no explicit mention of the implications of these findings for patients undergoing simultaneous tumor-directed therapy with EGFR TKIs alongside regimens of glucocorticoid-based palliative care, it stands to reason that staggered administration of the two drugs may be effective in achieving optimal EGFR inhibition (152). Taken together, these findings support a role for glucocorticoid treatment in NSCLC beyond just palliative care—it may simultaneously synergize with targeted EGFR inhibitors to further ablate associated downstream kinase signaling.

In addition to their clinical uses, glucocorticoids—predominantly hydrocortisone—are a component of standard culture media that is used to establish and perpetuate primary lung cancer cell lines (154,155). Our lab frequently establishes patient-derived primary NSCLC models simultaneously in RPMI-1640 and chemically-defined ACL-4 media, comprised of RPMI-1640 basal media supplemented with growth factors, including hydrocortisone (Table 4-1). While ACL-
4 media is broadly used in the culture of lung cancer cell lines (155), previous work in our lab has suggested that the presence of growth factors in the media—most notably EGF and insulin—may confound experimental readouts by masking drug efficacy sensitivity assays. As a result, in establishing primary cell lines, we have sought to strike a balance between culturing cells in media that will promote their survival and growth, while simultaneously trying to faithfully recapitulate cell lines’ drug sensitivity properties. To this end, each patient sample that is obtained is simultaneously established in ACL-4 media + 10% FBS (A10) and its less supplemented counterpart, RPMI-1640 + 10% FBS (R10).

Table 4-1: Composition of ACL-4 Media: RPMI-1640 + Factors (156)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>20 μg/mL</td>
</tr>
<tr>
<td>Transferrin</td>
<td>10 μg/mL</td>
</tr>
<tr>
<td>Sodium Selenite</td>
<td>25 nM</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>50 nM</td>
</tr>
<tr>
<td>Epidermal Growth Factor</td>
<td>1 ng/mL</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>100 pM</td>
</tr>
<tr>
<td>Phosphoryl-ethanolamine</td>
<td>10 μM</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>10 μM</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>2 mg/mL</td>
</tr>
<tr>
<td>HEPES buffer</td>
<td>10 mM</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2 mM</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>0.5 mM</td>
</tr>
</tbody>
</table>

While many lung cancer cell lines grow better in ACL-4 media, RPMI-1640 is preferred for validation of drug sensitivity without confounding factors present in culture media. Although we only used passages of the DFCI161 cell line that had been established in R10 media for the entirety of the MET dependency studies, we also had an isogenic model derived from the same precursor specimen that had been continually propagated in A10 media. Based on the results of our in vivo patient-derived xenograft studies, we are confident that the R10 conditions
adequately model a clinical scenario. Nonetheless, as part of our characterization of the MET dependency of these models, we were interested in interrogating our phenotype in both media conditions.

4.2. EGFR-mutant, MET-dependent models of NSCLC exhibit a culture media-dependent switch in drug sensitivity

Assaying the drug sensitivities of DFCI81 and DFCI161 cell lines in R10 versus A10 media, we observed a surprising culture media-dependent shift in the drug sensitivity profiles of these cells. In R10 basal media (the culture condition used in all studies described in Chapter 3), both cell lines exhibited resistance to EGFR inhibitors and sensitivity to MET inhibitors. Because this phenotype was consistent with xenograft-based in vivo models of the tumors, we surmised it was a representative model of the cells’ baseline oncogene dependencies. However, both DFCI81 and DFCI161 cells cultured long-term in A10 media exhibited crizotinib resistance. DFCI161 cells also exhibited a reversion to single agent gefitinib sensitivity in A10 media, a phenotype that was not observed in DFCI81 cells (Supplemental Figure III-1). While induction of drug resistance seemed attributable to the supplementary growth factors present in ACL-4 media, the observation of gefitinib sensitization—particularly in a growth factor-rich media—was highly unexpected.

After validating a common origin of the DFCI161 cells established in R10 and A10 media by STR analysis (data not shown), we sought to examine whether the distinct drug sensitivity profiles of the DFCI161/R10 cells and DFCI161/A10 cells were interchangeable. To this end, each cell line was plated in both culture media conditions and treated with a panel of EGFR and MET inhibitors to evaluate drug sensitivities. Remarkably, we observed that the culture media in which the cells were assayed was more predictive of their drug vulnerabilities than then media in which
the cell lines were established and chronically maintained: DFCI161/R10 cells plated and treated in A10 media exhibited a drug sensitivity switch to MET TKI resistance and EGFR TKI sensitivity, while DFCI161/A10 cells cultured acutely in R10 media shifted to MET TKI sensitivity and EGFR TKI resistance (Figure 4-1). An analogous acute switch in MET TKI sensitivity was conserved in DFCI81 cells, although A10 media did not affect gefitinib resistance in this model (data not shown). This property was unique to EGFR-mutant, MET dependent NSCLC models, and not observed in any other cell line tested (Supplemental Table III-1).

In published models of EGFR oncogene dependency, inhibition of EGFR phosphorylation is sufficient to ablate the activation of downstream kinase signaling—a process that ultimately triggers apoptosis (117,157,158). In our MET-dependent models, we have characterized an analogous dependency of activation of downstream kinases on MET activation. Considered in this context, the ACL-4-mediated drug sensitivity switch was hypothesized to induce a switch in the upstream oncogenic regulator of downstream kinase phosphorylation. In other words, while single-agent MET inhibitor treatment inhibited the phosphorylation of ERK1/2, Akt, and S6 ribosomal protein in R10 media, we anticipated that EGFR inhibitor treatment would be sufficient to ablate the phosphorylation of these downstream pathways in A10 media.

To test this hypothesis, we treated DFCI161/R10 cells and DFCI161/A10 cells, each plated in the corresponding media condition in which they were maintained, with dose ranges of gefitinib and crizotinib. Strikingly, we observed that phosphorylation of downstream ERK1/2 and Akt remained fully dependent on MET activation, regardless of media condition (Figure 4-2). This disconnect is unfamiliar in EGFR-driven models, and was difficult to reconcile with the culture media-dependent drug sensitivity switch we observed. Although unusual, this discrepancy in
downstream kinase signaling being modulated seemingly independently of oncogenic driver was also conserved the crizotinib resistant DFCI161 subclones, and may be another idiosyncratic artifact of this cell line (Supplemental Figure II-11b).

Figure 4-1: Observation of a culture media dependent switch in drug sensitivity of DFCI81 and DFCI161 cell lines

Regardless of the culture media in which the cell line was established and maintained, DFCI161 cells assayed in R10 media are gefitinib tolerant and crizotinib resistant, are gefitinib sensitive and crizotinib resistant in A10 media. Acutely culturing DFCI161 cells, regardless of origin, in R10 or A10 media over the course of a 96-hour MTS assay is sufficient to switch their oncogene dependencies. For these studies, the panel of EGFR and MET inhibitors tested in Chapter 3 was used to corroborate cell oncogene dependencies, with one exception: an experimental MET inhibitor, PHA665752 (103), which was used in place of merestinib.
DFCI161 cells established and cultured in RPMI-1640 (DFCI161/R10) and in ACL-4 (DFCI161/A10) media both exhibit crizotinib sensitive modulation of downstream kinases Akt, ERK1/2, and S6 ribosomal protein, although only the DFCI161/R10 cells appear MET dependent in drug sensitivity assays. EGFR-dependent DFCI161/A10 cells show significant downregulation of phospho-MET levels compared to DFCI161/R10 counterparts.

Western blot analysis of DFCI161 cells cultured in R10 versus A10 media also revealed significant downregulation of phosphorylated MET levels in the EGFR inhibitor sensitive DFCI161/A10 cells compared to the MET inhibitor sensitive DFCI161/R10 cells (Figure 4-2). This observation is consistent with the EGFR: MET stoichiometry-dependent switch in oncogene addiction characterized in Chapter 3. Based on these findings, we postulated that some factor(s)
in ACL-4 media are inducing downregulation of MET activation, thereby shifting the activated EGFR: MET ratio in DFCI161 cells. As noted previously, modulating the EGFR: MET expression and activation ratio is sufficient to confer resistance to single-agent MET inhibition, and may promote a switch to EGFR inhibitor sensitivity.

4.3. Glucocorticoids induce culture media-associated drug sensitivity switch in DFCI161 cells and downregulate MET expression and phosphorylation

We next set out to identify the factor in ACL-4 media that was inducing the drug sensitivity switch observed in DFCI161 cells. Through a media reconstitution MTS assay, in which each highlighted component of ACL-4 media (Table 4-1) was added individually to cells in RPMI-1640 media, we identified the glucocorticoid hydrocortisone as the driver of the ACL-4 associated drug sensitivity phenotypes (Figure 4-3). Cells that exhibited crizotinib sensitivity and gefitinib resistance in R10 media became crizotinib tolerant and gefitinib sensitized when treated in the presence of either ACL-4 media or R10 supplemented with hydrocortisone (Figure 4-3a). DFCI81 cells also exhibited glucocorticoid-induced crizotinib resistance, although they did not become gefitinib sensitive in the presence of steroid (data not shown). Addition of hydrocortisone to R10 media was also sufficient to downregulate both total and phosphorylated MET levels in DFCI161 cells, supporting the putative mechanism of MET downregulation as the basis of drug sensitivity switching (Figure 4-3b).

Interested in whether other classes of steroid hormone also affected the drug sensitivities of DFCI81 and DFCI161 cells, we tested other steroids including estrogen, progesterone, aldosterone, and dexamethasone to assess whether any of them phenocopied the oncogene dependence switch observed following hydrocortisone treatment. Unsurprisingly, we found that
dexamethasone, another glucocorticoid, also induced crizotinib resistance in DFCI81 and DFCI161 cells, and gefitinib sensitivity in DFCI161 cells, when supplemented to R10 (Figure 4-3a). Aldosterone, a mineralocorticoid that retains some affinity for the glucocorticoid receptor, induced a similar but more modest drug sensitivity phenotype compared to hydrocortisone and dexamethasone (data not shown). The remaining steroid hormones, however, had no effect on gefitinib sensitivity or crizotinib resistance when added to R10 basal media (data not shown).

Through transient glucocorticoid receptor knockdown, we demonstrated that dexamethasone acts through its target receptor, the glucocorticoid receptor (NR3C1 gene), to induce the associated drug sensitivity phenotype. DFCI161 cells transfected with non-targeting control siRNA exhibited crizotinib resistance and gefitinib sensitization in the presence of dexamethasone. By contrast, transfecting cells with siNR3C1 (an siRNA pool targeting glucocorticoid receptor transcript) counteracted dexamethasone-associated drug sensitivity switch, reverting DFCI161 cells to baseline crizotinib sensitivity and gefitinib resistance (Figure 4-3c). The dexamethasone-mediated oncogene dependence switch was shown to be transient, reversible upon one week of steroid withdrawal (Supplemental Figure III-2).
Figure 4-3: Hydrocortisone in ACL-4 media induces A10-associated drug sensitivity phenotype
(a) Incubation and treatment of cells in R10+hydrocortisone (at 1X and 10X the levels present in A10 media), as well as another synthetic glucocorticoid, dexamethasone, is sufficient to induce a switch to crizotinib resistance and gefitinib sensitivity, identical to the switch observed when cells are treated in A10 media. (b) Hydrocortisone treatment also alters protein expression levels in a manner similar to incubation in A10 media—most notably by downregulating MET expression and phosphorylation. (c) Glucocorticoids modulate drug sensitivity through activation of the glucocorticoid receptor; siRNA knockdown of the receptor reverses the dexamethasone-associated drug sensitivity profile.

Intriguingly, the trends observed following dexamethasone treatment of both DFCI161 and DFCI81 cells mirror the decitabine-associated phenotype observed in Chapter 3. Like the DNA
methyltransferase inhibitor, dexamethasone also induces complete drug sensitivity reversion in DFCI161 cells, inducing both crizotinib resistance and gefitinib sensitivity, but only promotes crizotinib resistance without affecting gefitinib sensitivity in the DFCI81 model. This may be attributable to the differences in baseline EGFR expression between the two cell lines. Although DFCI161 cells express significantly lower levels of EGFR compared to EGFR/MET-codependent control cells, they express significantly more EGFR than DFCI81 cells. Consequently, it is possible that DFCI161 cells revert more readily to gefitinib sensitivity when the EGFR: MET expression ratio is shifted, as compared to DFCI81 cells which have very low EGFR expression, and likely shift to other oncogene dependencies in the absence of MET signaling.

In the case of the dexamethasone-treated DFCI161 cells, the shift to crizotinib resistance was accompanied by downregulation of MET and HER3 phosphorylation levels across all treatment conditions. As observed in ACL-4 media, downstream kinase activation in DFCI161 cells remained MET dependent in the presence of dexamethasone, even as the cells became less sensitive to MET inhibition (Figure 4-4a). Paradoxically, while the phosphorylation of downstream kinases (AKT and ERK1/2) was reduced in response to MET inhibition by crizotinib (regardless of dexamethasone co-treatment), phosphorylation levels of these same kinases were not impacted by dexamethasone treatment alone. While both crizotinib and dexamethasone reduce phosphorylation levels of MET and HER3, there is a disconnect that appears between dexamethasone-mediated MET inhibition and the transmission of this inhibition to affect downstream signaling. This likely occurs because dexamethasone does not affect the kinase activity of MET, such that even in the presence of dexamethasone, downstream ERK1/2 and Akt phosphorylation continues to be crizotinib responsive. However, in this context, the cells appear
to have developed a state of oncogene indifference, because their survival no longer seems dependent on activation of canonical EGFR-downstream signaling.

Figure 4-4: Glucocorticoid-mediated downregulation of MET expression and activation may be sufficient to induce GC-associated drug sensitivity phenotype in DFCI161 cells

(a) Treatment of DFCI161 cells with EGFR and MET inhibitors in the presence versus absence of dexamethasone does not appear to affect the upstream oncogenic dependencies of downstream kinase cascades. Dexamethasone does, however, downregulate the expression of phosphorylated MET and phosphorylated HER3 (b) MET downregulation observed in dexamethasone-treated DFCI161 cell lines may be an upstream driver of the drug resistance switch phenotype, as transient MET knockdown with siRNAs is sufficient to induce crizotinib resistance and gefitinib sensitization in DFCI161 cells.

Given our observations in Chapter 3 that affecting EGFR:MET expression stoichiometry can influence the drug resistance profiles of DFCI81 and DFCI161 cells, we were interested if the
downregulation of MET expression and phosphorylation secondary to glucocorticoid treatment could be accountable for the observed drug sensitivity switching. Intriguingly, we found that transient MET knockdown in DFCI161 cells appears sufficient to recapitulate the dexamethasone-associated phenotype of crizotinib resistance and gefitinib sensitivity in R10 media (Figure 4-4b). While further mechanistic studies are warranted to elucidate the underlying basis of dexamethasone-mediated MET downregulation, the observation that perturbing EGFR: MET ratios contribute to affecting oncogene dependency fortifies our model of the processes underlying drug sensitivities in EGFR-mutant, MET-dependent cell lines, and emphasizes the plasticity of these oncogene dependent models.

4.4. Glucocorticoid treatment induces growth retardation and a reversible senescence-like phenotype in steroid-sensitive models

Perplexingly, although the cell lines we had been characterizing were exquisitely sensitive to small molecule MET inhibitors, we had found that we were able to downregulate MET expression and phosphorylation through glucocorticoid treatment and transient knockdown without notably affecting downstream signaling or killing the cells. Perhaps unsurprisingly given the underlying oncogenic dependency of DFCI161 cells, we observed that dexamethasone treatment induced a unique morphological change accompanied by significant growth retardation in these cells (Figure 4-5a). We also noted that dexamethasone treatment triggers a low-grade apoptotic phenotype, resulting in caspase activation and Bim upregulation (data not shown). However, the cells are able to remain viable in dexamethasone for long periods of time, and invariably rebound to their normal growth kinetics following dexamethasone withdrawal.
Figure 4-5: Dexamethasone induces cell cycle arrest and senescence-associated properties

(a) Dexamethasone induces dramatically slowed growth in DFCI161 cells pre-incubated in steroid compared to DFCI161 cells cultured without steroid (b) In keeping with the slowed growth observed and upregulation of G1 arrest genes, flow cytometry PI analysis revealed a substantial increase in growth arrested cells, and virtually no cells in S phase in DFCI161 cells cultured in dexamethasone (c) β-galactosidase staining revealed that dexamethasone treatment induced substantial upregulation of senescent or quiescent β-gal positive DFCI161 cells. The β-galactosidase activity observed in dexamethasone treated cells was comparable to levels in positive control DFCI161 cells treated with sub-lethal cisplatin to induce senescence. As with the drug sensitivity phenotype, the senescent/quiescent state of DFCI161 cells was reversed by dexamethasone withdrawal and washout (d) Dexamethasone treatment is sufficient to upregulate expression of growth arrest and senescence-associated proteins p15, p21, and p27.
Cell cycle profiling of DFCI161 cells in the presence or absence of dexamethasone revealed a drop from 20% proliferating S phase cells at baseline to under 5% in cells pre-cultured in dexamethasone for one week, further supporting the observation that dexamethasone may induce cell cycle arrest (Figure 4-5b). Of note, cells cultured in dexamethasone exhibited significantly increased senescence-associated lysosomal β-galactosidase activity compared to their counterparts, an upregulation that was reversible by dexamethasone withdrawal (Figure 6c). Finally, analysis of protein expression revealed significant upregulation of p21\(^{WAF1}\) and p16 in the presence of dexamethasone, further suggesting the induction of cell cycle arrest in the presence of steroid (Figure 4-5d).

Dexamethasone-mediated drug sensitivity switching was not observed in a preliminary in vivo study. While DFCI161 xenograft tumors consistently showed crizotinib sensitivity and erlotinib resistance as we would expect, co-treatment of mice with dexamethasone did not affect tumor drug sensitivity (Figure 4-6a). Western blot analysis revealed that while the dexamethasone dosing regimen administered was sufficient to activate the glucocorticoid receptor, the relative level of activation was not as robust that achieved in vitro. Quantification of known GR-downstream genes in xenograft tumors also revealed modest changes following dexamethasone treatment, although all trends supported some extent of GR activation (data not shown). It is possible that this is due to endogenous regulation of corticosteroid signaling through antagonism or another mechanism in the animal that limits the efficacy of glucocorticoid signaling to switch drug sensitivity. Furthermore, dexamethasone treatment in vivo did not downregulate levels of phospho-MET, a prominent feature observed in the DFCI161 cell line.
model which appears to play a mechanistic role in the process of oncogene switching in vitro (Figure 4-6b).

Figure 4-6: Dexamethasone treatment of mice bearing patient-derived DFCI161 xenografts did not induce any change in the drug sensitivity of the tumors

(a) Mouse tumor xenograft models of DFCI161 are erlotinib resistant and crizotinib sensitive, regardless of the presence of concurrent dexamethasone treatment (b) Dexamethasone treatment results in moderate activation of the glucocorticoid receptor in tumor cells, but fails to induce downregulation of phospho- or total MET as it does in the DFCI161 cell line model following dexamethasone treatment

4.5. Discussion of ongoing and future directions

Glucocorticoid-associated changes to drug sensitivity were intriguing, and relevant because glucocorticoids, including hydrocortisone and dexamethasone, are widely used in palliative care to mitigate cancer- and treatment-associated inflammation. As glucocorticoids are frequently administered concurrently with small molecule kinase inhibitors, it is important to understand the potential mechanisms through which they may influence resistance and sensitivity to targeted therapies in NSCLC.
Here, we describe the profound effect of glucocorticoids on the drug sensitivity profiles of EGFR-mutant, MET dependent cell line models. Ultimately, this phenotype appears to be intimately linked to glucocorticoid-induced downregulation of MET signaling, a feature unique to this NSCLC subset. Future studies are warranted to elucidate the mechanisms through which dexamethasone induces MET downregulation concurrent with gefitinib sensitivity and crizotinib resistance in DFCI161 and DFCI81 cell lines. Concurrently, dexamethasone + TKI xenografts studies will also be repeated to reaffirm whether the mechanisms observed in our in vitro models also hold true in vivo. It will be important to establish whether the observed steroid-mediated switches in drug sensitivity translate to the clinic, and if so, how they can be harnessed to modulate and potentiate patient drug sensitivity and response. Results from this study suggest that even if glucocorticoid treatments do not affect in vivo drug sensitivities, it is worthwhile to understand how this regulation is overcome, as targeting this pathway may provide new biological insights into the interplay between glucocorticoid and MET signaling in vivo.
Chapter 5: Discussion and Conclusions
In Chapter 2, we applied CRISPR-Cas9 genome editing to create a panel of cell lines expressing the osimertinib resistance mutation C797S, for which there is currently no endogenous lung cancer model. In doing so, we developed an essential platform for the screening and validation of potential next-line cancer treatments on a presently untargetable subset of triple mutant EGFR driven NSCLC. Prior to the development of these models, we and others had been using Ba/F3 cells overexpressing resistance mutations for drug screening. However, Ba/F3 models are limited, not only because they are a non-human, non-cancer cell line, but also because they lack expression of wild type EGFR or other RTKs for mutant EGFR to dimerize with. In lung cancer, mutant EGFR may not only homodimerize, but also heterodimerize with wild type EGFR alleles or other receptors, yet most Ba/F3 models are only representative of mutant EGFR homodimers. Consequently, Ba/F3 models also discount the kinase activity of other receptors not only being phosphorylated by, but potentially also phosphorylating EGFR in endogenous lung cancer contexts. Furthermore, overexpression models do not always recapitulate biological processes representative of what happens at endogenous expression levels. Finally, likely resulting from a combination of all these factors, targeted inhibitors appear more efficacious in Ba/F3 cells than they are observed to be in endogenous lung cancer contexts. To more accurately predict the clinical efficacy of novel classes and combinations of targeted therapies for C797S mutant NSCLC, we have developed lung cancer cell line models of C797S, and T790M in cis with each of the two most prevalent activating EGFR mutations, del19 and L858R.

Moreover, we sought to characterize the EGFR T790I gatekeeper mutation, which we believe may represent a rare and still uncharacterized mutational resistance mechanism in EGFR-driven NSCLC. The minor allele that would be expected to give rise to T790I is extremely rare in
the population, which is likely why this mutation has yet to come to clinical attention (159). Even so, it is essential to assess whether EGFR T790I can develop and confer resistance, and understand its biology in case it ever does come to clinical attention.

The importance of these studies lies mostly in the development of new models to be used in preclinical screening. Establishment of these models will allow lab members and collaborators to apply them toward characterizing novel preclinical drug candidates. We have used the CRISPR model PC9 del19/T790M/C797S for an in vivo PDX study to evaluate the efficacy of the FDA-approved, EGFR-directed antibody therapy Necitumumab in EGFR triple mutant tumors. We hope to further harness this tool to evaluate the prospect of targeting HER3 in treatment-refractory EGFR driven NSCLC. In parallel, once established, the H3255 L858R/T790M/C797S cells will provide a novel model for screening. Preclinical allosteric inhibitors have shown promise in L858R/T790M double mutant NSCLC models, but their activity against the C797S triple mutant in a stoichiometrically representative lung cancer context remains to be determined.

In Chapter 3, we observed a novel oncogenic switch paradigm, whereby a model harboring an activating EGFR mutation developed MET dependency, which has not previously been documented in lung cancer. Understanding the basis of MET dependency in EGFR mutant lung cancers would provide novel therapeutic opportunities, as an ability to treat select patients with single agent crizotinib instead of a TKI combination would improve drug tolerability. Moreover, our observations underscore the need to develop clinical tests beyond sequencing to understand the wiring and oncogene dependencies of cancer cells. In the absence of any genomic signature to indicate a possible MET dependency, patients with MET-dependent tumors cannot receive the full clinical benefit of MET inhibitor monotherapy. Our hope is that elucidating the
basis of MET addiction in EGFR mutant lung cancer models can contribute to the development of novel diagnostic tests to identify and exploit drug vulnerabilities.

Switches in oncogene addiction as a mechanism of drug resistance, as observed in the patient primary cell lines DFCI81 and DFCI161, are largely undocumented in EGFR driven NSCLC. Evaluating the mechanistic underpinnings of the observed resistance switch and understanding the broader implications of switches in oncogene addiction in NSCLC may have important consequences for the future classification and treatment of EGFR mutant lung cancer. Through characterization of patient-derived cell lines and xenografts, we have identified three EGFR inhibitor resistant models—DFCI81, DFCI161, and DFCI307—that exhibit a unique sensitivity to single agent MET inhibition, a phenomenon which has not previously been observed in EGFR mutant lung cancers.

At present, patients who reveal genomic upregulation of MET kinase concurrent with EGFR activating mutations receive combination treatment with EGFR and MET inhibitors. The limitations of combinatorial kinase therapy with EGFR and MET inhibitors are threefold. First, it restricts the tolerability of the drugs by increasing dose-limiting toxicities—since both EGFR and MET converge on the same downstream signaling pathways. Concurrent doses of EGFR and MET inhibitors need to be reduced in combination therapy situations to improve clinical tolerability. These dosing limitations particularly become a disadvantage in situations where a tumor is predominantly driven by one of the oncogenes (in this case, MET). For example, patient with an EGFR-mutant yet MET dependent tumor could be receiving 250 mg, twice daily, of crizotinib (144). However, because current thoracic oncology practice relies on genomic data in determining patient treatment regimen, a MET dependent patient exhibiting an EGFR mutation
would be limited to treatment with 150 mg, twice daily, of MET inhibitor concurrent with an EGFR inhibitor (143). In this scenario, combination EGFR inhibitor treatment not only offers no clinical benefit, but it also reduces the dose of the therapeutically efficacious drug a patient can receive.

Second, development of resistance to targeted kinase inhibitors is well documented across cancers and is inevitable in most cases. As with other oncogene driven tumors, we would expect MET-dependent tumors to eventually become drug resistant, regardless of whether they were treated with single agent MET inhibitor or a combination regimen of EGFR inhibitor + MET inhibitor. However, developing resistance to combination therapy substantially restricts targeted treatment opportunities at progression. Based on the presence of an EGFR mutation in the genomes of our three MET-dependent cases, we may readily infer that at some point during cancer progression, each of these tumor lineages were EGFR dependent—as each model presents a typical activating mutation, we have discounted the possibility of these mutations being passenger events. We may then hypothesize that the presence of an EGFR activating event in the tumors’ genomic history may predispose them to develop crizotinib resistance through an EGFR-mediated mechanism—or revert to co-dependency. Should this happen, it would allow patients to continue receiving some combination of EGFR and MET inhibitor therapy following resistance to MET inhibitors (clinical scenarios presented in Figure 5-1). It should be noted that these scenarios are theoretical, and shown to emphasize the importance of further characterizing and understanding the basis of a switch to MET dependency rather than EGFR-MET codependency as a mechanism of EGFR inhibitor resistance. At present, there are no available or clinically approved means of predicting response to single-agent MET inhibitors de novo. However, we envision these scenarios will become relevant with a greater understanding of the
processes and mechanisms underlying a switch to MET dependency, and identification of clinical predictors thereof.

**Current Standard of Care for EGFR/MET-codependent patients:**

| EGFR inhibitor (9-18 months) | EGFR + MET TKI Combination (~6 months) | Chemotherapy (5 months) | PD, Hospice |

**Predicted Therapeutic Approach for EGFR Mutant Patients with MET Dependent Tumors:**

| EGFR inhibitor (9-18 months) | MET Inhibitor (7-8 months) | EGFR inhibitor (if resensitized) | MET Inhibitor (if resensitized) | EGFR + MET TKI Combination (~6 months) |

**Figure 5-1: Predicted clinical benefits of first-line MET inhibitor monotherapy for patients whose EGFR-mutant tumors exhibit a switch to MET oncogene dependency**

Our experimental evidence suggests that *EGFR* mutant, MET-dependent models may develop resistance to MET inhibitor monotherapy through either a complete reversion to EGFR dependency (as illustrated here), or through establishment of codependency. If a tumor from this cancer subtype reverts to EGFR dependency clinically, a patient would not only be eligible for single agent treatment with EGFR inhibitors, but would also benefit from a MET inhibitor drug holiday. There are well established precedents for therapeutic resensitization during a drug holiday (160), and our preclinical evidence also supports that MET-driven tumors may have a tendency towards resensitization during crizotinib withdrawal, which would open the door to treating patients with better tolerated alternating sequential therapy rather than combination treatment. In either scenario, successfully identifying patients with this oncogenic dependency and treating them with single agent MET inhibitor rather than immediately prescribing combination treatment with concurrent EGFR and MET inhibitors would improve clinical outlook by postponing combination treatment as a potential next-line therapeutic avenue upon progression.

A final limitation of combination therapeutic regimens is that the mechanisms of resistance that inevitably emerge from them have proven therapeutically less tractable (161). These include EMT or SCLC transition—mechanisms for which there are not yet effective
combinatorial approaches. These potential clinical benefits of identifying patients who may respond to single-agent MET inhibitor therapy instead of combinatorial blockade of both EGFR and MET pathways highlight the importance of understanding and predicting this novel subtype of EGFR-mutant NSCLC.

In Chapter 4, we identified and characterized a novel glucocorticoid-mediated switch in oncogene dependency that appears to be specific to the newly discovered EGFR mutant, MET-dependent subset of NSCLC that was characterized in Chapter 3. Corticosteroids are broadly prescribed concomitant with chemotherapy and targeted therapy to mitigate treatment-associated inflammation in lung cancer. Our dexamethasone responsive lung cancer models are currently limited to DFCI81 and DFCI161, yet the steroid responsive drug resistance phenotypes appear robust and reproducible. Of note, 2/2 of our EGFR-mutant, single agent MET inhibitor sensitive cell line models exhibit steroid responsiveness, raising the possibility that these phenotypes may be interconnected.

Because of the broad use of glucocorticoids in lung cancer, and because they appear to confer reversible crizotinib resistance in DFCI161 and DFCI81 cells, it will be important to identify the mechanism through which they are modulating drug vulnerabilities in this context. It is interesting to consider that drugs commonly prescribed to manage treatment-associated complications may be affecting disease progression and the efficacy of oncogene targeting. Because glucocorticoids are already prescribed in lung cancer, it is tempting to consider utilizing them not just to manage inflammation, but also to affect drug sensitivities of a patient’s tumor. While this possibility will require significant exploration, the glucocorticoid axis is druggable and already targeted in many cancer patients. Identification of this pathway as a possible mediator
of oncogene dependency in a subset of cancer patients may impact how we consider treating chronic inflammation during targeted NSCLC therapy.

Herein, we have used an arsenal of genetic and pharmacological approaches to model and modulate oncogene dependency in lung cancer. We have generated CRISPR-edited models of resistance to next-generation EGFR inhibitors, and identified a subgroup of patients whose EGFR mutant tumors exhibit a previously uncharacterized vulnerability to MET inhibitor monotherapy. We have further shown that the drug sensitivities of cell line models of EGFR mutant, MET dependent NSCLC may be modulated by exogenous glucocorticoid treatment. Taken together, we have established and characterized new models of resistance and sensitivity to targeted therapies in EGFR-driven NSCLC.
Appendices
Appendix I: Supplemental Materials for Chapter 2

Supplemental Figure I-1: CRISPR construct design

CRISPR donor oligo for introducing *EGFR* resistance mutations. The white region of the construct corresponds to an intron, the yellow part aligns with exon 20 of *EGFR*. Construct shown is the donor construct for introducing T790M/C797S, with both the codons harboring the resistance mutations highlighted in red. Additional silent mutations that comprise the donor oligo’s signature are indicated in purple, with the corresponding wild type codon indicated in yellow above the oligo strand. CRISPR crRNA alignment with the donor and corresponding PAM sequence are also indicated.
Supplemental Figure I-2: Rate of spontaneous mutation in CRISPR-edited PC9 cells

Identity and frequency of spontaneous point mutations that emerged over the course of gefitinib and osimertinib selection following CRISPR editing of PC9 cells to introduce point mutations conferring drug resistance. Analysis of sequencing amplicons reveals that, while mutations introduced by homologous recombination are most prevalent in the PC9 cells, spontaneous acquisition of the L798 silent PAM sequence mutation is prevalent across all three cell populations. Spontaneous development of C797S is also a common event occurring in response to osimertinib selection. The PC9 cells are heterozygous with a SNP at Q787, so this alteration is not mutational in nature, although its concurrence with both L798 and C797S mutations is a testament to the ubiquity of the spontaneous emergence of these latter two mutations.
Supplemental Table I-1: Frequencies of Donor Sequence Integration versus Spontaneous Mutation in Post-Selection CRISPR Models

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Shaded cells represent the only alleles that are of interest in the population, while non-shaded cells are passenger events. Subclones of these populations will be identified for use in future studies on the basis of harboring these parental and donor alleles (introduced by CRISPR), but none of the other passenger events.
Supplemental Figure II-1: DFCI81 and DFCI161 cells are unique in their sensitivity to single-agent crizotinib treatment

Treatment with single-agent MET inhibitor crizotinib is sufficient to induce apoptosis in MET-dependent DFCI81 and DFCI161 cell lines. By contrast, gefitinib treatment induces apoptosis in the EGFR-driven cell line HCC827, and combination treatment with gefitinib and crizotinib is required to induce apoptosis. Caspase 3/7 activation was measured as a surrogate of apoptosis, using a fluorescent active caspase-specific dye, and imaged continually for four days using the IncuCyte® Live Cell Analysis system. Data are shown as change in caspase activity normalized to confluence change over the four-day assay.
Supplemental Figure II-2: DFCI81 and DFCI161 cells are unique in their sensitivity to single-agent crizotinib treatment

A panel of EGFR mutant and MET amplified cell lines was assayed in search of additional models that may have undergone a switch to single agent crizotinib sensitivity. The classes of cell lines are coded by color: NSCLC lines indicated in blue (H3255, HCC827, HCC2935, and PC9) harbor activating EGFR mutations, without concurrent MET amplification. As predicted, these lines exhibit gefitinib sensitivity and crizotinib resistance. The lines shown in purple harbor concomitant EGFR activating mutation and MET upregulation, in most cases through amplification. Of these lines, our patient-derived models DFCI81 and DFCI161 display our phenotype of interest: resistance to gefitinib, sensitivity to single agent crizotinib. However, each of the other EGFR mutant MET amplified lines tested—including HCC827GR6 alongside two additional patient-derived lines, DFCI202 and DFCI110—exhibited resistance to both single agent crizotinib and gefitinib. Finally, EBC-1 was included as a control MET amplified, EGFR wild type line known to be MET-dependent. Intriguingly, comparing DFCI81 and DFCI161 crizotinib sensitivity to that of a line known to be MET revealed the extent of their sensitivity.
Supplemental Figure II-3: DFCI307 xenografts exhibit MET-dependent activation of HER3 and EGFR-downstream signaling

(a) DFCI307 patient-derived xenograft tumors were treated with the vehicle control, the EGFR inhibitor osimertinib, the MET inhibitor savolitinib, or combined osimertinib + savolitinib. Three mice were treated for each arm of the study. While each kinase inhibitor effectively inhibited its target, with osimertinib downregulating EGFR phosphorylation and savolitinib hindering MET phosphorylation, savolitinib treatment alone was sufficient to ablate the phosphorylation and activation of downstream kinases ERK1/2 and Akt. Single agent savolitinib treatment was also sufficient to inhibit HER3, downregulating the ratio of active to total receptor.
Supplemental Figure II-4: Expression of growth factors and receptor tyrosine kinases in DFCI81 and DFCI161 cell line models

(a) MET-dependent cell lines exhibit reduced expression of the ERBB family ligands epiregulin and neuregulin compared to the EGFR/MET co-dependent control line HCC827GR6, as quantified by qRT-PCR. (b) DFCI81 in particular, but also DFCI161 exhibit dramatically reduced expression of total EGFR compared to their EGFR/MET codependent counterpart HCC827GR6.
Supplemental Figure II-5: EGFR involvement in ERBB ligand-induced crizotinib resistance

NRG1-induced crizotinib resistance appears independent of EGFR activation in DFCI81 cells. In DFCI161 cells, on the other hand, EGFR appears to mediate drug resistance conferred by not only recombinant NRG1 treatment, but also treatment with EGF and EPR. Because growth factor-mediated crizotinib resistance in DFCI161 cells is gefitinib dependent, it is reversed by treatment with gefitinib. All graphs were normalized to untreated control, with error bars representing standard deviation.
Supplemental Figure II-6: Ectopic overexpression of wild type EGFR in DFCI81 cells is sufficient to induce EGFR-MET codependency following ERBB ligand treatment

(a) Unlike DFCI161 cells shown above in Supplemental Figure II-5, parental DFCI81 cells do not respond to EGFR activation by becoming crizotinib resistant (b) When wild type EGFR is retrovirally overexpressed in DFCI81 cells, the cells develop crizotinib resistance upon ERBB ligand stimulation. These cells become artificially induced to EGFR/MET codependency, as they retain sensitivity to a combination of EGFR and MET inhibitors, even in the continued presence of ligand.
Supplemental Figure II-7: Prevalence of EGFR-HER3 dimers is unaffected by ligand treatment

Although western blot analysis showed that treatment with ERBB ligands induced sustained HER3 and/or phosphorylation in the presence of ligand, as a putative mechanism of circumventing crizotinib sensitivity, there are no detectable changes to dimerization between either total or phosphor HER3, and either wild type or mutant EGFR.

Supplemental Figure II-8: p85-HER3 dimerization dynamics

As previously reported, treatment with gefitinib was shown to disrupt dimerization between p85 and HER3 in HCC827 parental cells, while combination treatment with gefitinib + crizotinib was required to disrupt p85-HER3 dimers in MET-amplified HCC827GR6 cells (103). By contrast, pulldown with p85 in DFCI161 cells showed slightly diminished HER3 interaction as a result of single-agent crizotinib treatment, supporting that MET is predominantly functioning through HER3-mediated PI3K-Akt signaling activation in this MET-dependent context. Intriguingly, neither DFCI81 cells nor DFCI161 cells show any interaction between p85 and EGFR at baseline.
Supplemental Figure II-9: Mini-screen of Epigenetic Modifiers in DFCI81 and DFCI161 cells

A panel of histone post-transcriptional modifiers and epigenetic regulators were tested to assess whether they could be used to modulate EGFR expression levels, and thereby drug sensitivity. Compounds tested included the EZH2 histone methyltransferase inhibitors GSK126 and EPZ6438, the histone deacetylase inhibitors MGCD103, SAHA, and MS275, and the DNA methyltransferase inhibitor decitabine (162-165). Of these epigenetic regulators, only decitabine induced crizotinib resistance in both DFCI81 and DFCI161 cell lines. Additionally, decitabine sensitized DFCI161, but not DFCI81, cells to gefitinib (shown on next page). Quantitative confirmation of changes to EGFR and MET expression levels as potential modulators of these drug sensitivity switches is underway. All standard curves are plotted as mean +/- SEM.
Supplemental Figure II-9 (Continued)
Supplemental Figure II-10: Decitabine induces EGFR-mediated resistance to crizotinib in DFCI81 cells

Although decitabine did not sensitize DFCI81 cells to single-agent gefitinib treatment, as it did in the case of DFCI161 cells, it did induce crizotinib resistance relative to control. Moreover, this crizotinib resistance appeared to be mediated by a shift to oncogenic codependency on EGFR, because the crizotinib resistant DFCI81 cells in the presence of decitabine retained sensitivity to combination treatment gefitinib + crizotinib. Statistical significance was evaluated by one-way ANOVA test of variance.
Supplemental Figure II-11: Characterization of crizotinib resistant DFCI161 populations and clones

(a) The crizotinib resistant bulk populations, DFCI161CR Bulk A and DFCI161CR Bulk B exhibit resistance to single-agent treatment with gefitinib and crizotinib, but retain sensitivity to gefitinib + crizotinib combination treatment. This drug sensitivity profiles implies that the DFCI161CR Bulk populations have developed EGFR mediated crizotinib resistance (b) Western blot analysis reveals downregulation of both total and phosphorylated MET in crizotinib resistant, gefitinib sensitive DFCI161CR B4 and DFCI161CR B5 clones. Surprisingly, even while robustly crizotinib resistant, all of the resistant populations appear to retain MET dependent control over most downstream kinase signaling pathways.
Supplemental Figure II-12: Identification of alternative mechanisms of resistance development in DFCI81 cells

(a) DFCI81CR-Bulk A and DFCI81CR-Bulk B cells show resistance to crizotinib, gefitinib, and to combination treatment with crizotinib + gefitinib (b) Although DFCI81CR-Bulk A cells exhibit crizotinib resistance, the MET-downstream kinases remain inhibited by crizotinib treatment. Conversely, western blot analysis of DFCI81CR-Bulk B lysates shows reactivation of downstream kinases, likely driving survival in crizotinib (c) Phospho-RTK array reveals phosphorylation of Axl in the DFCI81CR-Bulk B cells in the presence of crizotinib. Because Axl is not phosphorylated in the parental cells control treated with crizotinib, it will be examined further as a potential driver of crizotinib resistance in this population of cells.
Appendix III: Supplemental Materials for Chapter 4

Supplemental Figure III-1: Media-associated modulation of drug sensitivity in EGFR mutant, MET-dependent cell lines

DFCI161 and DFCI81 cells cultured and assayed in RPMI-1650 media + 10% FBS (R10) are gefitinib tolerant and crizotinib resistant. On the other hand, DFCI161 and DFCI81 cells cultured in ACL-4 media + 10% FBS (A10) are gefitinib sensitive and crizotinib resistant.
Supplemental Table III-1: Media-dependent drug sensitivity profile appears to be unique to MET-dependent models

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<th>Cell Line</th>
<th>EGFR mutation</th>
<th>MET mutation</th>
<th>Gefitinib sensitivity</th>
<th>Crizotinib sensitivity</th>
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Supplemental Figure III-2: Dexamethasone-associated switches in drug sensitivity are transient and reversible

(a) Timeline of dexamethasone exposure and washout (b) Western blot analysis reveals that both total and phosphorylated MET levels revert to baseline following one week of dexamethasone washout (c) Reversion of MET phosphorylation levels to baseline coincides with reversion of cells from crizotinib resistance and gefitinib sensitivity in the presence of dexamethasone to crizotinib sensitivity and gefitinib resistance in its absence.
Materials and Methods

Establishment and Maintenance of Primary Cell Lines: Patient-derived pleural effusions were processed to isolate and propagate cancer cells as follows: after receiving samples, effusions were cleared with red blood cell lysis buffer, spun down, and sorted for EPCAM to separate cancer cells from tumor-associated fibroblasts. Cells were then plated into flasks and cultured in either RPMI-1640 or ACL-4 media, supplemented with 10% FBS. Cells were continually split once confluent. As patient-derived cell lines derived from these effusions and biopsies already harbor oncogenic drivers, there is no need to transform them with ectopic oncogenic drivers such as SV-40 for immortalized growth ex vivo.

Xenograft Studies: Xenograft models studied were generated either straight from patient specimens, of from cultured cell lines. In the case of patient-derived xenograft models, cancer specimens from either pleural effusions or biopsy specimens were purified and implanted subcutaneously into mice. These cells were exclusively passaged in immune compromised NSG mice and never exposed to cell culture. For cell line-derived xenograft studies, cells were dissociated and counted, and kept on ice until implantation. 10 million cells were transplanted subcutaneously into each NSG mouse and monitored closely. Changes in the body weights of mice were closely monitored and documented to ensure tolerability of targeted inhibitor treatment regimens (data not shown). At collection, mice were euthanized, and tumors were dissected from the transplant site and flash frozen for western blot and RNA analysis, unless otherwise specified.
CRISPR/Cas9 Genome Editing: Cas9 targeting crRNAs to target *EGFR* exon 20 were designed using the MIT CRISPR design tool (121). Homologous repair template was supplied as a short single stranded piece of linear DNA. Silent mutations were incorporated into the donor strand where possible to protect the template from re-cleavage by Cas9 following integration, and to assess editing efficiency using exome sequencing. To minimize the effects of codon bias on translational efficiency, silent mutations incorporated within the donor template were either natural variants, or correspond to codons encoding tRNAs found at comparable frequency in the cell as those encoded by the endogenous codon. Targeting constructs and donor constructs were electroporated into cells alongside nuclear-targeted recombinant active Cas9 protein. Following gene editing, engineered cell lines were selected using appropriate EGFR TKIs, beginning 72 hours after nucleofection. Colonies were picked by hand under a light microscope and expanded in separate wells, under continual EGFR TKI drug selection.

Lentiviral Infection: Retroviral and lentiviral particles containing constructs of interest were produced in HEK293T cells. Lentiviral supernatant was collected and exposed to recipient cells overnight before removal of media. Cells were initiated on antibiotic (puromycin) treatment to select for successfully infected cells. Media changed every 48-72 hours, and cells were passaged remove residual viral particles prior to use. Post-infection, cells were maintained in culture under continual puromycin selection, except during experimentation.

Selection of Crizotinib Resistant Cells: Cells were seeded sparsely at 100,000 cells/plate in 15-cm² dishes and allowed to attach overnight. The following day, cells were treated with 100 nM crizotinib. Cells were continually incubated in the presence of 100 nM crizotinib with weekly
media changes until colonies were observable. Once colonies were visible, they were picked by hand under a light microscope, and perpetuated separately under constant drug selection. The remaining cells that were not picked as colonies continued to be cultivated and selected as the “Bulk” population models of drug resistance. As cells developed resistance to 100 nM crizotinib, their drug dose was escalated step-wise: first to 250 nM, then 500 nM, then finally to 1 μM. Once cells were able to grow unhindered in 1 μM of crizotinib, they were deemed ready to be used in experiments to identify mechanisms of drug resistance. While all crizotinib resistant populations and clones continued to be cultured in constant drug selection, a minimum of a one-week washout was imposed prior to any experimentation to ensure that residual crizotinib in culture media would not confound experimental observations.

**MTS Assay:** MTS assays are used to measure NAD(P)H production by cells as a proxy for cell survival (166). Cells were plated at 3,000-5,000 cells/well, depending on the cell type, in 96-well plates and treated the following day with inhibitor dose response curves ranging from 0.001-10 μM (unless otherwise specified), with n=6 replicates per treatment per dose. Replicates were subsequently used to calculate IC$_{50}$ values across different cell lines, inhibitors, and culture conditions.

**Western Blot:** Cells were plated at 500,000 cells per plate and allowed to attach overnight. The following day, they were treated as described in the results sections. Lysates were collected in RIPA buffer containing phosphatase and protease inhibitors, and stored at -81°C until used. All antibodies used for western blot were purchased from Cell Signaling Technologies, except from tubulin, which was from Sigma, and HSP90, which came from Santa Cruz. All phosphorylated
EGFR blots were probed with pY1068 residue-specific antibody, phosphorylated MET was probed for pY1243/5, phosphorylated Akt blots were probed with pS423, and phosphorylated ERK1/2 bolts were probed for pT202/Y204, unless otherwise noted.

**Immunoprecipitation:** 1.5 – 2 million cells were seeded per 15 cm² plate, often with two 15 cm² plates per experimental condition. Cells were treated the following day, and lysed 16-20 hours after treatment in NP-40 buffer with phosphatase and protease inhibitors. 500 µg – 1 mg of protein per pulldown in 600 µL to 1 mL of NP-40 and incubated overnight with primary antibodies. Antibody-protein-protein complexes were isolated using Protein A/G coated agarose beads and resuspended in sample buffer for western blot analysis.

**Polymerase Chain Reaction:** Cells were lysed, and RNA was collected using phenol chloroform extraction. Following collection, cDNA was generated using the SuperScript III reagent. Real Time RT-PCR was performed using gene-specific Taqman Probes. Fold-changes were calculated using the ddCT method.

**IncuCyte Assays:** Cells were seeded at 5,000 cells per well in clear bottom 96-well plates. After being allowed to attach overnight, they were treated the following day as described. Caspase Glo reagent was added to cells and plates were incubated in the IncuCyte for imaging. Imaging was conducted immediately, and plates were imaged every 2 hours for the duration of the experiment. Caspase values were quantified and normalized to confluency.
Statistical Analysis: All error bars are representative of standard deviation within experiments, unless otherwise noted. Statistical significance was determined using one-way and two-way ANOVA tests, as appropriate. Although experiments were repeated at least three separate times (with some replicate studies underway), data was presented as one representative result, unless otherwise noted.
References:


with erlotinib and afatinib in a patient with EGFR-mutated non-small cell lung cancer (NSCLC) using sequential [18F]fluorothymidine (FLT-)PET. Lung cancer 77, 617-620


Inhibitors. Clinical cancer research : an official journal of the American Association for Cancer Research 21, 3913-3923


