



Genetic and Chemical Modifiers of EGFR Dependence in Non-Small Cell Lung Cancer

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Genetic and Chemical Modifiers of EGFR Dependence in Non-Small Cell Lung Cancer

A dissertation presented

by

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to

The Division of Medical Sciences

in partial fulfillment of the requirements

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Genetic and Chemical Modifiers of EGFR Dependence in Non-Small Cell Lung Cancer**ABSTRACT**

The term ‘oncogene addiction’ has been used to describe the phenomenon whereby tumor cells exhibit singular reliance on an oncogene or oncogenic pathway for their survival, despite the accumulation of multiple genetic lesions. In non-small cell lung cancer (NSCLC), this principle is perhaps best exemplified with the finding that epidermal growth factor receptor (*EGFR*) mutations predict response to EGFR-targeted therapies and thus represent a dependency in the subset of tumors harboring these alterations. Yet while *EGFR*-mutant tumors often respond dramatically to EGFR inhibition, nearly 30% of cases are refractory to therapy at the outset, and all responsive patients ultimately develop resistance to therapy. A deeper understanding of the genetic underpinnings of EGFR dependence, and of the mechanisms by which *EGFR*-mutant cells can overcome addiction to EGFR, may improve clinical outcomes.

In this work, we have applied systematic functional screening approaches to identify modifiers of EGFR dependence in NSCLC. Recent advances in large-scale functional screening libraries have made it possible to query a wide-range of genetic or chemical perturbations for their ability to modulate specific cellular phenotypes. Using the model of *EGFR*-mutant, erlotinib-sensitive NSCLC cells, we have performed systematic open reading frame (ORF)- and shRNA-based screens to identify genetic perturbations that can complement loss of EGFR in an EGFR-dependent context. Additionally, by integrating screening findings with an unbiased, gene-expression-based approach, we have identified chemical compounds that can modulate the degree to which cells rely on EGFR. Our findings indicate broad potential for EGFR substitution

in the setting of EGFR dependence, with compensatory mechanisms commonly conferring EGFR-independent activation of the phosphoinositide 3-kinase (PI3K)-AKT and MEK-ERK signaling pathways. These data support the idea that the EGFR-addicted state can be redundantly driven by diverse genetic inputs that commonly converge on shared downstream signaling nodes.

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For Mom, Dad, and Torfay

In memory of Dereck Chiu

INTRODUCTION

EGFR Dependence in Non-Small Cell Lung Cancer

Summary

Lung cancer is a wide-ranging term that encompasses several heterogeneous malignancies of the lung. While lung cancer was once treated as a single disease entity, the observation that lung tumors of distinct subtypes could display differential response to therapy has precipitated a refined classification of this disease and its treatment. It is now understood that specific genetic alterations can drive the growth and survival of these tumors, and in turn, render them highly susceptible to specific targeted therapies. In this manner, mutations in the epidermal growth factor receptor (*EGFR*) drive a subset of non-small cell lung cancers (NSCLCs) and predict responsiveness to EGFR-tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib. Indeed, *EGFR*-mutant NSCLCs exemplify the phenomenon of 'oncogene addiction' in their singular reliance on EGFR for activation of critical downstream signaling pathways and exquisite sensitivity to EGFR inhibition.

Yet while *EGFR*-mutant NSCLCs often respond dramatically to EGFR inhibitors, the development of resistance to EGFR-TKIs following an initial response (acquired resistance), or the failure to respond to begin with (primary resistance), confound clinical benefit. Several mechanisms of primary and acquired resistance to EGFR-TKIs have been identified, and these can be broadly categorized as either intrinsic or extrinsic to EGFR. In the latter case, primary and acquired resistance to therapy can be attributed to the existence of key genetic modifiers that can regulate the degree to which these tumors rely on EGFR. A deeper understanding of which genes or pathways are able to reduce dependency on EGFR, as well as which genes or pathways are required to maintain the oncogene-addicted state, may reveal new therapeutic opportunities in these cancers and will be the subject of this work.

Lung cancer classification and treatment paradigms

Overview

Lung cancer is the leading cause of death from cancer in the United States and across the world, estimated to cause over 160,000 deaths in the U.S. annually [1] and over 1.3 million deaths per year worldwide [2]. Cancers of the lung have been estimated to account for 28% of all cancer-related deaths in the United States in 2012, despite representing only 14% of new cancer diagnoses. Further illustrating the lethality of this disease, the 5-year survival rate for patients diagnosed with lung cancer is only 16%, considerably lower than the survival rates for cancers of the breast, colon, and prostate [1].

The high mortality rate of lung cancer is attributable to several factors, including the high incidence of the disease and the typically advanced stage at diagnosis: approximately two-thirds of patients present with locally advanced or metastatic disease [3]. Moreover, until recently, this clinically, histologically, and molecularly heterogeneous disease was treated as a single malignancy, with treatment based predominantly on clinical stage [4]. The recognition that lung cancer could be sub-stratified based on histological and molecular criteria, and that lung tumors of distinct subtypes could display differential response to therapy, has extended overall survival and improved response rates of patients with advanced disease [5,6,7]. Continued refinement of the distinct disease subsets represented within this cancer type holds promise in revealing new therapeutic opportunities or improving patient selection methods for specific therapies.

Histological classification of lung cancer

Lung cancer is a wide-ranging term that encompasses several heterogeneous malignancies of the lung, but can be broadly classified into small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the latter of which accounts for approximately 87% of lung cancer cases [8]. These two types of lung cancer are usually distinguished according to clinical, histological, and neuroendocrine features [9,10]. NSCLC can be further divided by histological criteria into three major subtypes: adenocarcinoma, squamous cell carcinoma, or large-cell carcinoma [9].

Adenocarcinomas are the most common subtype of NSCLC, accounting for 41% of all NSCLC cases [11]. These tumors typically arise peripherally, and can be further subdivided based on histological criteria into acinar, papillary, broncholoalveolar, solid adenocarcinoma with mucin formation, or mixed subtypes [12].

Conventional treatments for non-small cell lung cancer

Treatment for NSCLC varies by tumor type and stage, and may include surgery, radiation therapy, chemotherapy, or a combination of modalities. Surgical resection is the standard approach for patients with stage I and II NSCLC [13], while stage III cancers are often treated with combined chemoradiotherapy, with or without surgery [14]. For stage IV patients, palliation of symptoms and prolongation of survival are primary goals, and treatment may consist of chemotherapy, palliative radiotherapy, or targeted therapy [15,16,17].

For patients with advanced NSCLC, cytotoxic chemotherapy regimens commonly consist of treatment with a platinum-based doublet (e.g. carboplatin plus paclitaxel) [16]. A randomized clinical trial comparing four platinum-based doublet regimens (cisplatin-paclitaxel, cisplatin-gemcitabine, cisplatin-docetaxel, carboplatin-paclitaxel) reported median survival values of 7-8 months under each of the four regimens [18]. It has been reported that the addition of a third agent, the vascular endothelial growth factor (VEGF)-directed monoclonal antibody bevacizumab, can provide an overall survival benefit in non-squamous-cell NSCLC patients treated with carboplatin and paclitaxel, extending the median overall survival of patients to 12.3 months as compared to 10.3 months in the chemotherapy-alone group [19].

More recently, a phase III study has demonstrated that use of the antifolate pemetrexed in combination with cisplatin is superior to cisplatin plus gemcitabine in NSCLC patients with adenocarcinoma and large-cell carcinoma histology [5]. Intriguingly, pemetrexed-cisplatin treatment was predictive of poor outcome in patients with squamous cell histology in this study [5]. The observation that histology can be a predictive marker of treatment response has been

reported with other chemotherapy regimens in advanced NSCLC [6], and is now understood to be a critical component in treatment selection [16].

Oncogene addiction and targeted therapies

In recent years, further sub-classification of lung cancer based on molecular criteria has been precipitated by the understanding that specific genetic alterations can drive and maintain tumor growth, and that tumors harboring different driver mutations may display differential sensitivity to targeted therapies. The term 'oncogene addiction' has been used to describe the phenomenon whereby tumor cells exhibit singular reliance on an oncogene or oncogenic pathway for their survival, despite the accumulation of multiple genetic lesions [20] (Figure I-1). While the mechanisms underlying oncogene addiction are not fully understood, this principle provides a basis for pharmacologically targeting the required oncogene in these tumor cells and thereby achieving cancer-cell selectivity [21]. Indeed, oncogene addiction appears to underlie the observed clinical responses to BCR-ABL inhibition in chronic myeloid leukemias (CML) harboring *BCR-ABL* translocations [22]; HER2-targeted therapy in HER2-positive breast cancers [23]; KIT inhibition in KIT-positive gastrointestinal stromal tumors (GIST) [24], and mutant-BRAF inhibition in *BRAF*-mutant melanoma [25], among other examples. In lung cancer, the most extensively-studied example of oncogene addiction is that of EGFR dependence in *EGFR*-mutant NSCLC [26,27,28,29]. More recently, *ALK* rearrangements have been demonstrated to represent another tumor dependency mechanism in NSCLC [30].

Molecular classification of lung cancer

Alongside these findings, genome characterization efforts over the last several years have dramatically refined our understanding of the genetic changes that drive and maintain lung tumorigenesis. Since 1987, when recurrent driver mutations in *KRAS* were first identified in NSCLC [31], this disease has been increasingly subdivided on the basis of specific molecular

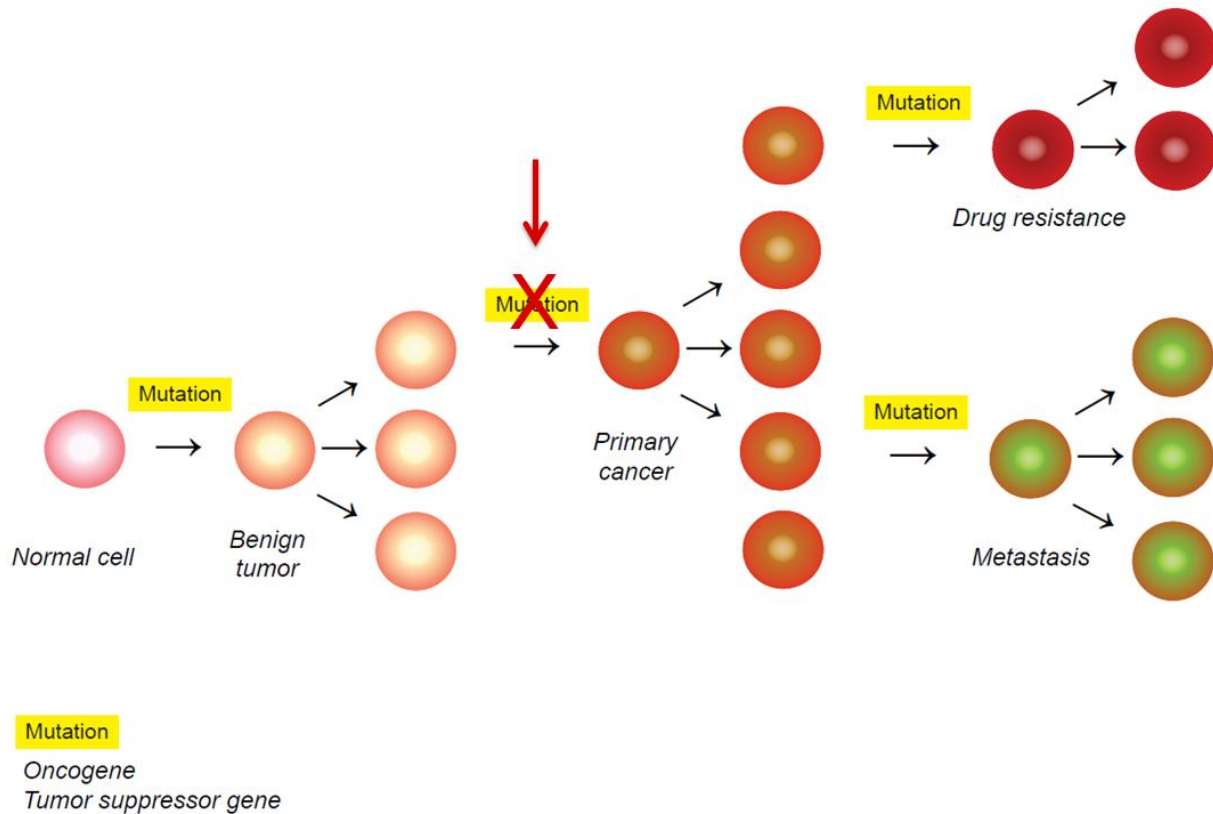


Figure I-1. The phenomenon of oncogene addiction. Cancers arise through a multistage process, in which a normal cell develops into a benign tumor, then a primary cancer, and ultimately an invasive metastasis (and possibly other subpopulation[s]), through the progressive accumulation of genetic mutations. These mutations can lead to the activation of oncogenes or the inactivation of tumor suppressor genes [32,33]. In oncogene-addicted cancers, tumors exhibit dependence not on the totality of these mutations, but rather on a single mutant protein (red arrow) for their growth and survival [20,21]. Inactivation of such a driver oncogene (red cross) can achieve tumor cell death and thus represents a therapeutic opportunity in these cancers. Figure adapted from [33].

criteria [7]. Examples of driver alterations identified in NSCLC within approximately the last decade include the aforementioned *EGFR* mutations [34,35,36] and *EML4-ALK* fusions [37], as well as *HER2 (ERBB2)* mutations [38,39,40], *BRAF* mutations [41,42,43], *PIK3CA* mutations [44,45], *AKT1* mutations [46], *MEK1* mutations [47], *NRAS* mutations [48,49], and translocations involving *ROS1* [50,51,52,53] or *RET* [53,54,55,56]. Recently, a study of 183 lung adenocarcinomas has also identified recurrent somatic mutations in the splicing factor gene *U2AF1* and truncating mutations in *RBM10* and *ARID1A* [57], though the functional consequence of these mutations has not yet been determined. In addition to mutations and translocations, copy number gains are also typical in lung adenocarcinomas, frequently occurring in *TERT*, *MYC*, *EGFR*, and *NKX2-1* [57,58].

Lung adenocarcinomas can also be annotated on the basis of loss-of-function mutations and deletions, though at present these alterations are less likely to be therapeutically tractable. Mutations in the *TP53* tumor suppressor, for example, are highly prevalent in lung adenocarcinoma, occurring at a frequency of approximately 50% [48,57,59]. Other tumor suppressor genes with loss-of-function mutations in lung adenocarcinoma include *STK11*, *RB1*, *NF1*, *ATM*, *CDKN2A*, *SMARCA4*, and *KEAP1* [48,57,60,61,62]. Copy number deletions are commonly observed in *TP53* and *CDKN2A*, among other genes [57,58].

Importantly, a large percentage of lung adenocarcinomas do not harbor a known oncogenic driver alteration [63], indicating that our understanding of the genetic underpinnings of lung adenocarcinoma is likely incomplete. It is possible that larger patient cohorts may be required to assign significance to low-frequency alterations [57], or that there exist driver alterations generated by mechanisms undetectable by current genome characterization methods.

EGFR oncogene addiction in non-small cell lung cancer

Perhaps the most well-studied driver alteration in NSCLC is mutation of the epidermal growth factor receptor (*EGFR*) gene. Activating kinase domain mutations in *EGFR* are present in 10-15% of Caucasian and 30-40% of Asian NSCLC patients erlotinib [34,35,36,64,65], and these alterations strongly correlate with clinical response to the EGFR-tyrosine kinase inhibitors gefitinib and erlotinib [26,27,28,29].

The ErbB family of receptor tyrosine kinases

EGFR (also known as ERBB1/HER1) belongs to the ErbB family of receptor tyrosine kinases, of which ERBB2/HER2, ERBB3/HER3, and ERBB4/HER4 are also members. Each of these ErbB receptors is structurally comprised of an extracellular ligand-binding domain, a single-pass transmembrane domain, and a cytoplasmic tyrosine kinase domain. Activation of these receptors, which under normal conditions are inactive and monomeric, can be mediated by binding of their ligands, members of the EGF-family of ligands, to the extracellular region of the receptor (reviewed in [66,67]). It is worth noting that ERBB3 lacks intrinsic kinase activity [68], and a soluble ligand for ERBB2 has not been identified [69]. Upon ligand-binding, these receptors undergo conformational changes that promote homo- and heterodimerization, leading to intrinsic kinase domain activation and autophosphorylation of key tyrosine residues in the carboxy-terminus. These phosphorylated tyrosine residues can then serve as docking sites for proteins containing Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains, leading to the activation of intracellular signaling pathways [66,67,70].

EGFR signaling and oncogene addiction

As described above, activating mutations in *EGFR* have been reported in a large subset of NSCLC patients cases [34,35,36,63,64], and the presence of these mutations predicts sensitivity to the EGFR-TKIs gefitinib and erlotinib [26,27,28,29]. In NSCLC, activating

mutations occur in exons 18-21 of *EGFR*, residing within the tyrosine kinase domain of the receptor [71]. The two most common types of *EGFR* activating mutations, together accounting for over 90% of cases, are an in-frame deletion in exon 19 (commonly deleting amino-acid residues 746-750), and a leucine-to-arginine substitution in exon 21 (L858R), though several other kinase domain mutations have been associated with inhibitor sensitivity, including substitutions of the glycine residue at position 719 [27,29,71].

On a structural level, it is thought that *EGFR* mutations alter the catalytic activity of EGFR by destabilizing the inactive conformation of the kinase [72]. Indeed, the crystal structure of the L858R *EGFR* mutant reveals a conformation very similar to the wild-type kinase in the activated conformation, but it appears that the substitution of a bulkier, charged arginine residue for leucine renders this mutation incompatible with the kinase's inactive conformation [72]. Locking the kinase in a constitutively active state is thought to explain the approximately 50-fold increase in catalytic activity observed for the mutant versus wild-type kinase [72]. Compared to the wild-type kinase, mutant *EGFR* can confer ligand-independent activation of EGFR and is transforming in both *in vitro* and *in vivo* systems [73,74].

EGFR exerts pro-survival and pro-proliferative effects predominantly via signaling through the phosphoinositide 3-kinase (PI3K)-AKT and RAS-RAF-MEK-ERK pathways, though Src kinase, PLC γ -PKC, and STAT signal transduction pathways are also known to be targets of activation [70,75]. In *EGFR*-mutant tumors, downstream signaling pathways are under the singular regulation of EGFR, rendering these cells dependent or 'addicted' to EGFR for their growth and survival [76,77]. When EGFR is inhibited in these mutant cells, critical downstream pathways, including those of AKT, STAT, and ERK, are suppressed and cells undergo apoptosis [76,77,78,79]. EGFR-TKI-mediated cell death in *EGFR*-mutant cells is induced the intrinsic, or mitochondrial, apoptotic pathway and depends on the pro-apoptotic BCL2 family member BIM, which is regulated by ERK signaling [80,81,82].

Despite these advances, the mechanisms underlying EGFR oncogene addiction are at present not fully understood. One model that has been proposed to explain the phenomenon of oncogene addiction, termed ‘oncogenic shock’, suggests that differential signal attenuation between more transient pro-survival signals and more lagging pro-apoptotic signals following oncogene inactivation permits a crucial time window for apoptosis in these cells [71,83]. Importantly, the expression of *EGFR* mutations in transfected cells is not sufficient to render cells dependent on EGFR signaling for survival [71], underscoring the role of cellular context in supporting the *EGFR*-addicted state.

Resistance to EGFR-targeted therapies

Despite the sometimes dramatic clinical responses of *EGFR*-mutant tumors to gefitinib or erlotinib, clinical responses are not universal, with the rate of objective response estimated to be approximately 71% [26,29]. Moreover, even patients who initially respond to EGFR inhibitors almost invariably acquire resistance to therapy [84]. The development of resistance to EGFR-TKIs following an initial response (acquired resistance), or the failure to respond to begin with (primary resistance), continue to be significant clinical problems and are active areas of research.

Primary resistance

Several mechanisms are known to mediate primary resistance to EGFR-TKIs, and these mechanisms can be either intrinsic or extrinsic to EGFR. Specific types of *EGFR* kinase domain mutations, such as in-frame duplication and/or insertion mutations in exon 20 of *EGFR*, are associated with reduced sensitivity to EGFR-TKIs [74,85]. It has been demonstrated that insertion mutations in exon 20, for example, can induce oncogenic transformation but require 100-fold higher concentrations of erlotinib to inhibit this phenotype than required for the L858R

or exon 19 deletion mutants [74]. Moreover, the T790M mutation in exon 20 (described below), though typically associated with acquired resistance, has been reported in drug-naïve patients with co-occurring sensitizing *EGFR* mutations [86,87] and corresponds to reduced progression-free survival in these patients following EGFR-TKI therapy [87].

Primary resistance to therapy may also be influenced by genetic factors extrinsic to EGFR. It has been suggested that activation of the insulin-like growth factor 1 receptor (IGF-1R) signaling pathway, for example, may contribute to the emergence of EGFR-TKI-tolerant cells in an otherwise drug-sensitive population [88]. Co-treatment of *EGFR*-mutant cells with an EGFR-TKI and an IGF-1R inhibitor is sufficient to prevent the development of these drug-tolerant cells [88]. Loss of phosphatase and tensin homolog (*PTEN*) has also been described as a mechanism underlying reduced sensitivity of *EGFR*-mutant cells to EGFR-TKIs [89], possibly through impaired PTEN-mediated ubiquitylation and degradation of EGFR [90]. Seemingly contradictory to these findings, a large study conducted in NSCLC patients treated with gefitinib did not find expression levels of IGF-1R or loss of PTEN to be associated with primary resistance to therapy [91], though this study was performed in an unselected patient population and thus may not reflect response rates when controlling for *EGFR* mutation status. More recently, it has been observed that lower BIM expression in treatment-naïve patients with *EGFR*-mutant lung cancers predicts worse progression-free survival following EGFR-TKI treatment [92]. Thus BIM expression may play a role in the observed heterogeneity of treatment response in *EGFR*-mutant lung cancers [92].

Acquired resistance

Secondary mutations in EGFR

As previously mentioned, among patients who initially respond to EGFR-TKIs, the development of acquired resistance to therapy is universal, with a median time to progression of

approximately 12 months [93]. In approximately 50% of acquired resistance cases, EGFR-TKI insensitivity can be attributed to the development of a secondary kinase domain mutation in *EGFR* itself, leading to the substitution of a methionine for a threonine at position 790 (T790M) [93,94,95,96]. Also termed the 'gatekeeper' mutation, T790M is analogous to resistance-associated mutations in *BCR-ABL* (T315I), *PDGFRA* (T6741), and *KIT* (T6701) following imatinib treatment [95,97,98,99]. While it was initially surmised that T790M sterically hinders drug binding, as occurs with T315I mutations in *BCR-ABL*, it now appears that the mutation reduces drug sensitivity by increasing the receptor's affinity for its natural substrate ATP [97,100]. Other studies have also shown that the presence of T790M *in cis* with a canonical activating mutation enhances the kinase activity and transforming potential of EGFR relative to either mutation alone [73].

Because *EGFR*-mutant tumors harboring the T790M mutation still depend on EGFR, the use of second- and third-generation EGFR inhibitors that retain activity against T790M have been pursued in these cancers. Second-generation, irreversible, quinazoline-based EGFR inhibitors afatinib and dacomitinib, for example, are effective in overcoming T790M-mediated resistance in preclinical models, presumably due to their covalent binding and thus greater ATP-binding site occupancy [100,101,102]. Third-generation, irreversible, pyrimidine-based EGFR inhibitors, such as WZ4002, have also been developed to increase potency and selectivity for T790M-positive EGFR [103]. Clinical trials assessing whether the efficacy of several irreversible EGFR inhibitors translates in patients are ongoing [104].

Other secondary *EGFR* mutations, such as D761Y, L747S, and T854A, have also been associated with acquired EGFR-TKI resistance, although these mutations occur very rarely in patients [105,106,107]. These secondary *EGFR* mutations also appear to reduce drug sensitivity much more modestly than T790M in *in vitro* assays [105,108,109].

Bypass mechanisms

Another common route by which *EGFR*-mutant tumors acquire resistance to EGFR-TKIs is through so-called 'bypass' mechanisms, in which activation of an alternative signaling effector can sustain signaling activation downstream of EGFR in an EGFR-independent fashion. The most well-studied bypass mechanism in *EGFR*-mutant NSCLC is amplification of the receptor tyrosine kinase *MET*, which accounts for approximately 20% of acquired resistance cases [110,111]. *MET* amplification is able to mediate resistance, in spite of EGFR inhibition, through persistent activation of PI3K in an ERBB3-dependent manner [110]. Elevated expression of the ligand for *MET*, hepatocyte growth factor or HGF, has also been identified as a mechanism of EGFR-TKI resistance [112]. Importantly, combined EGFR and *MET* inhibition can overcome *MET*-mediated EGFR-TKI resistance *in vitro* and *in vivo* [110,113].

More recent studies have implicated several additional bypass mechanisms in EGFR-inhibitor resistance. Mutations in *PIK3CA* have recently been reported in a small fraction (5%) of tumors with acquired resistance to EGFR-TKIs [93], providing clinical evidence for earlier *in vitro* studies demonstrating oncogenic mutant *PIK3CA* to be sufficient to confer resistance to gefitinib through sustained activation of PI3K signaling [114]. Another recent study reported increased expression of the receptor tyrosine kinase *AXL* in *in vitro* and *in vivo* *EGFR*-mutant lung cancer models with acquired resistance to erlotinib [115]. Clinical specimens resistant to EGFR-TKIs were found to harbor increased expression of *AXL* (20% of evaluable cases) and/or its ligand *GAS6* (25% of evaluable cases), with or without a co-occurring T790M mutation [115]. Furthermore, yet another potential bypass mechanism is amplification of the ErbB family member *HER2* (*ERBB2*), which has been observed in 12-13% of *EGFR*-mutant patient tumors with acquired resistance to EGFR-TKIs and can occur independently of the T790M secondary mutation [116,117]. Supporting the notion that these alterations could be causative in driving resistance, *HER2* overexpression is sufficient to induce resistance to erlotinib in an *in vitro*

context [116]. *In vitro* studies have also implicated overexpression of *CRKL*, which encodes an adaptor protein and is frequently amplified in NSCLC, as sufficient to induce resistance to gefitinib [118]. *CRKL* mediates gefitinib resistance through persistent MAPK and AKT signaling [118].

Bypass mechanisms have also been implicated in acquired resistance to newer, irreversible EGFR inhibitors. Ercan et al. have recently identified amplification of mitogen-activated protein kinase 1 (*MAPK1*) as a resistance mechanism to the third-generation EGFR inhibitor WZ4002 in models harboring *EGFR-T790M* [119]. Interestingly, in contrast to earlier work that had identified preferential amplification of the *EGFR-T790M* allele in *EGFR-T790M*-containing models with acquired resistance to dacomitinib [120], the findings with WZ4002 suggest that bypass mechanisms can occur secondarily to gatekeeper mutations in *EGFR* [119].

Collectively, these findings suggest that activation of alternative signaling proteins may represent a more general way in which cancers overcome dependence on a primary driver oncogene.

Related to this phenomenon of EGFR bypass, recent work has implicated FAS and members of the nuclear factor (NF)- κ B pathway as genetic modifiers of EGFR dependence in *EGFR*-mutant cells [121]. Bivona and colleagues have demonstrated that EGFR dependence is enhanced by downregulation of the FAS-NF- κ B pathway, NF- κ B activation confers EGFR-TKI resistance, and that high levels of NF- κ B activation (as measured by low expression of the NF- κ B inhibitor I κ B) predict worse progression-free and overall survival in *EGFR*-mutant NSCLC patients treated with an EGFR-TKI [121].

Histological transformation

Intriguingly, a recent study has reported histological changes in *EGFR*-mutant NSCLC tumors after the development of acquired resistance to EGFR-TKIs, potentially identifying a new class of resistance mechanism in this disease [93]. In analyzing pre-treatment and post-relapse NSCLC tumor specimens, Sequist et al. noted that some resistant cancers lacking known resistance mechanisms displayed morphological changes consistent with an epithelial-to-mesenchymal transition, which is associated with disease aggressiveness. Related to this notion of histological transformation, these authors also reported a surprising finding that 5 of 37 (14%) drug-resistant tumor biopsies had a diagnosis of SCLC at the time of TKI resistance, suggesting a histological transformation from NSCLC to SCLC during the course of TKI treatment. These SCLC specimens retained their original *EGFR* mutation and were in some cases responsive to SCLC chemotherapy [93]. These findings are consistent with other observations reporting *EGFR*-mutant, SCLC diagnoses after progression on EGFR-TKI therapy [122,123]. It is at present unclear if these histological changes reflect *bona fide* transformation from NSCLC to SCLC, or rather selection for pre-existing SCLC clones following EGFR-TKI treatment.

Context for the current work and thesis summary

EGFR mutations represent a clinically-relevant Achilles' heel in the subset of non-small cell lung cancers harboring these alterations. Typically, these tumors are singularly reliant on EGFR for their proliferation and survival and are thus responsive to EGFR-targeted therapies. Yet the phenomenon of EGFR oncogene addiction itself implies a unique cellular circuitry that may be exploited alongside EGFR. Moreover, primary and acquired resistance to therapy suggests the existence of key genetic modifiers that in some cases can regulate the degree to which these tumors rely on EGFR. The question of which genes or pathways are able to reduce

dependency on EGFR, as well as which genes or pathways are required to maintain the oncogene-addicted state— questions bearing important implications for the development of long-term therapeutic strategies and overcoming acquired drug resistance—will be the subject of this work.

In these studies, we have sought to identify modifiers of EGFR dependence in NSCLC by applying systematic functional screening approaches. The advent of large-scale functional screening libraries has made it possible to query a wide-range of genetic or chemical perturbagens for their ability to modulate specific cellular phenotypes. In Chapter 1, we describe an open reading frame (ORF)-based genetic complementation screen for loss of EGFR in an EGFR-dependent model. Our findings indicate broad potential for kinase substitution in the setting of EGFR dependence and nominate two genes that may modify EGFR dependence in the clinical setting. In Chapter 2, we integrate these screening findings with an unbiased, gene-expression-based connectivity approach to identify commonalities and differences among these EGFR bypass genes. We find that many EGFR bypass genes have shared transcriptional effects, commonly re-activate the PI3K-AKT and MEK-ERK pathways under EGFR inhibition, and can regain dependence on EGFR via PI3K-mTOR and MEK co-inhibition. Using this gene-expression-based approach, we also identify chemical compounds that can reduce dependence on EGFR. In Chapter 3, we describe a genome-scale shRNA-based screen to identify genes required for EGFR dependence. We identify numerous candidate genes for further investigation and present intersecting findings between loss- and gain-of-function approaches. Together, these studies aim to identify genetic and chemical modifiers of EGFR dependence as a means to gain insight into the molecular underpinnings of EGFR oncogene addiction.

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CHAPTER 1

Diverse Kinase Genes Can Induce EGFR-Inhibitor Resistance

Attributions:

This chapter is reprinted from a manuscript in preparation.

All experiments and analyses were performed by Tanaz Sharifnia except as follows:

Primary screening was performed by Tanaz Sharifnia, with assistance from Federica Piccioni and Mukta Bagul. Analyses represented in Figures 1-5A were performed and visualized by Andrew Cherniack and Marcin Imielinski. Analyses represented in Figures 1-5B, 1-6, and Table 1-2 were performed by Andrew Cherniack and Tanaz Sharifnia.

ABSTRACT

Epidermal growth factor receptor (*EGFR*) activating mutations in non-small cell lung cancer (NSCLC) can predict sensitivity to EGFR tyrosine kinase inhibitors (TKIs), but clinical benefit is limited by primary and acquired resistance. Activation of alternative driver kinases is a common route by which *EGFR*-mutant cancers develop resistance to EGFR-TKIs, but the full-range of kinases capable of mediating EGFR bypass has not been systematically studied. Using an open reading frame (ORF)-based screen, we identified 18 kinase and kinase-related genes whose expression promotes resistance to the EGFR-TKI erlotinib in *EGFR*-mutant lung cancer cells. Resistance-inducing genes identified include seven of nine Src-family kinases, *FGFR1*, *FGFR2*, *ITK*, *NTRK1*, *NTRK2*, *MOS*, *MST1R*, and *RAF1*. Pharmacological blockade of resistance-promoting kinases restores sensitivity to erlotinib. Genomic profiling of lung adenocarcinomas reveals significantly co-occurring alterations in *EGFR* and resistance-inducing genes *FGR* and *LCK*, suggesting a role for these genes in modifying dependence on EGFR in the clinical setting. These data uncover a broad spectrum of kinases capable of circumventing EGFR inhibition in the setting of EGFR dependence.

INTRODUCTION

Lung adenocarcinomas harboring activating mutations in the epidermal growth factor receptor (*EGFR*) represent a common molecular subset of non-small cell lung cancer cases [1,2,3,4,5], and the presence of these alterations strongly correlates with clinical response to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib [6,7,8,9]. Yet while most *EGFR* mutation-positive NSCLC patients experience marked tumor regression upon treatment with a TKI, clinical responses are not universal even within this genetically-defined cohort, with the rate of objective response estimated to be approximately 71% [6,9]. Furthermore, the overwhelming

majority of patients who initially respond to EGFR inhibitors ultimately acquire resistance to therapy [10].

In many instances, EGFR-TKI resistance can be attributed to the development of a secondary mutation in *EGFR* itself (T790M, or the 'gatekeeper' mutation), which occurs in approximately 50% of acquired resistance cases and reduces drug sensitivity by increasing the receptor's affinity for its natural substrate ATP [11,12,13,14]. In other cases, primary or acquired refractoriness to therapy may stem from the activity of key genetic modifiers that can reduce these cancers' dependence on EGFR [15]. Examples include amplification of the *MET* receptor tyrosine kinase (RTK) [16,17] or activation of MET via its ligand, hepatocyte growth factor or HGF [18], activation of the nuclear factor (NF)- κ B pathway [15], amplification of the *HER2* (*ERBB2*) RTK [19], amplification of the *CRKL* gene [20], and activation of the AXL kinase [21]. Notably, and consistent with these findings, MET bypass can be reciprocally achieved via EGFR activation in MET-dependent cells [22], and analogous examples of reciprocal kinase switching have been reported in other kinase-driven cancer models [23,24]. These and other findings suggest that compensatory kinase switching may be a more general way in which oncogene-dependent cancers overcome reliance on their primary driver kinase [23,25].

Importantly, there remain many cases of primary and acquired EGFR-TKI resistance in which the underlying mechanism(s) are not understood [14,26]. We hypothesized that knowledge of the spectrum of kinases capable of mediating EGFR-inhibitor resistance could potentially identify novel mechanisms of resistance as well as elucidate shared features of kinase-driven EGFR bypass.

Discovery and characterization of known EGFR-TKI resistance mechanisms have been enabled by various experimental approaches to studying drug resistance, including the creation of isogenic drug-sensitive and resistant cell line pairs via stepwise selection methods, *in vivo* studies using genetically engineered mouse models, and direct profiling of pre-treatment and post-relapse human tumor samples [10,27]. Identification of new RTK bypass genes has been

facilitated by the advent of large-scale functional screening libraries, which make it possible to query a wide-range of genetic events sufficient to drive resistance to a given therapeutic agent [28,29]. This study has applied an unbiased, systematic cDNA screening approach to identify the range of kinase and kinase-related genes that, when highly expressed, are able to circumvent EGFR inhibition and promote drug resistance in *EGFR*-mutant cells. This approach has simultaneously enabled the recovery of previously characterized, clinically-validated mechanisms of erlotinib resistance as well as the identification of novel mediators of EGFR bypass in *EGFR*-mutant NSCLC.

RESULTS

An ORF-based screen identifies 18 kinase and kinase-related genes whose expression is sufficient to confer resistance to EGFR inhibition

To identify genes that rescue EGFR tyrosine kinase inhibition in the setting of EGFR dependence, we performed an open reading frame (ORF) overexpression screen in an EGFR-dependent NSCLC cell line in the presence of erlotinib. A library of 589 open reading frames (ORFs) encoding human kinases and kinase-related proteins (Center for Cancer Systems Biology (CCSB)/Broad Institute Kinase ORF Collection) [28,29] was expressed via lentiviral transduction in the *EGFR*-mutant NSCLC cell line, PC9, which is sensitive to EGFR-TKIs with a half-maximal inhibitory concentration (IC_{50}) of approximately 30 nM [30]. ORF-expressing PC9 cells were treated with either 300 nM erlotinib, 3 μ M erlotinib, or DMSO for 72h before being assayed for cell viability. Experimental ORFs were screened alongside positive control *EGFR* ORFs, encoding the T790M gatekeeper mutation *in cis* with a canonical *EGFR* activating mutation (*EGFR*- Δ (E746-A750)-T790M and *EGFR*-L858R-T790M) [31], as well as ORFs encoding activating alleles of several mitogen-activated protein kinase (MAPK) family members.

Cell viability under screening conditions was very low; the median relative viability for all experimental ORFs was 12% at the 300 nM dose and 8% at the 3 μ M dose (Figure 1-1). Overexpression of nineteen ORFs, of the 589 tested, led to a significant increase in viability of erlotinib-treated PC9 cells, with viability of at least 39% in 300 nM erlotinib and at least 31% in 3 μ M erlotinib (Figure 1-1). The genes whose ectopic expression led to greater viability in the presence of erlotinib include genes previously implicated in driving EGFR-inhibitor resistance (*AXL*, *ERBB2*, *CRKL*) as well as genes and gene families that are newly identified candidate drivers of EGFR-inhibitor resistance in *EGFR*-mutant lung cancer cells, including eight of the nine Src-family kinase members, as well as *FGFR1* and *FGFR2*, *ITK*, *NTRK1* and *NTRK2*, *MOS*, *MST1R*, and *RAF1*. The 19 genes whose expression led to apparent erlotinib resistance in the primary screen were selected for validation and functional characterization.

cDNA vectors corresponding to the 19 candidate resistance genes were sequence-verified and confirmed to express protein by immunoblot (Figure 1-2A). PC9 cells ectopically expressing each of these ORFs were assayed for their sensitivity to erlotinib across multiple drug doses using 72h growth inhibition assays (Figure 1-2B). For comparison, the erlotinib-resistant *EGFR*-mutant positive controls *EGFR*- Δ (E746-A750)-T790M (henceforth referred to as *EGFR*-ex19del-T790M) and *EGFR*-L858R-T790M were tested in parallel. Ectopic expression of 18 of the 19 candidate resistance ORFs was capable of reducing sensitivity to erlotinib relative to cells transduced with an inert ORF (Figure 1-2B). This reduction in erlotinib sensitivity corresponded to a >2-fold to >400-fold shift in IC_{50} values (Table 1-1). One primary screening hit, *YES1*, failed to confer erlotinib resistance in our validation studies, leaving seven of nine Src-family kinase genes confirmed to confer the resistance phenotype (Figure 1-2B and Table 1-1); and notably, among ORFs selected for validation, this gene scored nearest to the cutoff we used to select hits (Figure 1-1).

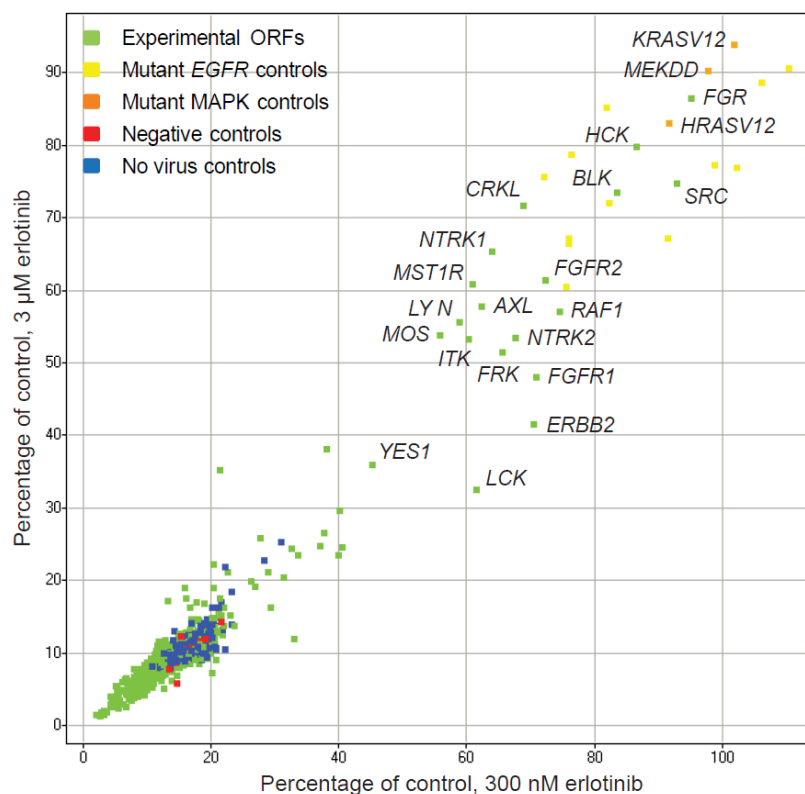


Figure 1-1. An ORF-based screen identifies 18 kinase and kinase-related genes sufficient to mediate resistance to EGFR inhibition. Screening results for PC9 cells transduced with a library of kinase ORFs, treated with 300 nM or 3 μM erlotinib or vehicle, then assayed for cell viability after 72h using CellTiter-Glo. Experimental ORFs (green points) were screened alongside *EGFR*-ex19del-T790M and *EGFR*-L858R-T790M positive controls (yellow points); activating alleles of the MAPK family (orange points); inert gene controls (red points); and no virus controls (blue points). Data are expressed as percent viability relative to vehicle. Nineteen candidate drivers of erlotinib resistance were selected for validation.

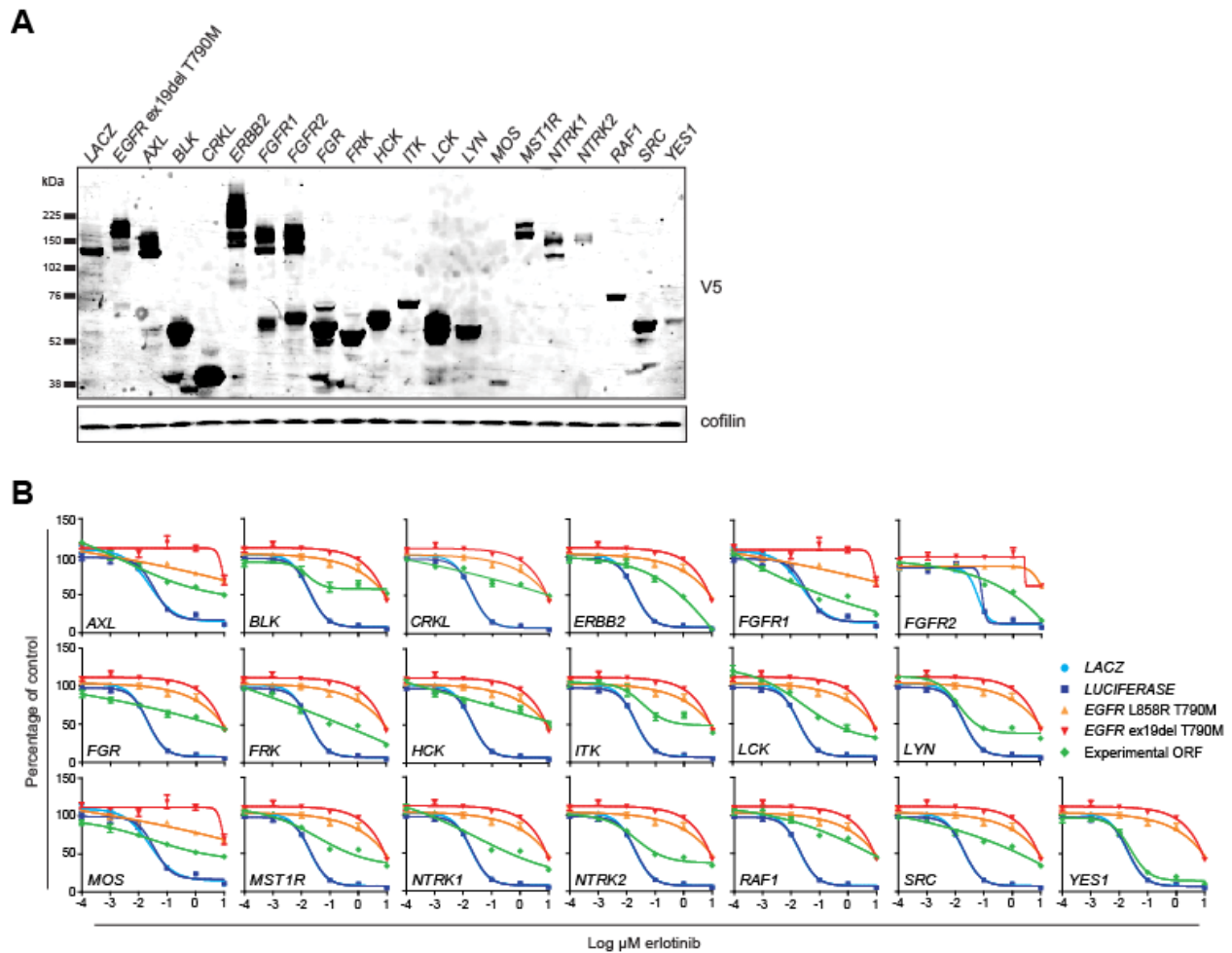


Figure 1-2. Validation of screening data. (A) Protein expression of ectopically-expressed ORFs. Immunoblot analysis of PC9 cells overexpressing candidate resistance-mediating ORFs and controls following treatment with DMSO for 6h. Cells were incubated with 0.5% serum media 18h before and during DMSO treatment. Total cell lysates were immunoblotted for ectopic protein expression using a V5-directed antibody. (B) PC9 cells expressing candidate resistance-inducing ORFs (green curve), *EGFR*-ex19del-T790M and *EGFR*-L858R-T790M positive controls (red and orange curves, respectively), and inert gene controls (light and dark blue curves) were treated with increasing concentrations of erlotinib and assayed for cell viability after 72h using CellTiter-Glo. Data are expressed as percent viability relative to vehicle-

Figure 1-2 (Continued)

treated cells and represent the mean \pm SD of 4 replicates. Graphs with identical control curves reflect experiments performed in parallel on the same day.

Table 1-1. IC₅₀ values of ORF-screen validation experiments. Absolute IC₅₀ values corresponding to validation experiments described in Figure 1-2B.

		IC ₅₀ value (μ M)				Experimental ORF
		LACZ	LUCIFERASE	EGFR L858R T790M	EGFR ex19del T790M	
Validation experiment	AXL	0.042	0.046	>10	>10	>10
	BLK	0.022	0.023	8.7	8.4	>10
	CRKL	0.022	0.023	8.7	8.4	>10
	ERBB2	0.022	0.023	8.7	8.4	0.79
	FGR	0.022	0.023	8.7	8.4	3.6
	FGFR1	0.042	0.046	>10	>10	0.19
	FGFR2	0.057	0.082	>10	>10	1.2
	FRK	0.022	0.023	8.7	8.4	0.29
	HCK	0.022	0.023	8.7	8.4	>10
	ITK	0.022	0.023	8.7	8.4	1.4
	LCK	0.022	0.023	8.7	8.4	0.34
	LYN	0.022	0.023	8.7	8.4	0.054
	MOS	0.042	0.046	>10	>10	2.3
	MST1R	0.022	0.023	8.7	8.4	0.53
	NTRK1	0.022	0.023	8.7	8.4	0.41
	NTRK2	0.022	0.023	8.7	8.4	0.11
	RAF1	0.022	0.023	8.7	8.4	6.2
	SRC	0.022	0.023	8.7	8.4	1.7
	YES1	0.022	0.023	8.7	8.4	0.031

Pharmacological blockade of resistance-promoting kinases restores sensitivity to erlotinib, and combination treatment is associated with downregulation of phospho-AKT

As both the screening set and the candidate erlotinib-resistance genes were enriched for human kinase genes, we sought to determine whether the observed resistance phenotypes were kinase-dependent, as well as to verify the specificity of the ORF-induced phenotype, by testing whether enzymatic inhibition of resistance-promoting kinases could restore sensitivity to erlotinib. To this end, PC9 cells transduced with kinase ORFs were treated with erlotinib alone, the relevant resistance-kinase inhibitor (where available) alone, or their combination for 72h, then assayed for cell viability with CellTiter-Glo (Figure 1-3 and Figure 1-4A). Inhibitors tested included dasatinib (targeting the Src-family kinases); XL880 (targeting AXL and MST1R); NVP-BGJ398 (hereafter referred to as BGJ398) (targeting FGFR1 and FGFR2); lestaurtinib (targeting NTRK1 and NTRK2); lapatinib (targeting ERBB2); and AZ628 (targeting RAF1).

We observed that cells expressing Src-family kinase genes could indeed be rendered sensitive to erlotinib when treated in combination with the Src-family kinase inhibitor dasatinib, whereas these cells were not similarly sensitive to either agent alone (Figure 1-3A and Figure 1-4A). In contrast, enhanced drug sensitivity under combined erlotinib/dasatinib treatment was not achieved in cells expressing *EGFR-ex19del-T790M*. Gene-specific rescue of erlotinib sensitivity was similarly observed for *AXL*, *MST1R*, *FGFR1* and *FGFR2*, *NTRK1* and *NTRK2*, *ERBB2*, and *RAF1* upon co-treatment with their respective inhibitors (Figure 1-3B-F and Figure 1-4A). Together, these data suggest that the resistant phenotype conferred by these ORFs is indeed specific to the encoded gene and requires the kinase activity of the expressed protein.

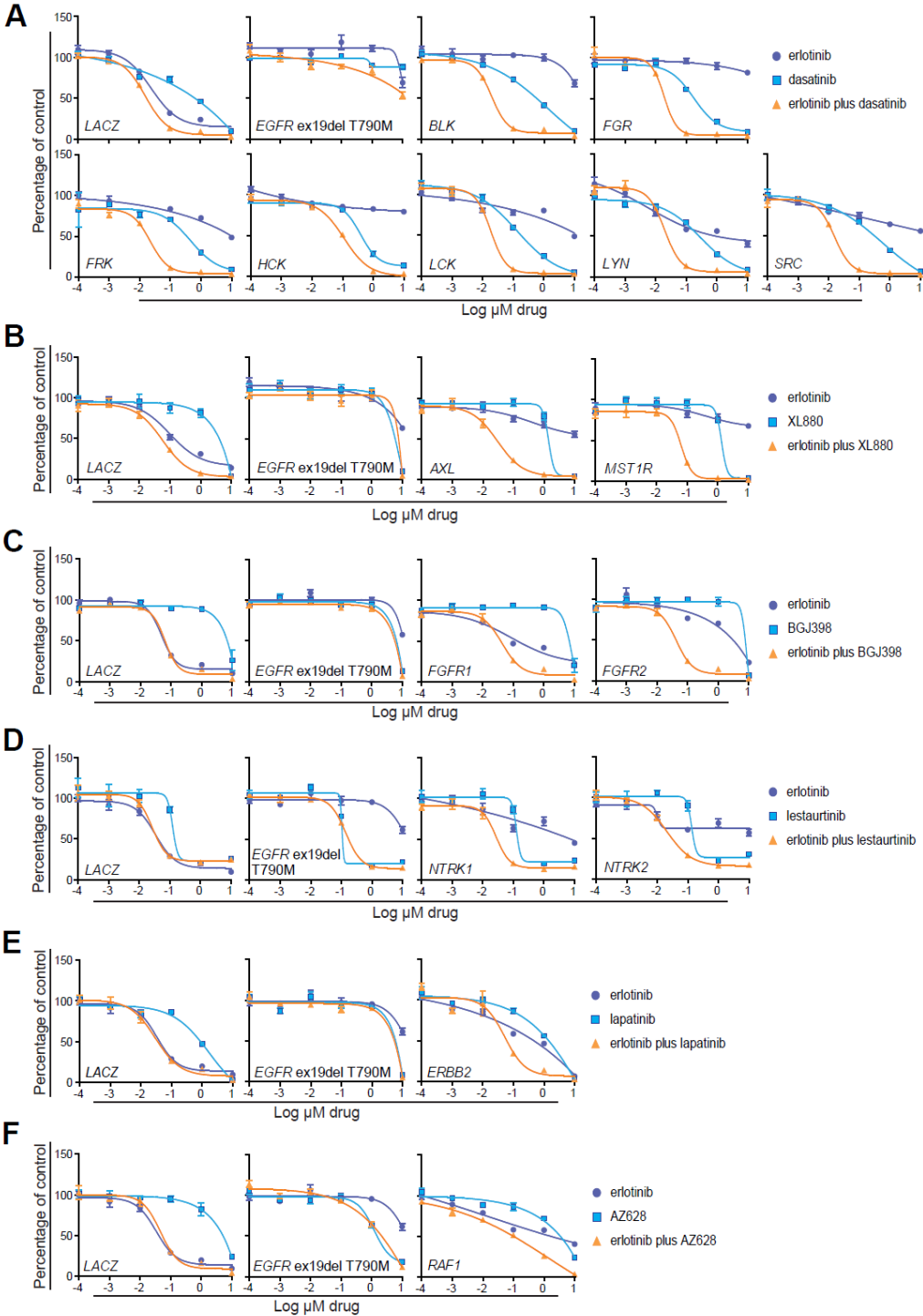


Figure 1-3. Pharmacological blockade of resistance-promoting kinases restores sensitivity to erlotinib. Cell viability of PC9 cells overexpressing indicated resistance-inducing kinases and controls following treatment with increasing concentrations of erlotinib (purple

Figure 1-3 (Continued)

curve), the relevant kinase inhibitor (light blue curve), or their combination (orange curve) for 72h. Cell viability was assayed with CellTiter-Glo. Data are expressed as percent viability relative to vehicle-treated cells and represent the mean \pm SD of ≥ 3 replicates. These dose-response curves were used to generate area under curve (AUC) values, plotted in Figure 1-4A. Kinase inhibitors tested included (A) dasatinib for Src-family kinases; (B) XL880 for AXL and MST1R; (C) BGJ398 for FGFR-family kinases; (D) lestaurtinib for NTKR-family kinases; (E) lapatinib for ERBB2; and (F) AZ628 for RAF1. Graphs with identical control curves reflect experiments performed in parallel on the same day.

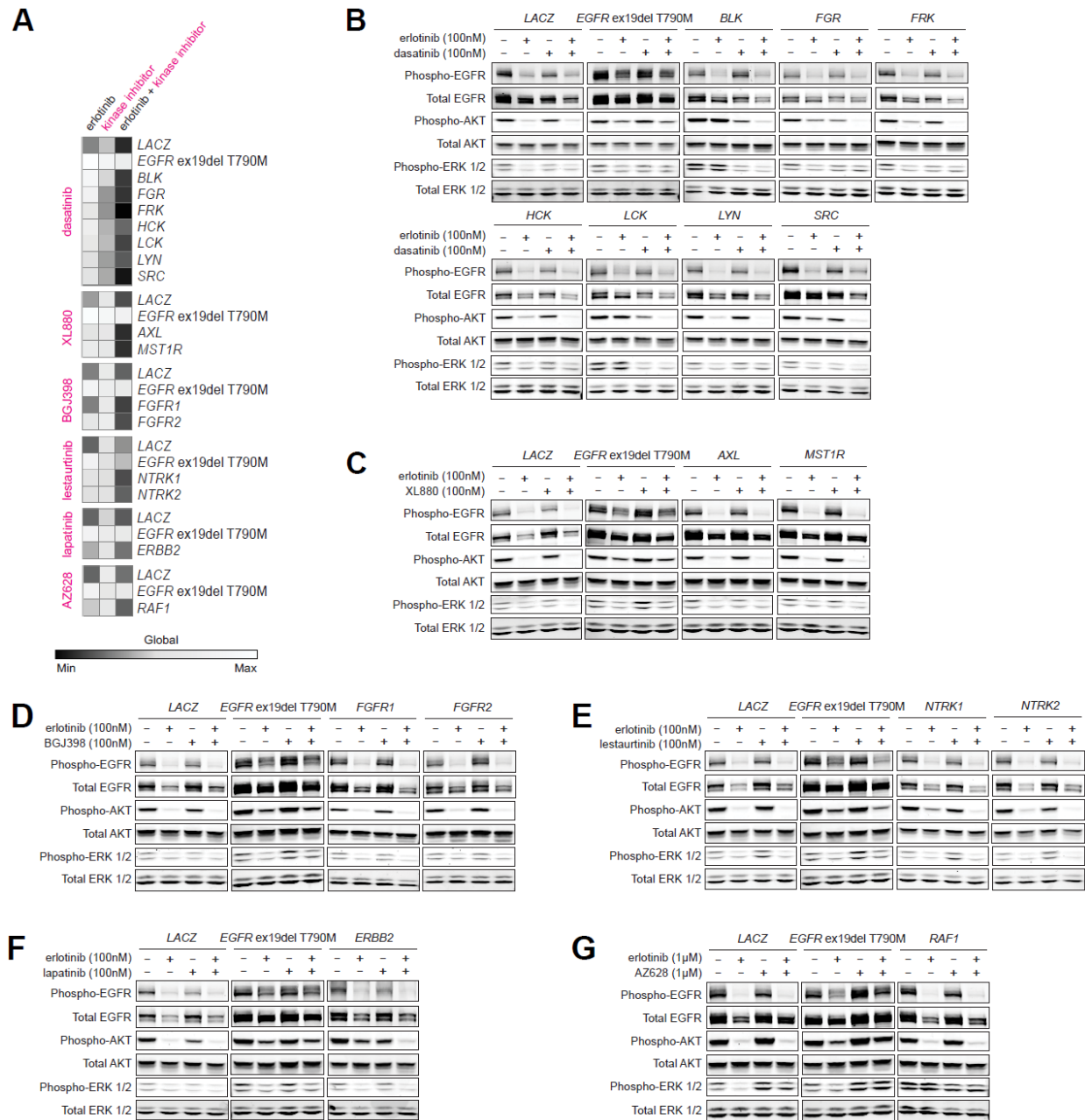


Figure 1-4. Pharmacological blockade of resistance-promoting kinases restores erlotinib sensitivity, and combination drug treatment is associated with downregulation of phospho-AKT. (A) Inhibitor sensitivity of PC9 cells expressing indicated resistance-inducing kinases and controls following treatment with multiple doses of erlotinib, the relevant kinase

Figure 1-4 (Continued)

inhibitor, or their combination for 72h. Cell viability was assayed with CellTiter-Glo, and the resultant dose-response curves (Figure 1-3) were used to generate area under curve (AUC) values, plotted. Darker/lighter squares represent smaller/larger AUC values. (B-G) Immunoblot analysis of PC9 cells expressing resistance-inducing kinases and controls under combination drug treatment. Transduced cells were treated with indicated doses of erlotinib, the relevant kinase inhibitor, or a combination for 6h. Cells were incubated with 0.5% serum media 18h before and during drug/DMSO treatment. Total cell lysates were immunoblotted for the indicated proteins. Kinase inhibitors tested included (B) dasatinib for Src-family kinases; (C) XL880 for AXL and MST1R; (D) BGJ398 for FGFR-family kinases; (E) lestaurtinib for NTKR-family kinases; (F) lapatinib for ERBB2; and (G) AZ628 for RAF1.

A few kinase inhibitors showed somewhat different patterns of growth-inhibition or rescue. We noted that lestaurtinib alone possesses some activity in *LACZ*- and *EGFR*-ex19del-T790M-transduced cells (Figure 1-3D and Figure 1-4A), though only *NTRK1*- and *NTRK2*-expressing cells exhibit enhanced sensitivity under combination treatment. We also observed that re-sensitization to erlotinib occurs only modestly in the case of *RAF1*-transduced cells treated with AZ628 (Figure 1-3F and Figure 1-4A), a finding that is consistent with previous work identifying overexpression of *RAF1* itself as a mechanism of resistance to RAF inhibition [29].

Next, since EGFR-TKI treatment in *EGFR*-mutant cells typically elicits downregulation of the phosphoinositide 3-kinase (PI3K)-AKT and MEK-ERK signaling pathways [2], we sought to determine whether the observed rescue of drug sensitivity using combination treatment versus monotherapy was associated with a distinct PI3K-AKT and/or MEK-ERK signaling profile. ORF-expressing cells were treated with erlotinib, the relevant kinase inhibitor, or their combination for 6h, and assayed by immunoblotting for activation of downstream signaling proteins (Figure 1-4B-G). As displayed in Figure 1-4B, *LACZ*-expressing cells undergo the expected downregulation of phospho-EGFR, phospho-AKT, and phospho-ERK1/2 in the presence of erlotinib, in contrast to *EGFR*-ex19del-T790M-expressing cells. We observed that cells transduced with Src-family kinases, however, maintain AKT phosphorylation in the presence of 100 nM erlotinib, and this effect is reversed only upon co-treatment with dasatinib. Selective loss of AKT phosphorylation under combination-treatment was similarly observed in cells transduced with *ERBB2*, *NTRK1* and *NTRK2*, and to a lesser extent with *FGFR1* and *FGFR2*, *AXL*, and *MST1R* (Figure 1-4C-F). Together these data suggest that AKT activation is associated with resistance in these cells, and raise the possibility that ORF-driven resistance may be mediated by the PI3K-AKT pathway. Further inquiry into these findings will be discussed in Chapter 2.

We did not observe selective abrogation of phospho-AKT levels in *RAF1*-expressing cells co-treated with erlotinib and AZ628 (Figure 1-4G), though as previously mentioned, this combination was also not sufficient to fully sensitize these cells (Figure 1-3F). We also noted that combined erlotinib/AZ628 treatment does not abolish phospho-ERK activation in these cells (Figure 1-4G), potentially explaining *RAF1*'s partial re-sensitization phenotype. Incidentally, treatment with AZ628 in *LACZ*- and *EGFR*-ex19del-T790M-expressing cells, but not *RAF1*-expressing cells, paradoxically induces activation of ERK (Figure 1-4G), a finding that has previously been observed with RAF inhibitors in other *RAF*^{WT} models [32,33].

Lung adenocarcinomas harboring *LCK* and *FGR* copy number gain are significantly more likely to possess concomitant *EGFR* mutations and amplifications

To evaluate whether *in vitro* erlotinib-resistance genes are altered in primary human tumors, we next queried somatic mutation and copy number alteration data from 230 lung adenocarcinoma cases, profiled as part of The Cancer Genome Atlas (TCGA), for the presence of alterations in genes identified in our ORF screen (Broad Institute TCGA Firehose, [34]). We hypothesized that alterations in these genes, if co-occurring with *EGFR* mutations, could drive *de novo* TKI resistance in patients with such tumors or represent pre-existing mechanisms of acquired resistance. In order to help distinguish tumors with *bona fide* *EGFR* dependency from those harboring 'passenger' mutations in *EGFR*, we defined '*EGFR* activating mutations' to include only those known or predicted to have an activating function (see Table 1-2, and Materials and Methods).

In comparing alterations in *EGFR* and our panel of resistance-promoting genes, we noticed a pattern of co-occurrence between alterations in *EGFR* and copy number gain of two resistance-inducing genes, *FGR* and *LCK* (Figure 1-5A, top panel), which reside less than 5 Mb

Table 1-2. EGFR mutations present in lung adenocarcinoma primary tumors and classification by predicted function. Non-silent somatic EGFR mutations present in (A) TCGA cohort of 230 patients and (B) validation cohort of 182 patients. Mutations were classified as ‘activating’ or ‘non-activating missense’ using criteria described in Materials and Methods.

A

Patient ID	EGFR mutation	Predicted function
TCGA-55-6980-01A-11D-1943-01	p.709_710ET>D	Activating
TCGA-69-7760-01A-11D-2166-01	p.709_710ET>D	Activating
TCGA-50-6673-01A-11D-1943-01	p.773_774insH	Activating
TCGA-75-6212-01A-11D-1752-01	p.ELR746del	Activating
TCGA-49-4501-01A-01D-1204-01	p.ELREA746del	Activating
TCGA-38-6178-01A-11D-1752-01	p.ELREA746del	Activating
TCGA-75-7025-01A-12D-1943-01	p.ELREA746del	Activating
TCGA-44-2661-01A-01D-1877-01	p.ELREA746del	Activating
TCGA-38-4628-01A-01D-1204-01	p.ELREA746del	Activating
TCGA-75-6207-01A-11D-1752-01	p.ELREA746del	Activating
TCGA-50-6595-01A-12D-1854-01	p.G719A, p.S768I	Activating
TCGA-55-7573-01A-11D-2035-01	p.K754E, p.ELR746del	Activating
TCGA-78-7147-01A-11D-2035-01	p.L387M, p.L861Q, p.D1083fs	Activating
TCGA-38-4627-01A-01D-1549-01	p.L62R, p.L858R	Activating
TCGA-05-5423-01A-01D-1624-01	p.L833F, p.L861Q	Activating
TCGA-67-6217-01A-11D-1752-01	p.L858R	Activating
TCGA-67-3772-01A-01D-0944-01	p.L858R	Activating
TCGA-64-1681-01A-11D-2062-01	p.L858R	Activating
TCGA-50-5944-01A-11D-1752-01	p.L858R	Activating
TCGA-49-4490-01A-21D-1854-01	p.L858R	Activating
TCGA-91-6835-01A-11D-1854-01	p.L858R	Activating
TCGA-91-6847-01A-11D-1943-01	p.L861Q	Activating
TCGA-55-6981-01A-11D-1943-01	p.LREAT747del	Activating
TCGA-78-7155-01A-11D-2035-01	p.Q432H, p.K754I, p.G901V	Activating
TCGA-44-6147-01A-11D-1752-01	p.S768I, p.V769L	Activating
TCGA-49-4494-01A-01D-1204-01	p.T790M, p.L858R	Activating
TCGA-05-4402-01A-01D-1204-01	p.TSPKANKE751del	Activating
TCGA-95-7947-01A-11D-2183-01	p.I91V, p.R1052I	Non-activating missense
TCGA-78-7158-01A-11D-2035-01	p.L907M	Non-activating missense
TCGA-69-7765-01A-11D-2166-01	p.Q486*	Non-activating missense
TCGA-05-4382-01A-01D-1204-01	p.R222L, p.E545Q	Non-activating missense
TCGA-05-4410-01A-21D-1854-01	p.R377S	Non-activating missense
TCGA-95-7039-01A-11D-1943-01	p.S921R	Non-activating missense

B

Patient ID	EGFR mutation	Predicted function
LUAD-E00163	p.709_710ET>D	Activating
LUAD_E01166	p.A237Y, p.G719A	Activating
LUAD-NYU947	p.ELREA746del	Activating
LUAD-NYU802	p.ELREA746del	Activating
LUAD-QCHM7	p.ELREA746del	Activating
LUAD-S01362	p.ELREA746del	Activating
LUAD-RT-S01770	p.ELREA746del	Activating
LUAD-D00147	p.ELREA746del	Activating
LUAD-NYU848	p.ELREA746del	Activating
LUAD-E01014	p.G719A	Activating
LUAD-NYU696	p.G719A	Activating
LUAD-NYU184	p.G857V	Activating
LUAD-RT-S01702	p.L633F, p.769_770insASV	Activating
LUAD_E01419	p.L858R	Activating
LUAD-NYU704	p.L858R	Activating
LUAD-NYU1210	p.L858R	Activating
LUAD-NYU252	p.L858R	Activating
LUAD-F00134	p.L858R	Activating
LUAD-RT-S01856	p.L858R	Activating
LUAD-NYU1101	p.L861Q	Activating
LUAD-NYU1093	p.L861Q	Activating
LUAD-RT-S01771	p.R776H, p.L858R	Activating
LUAD-CHTN-3090346	p.R836S	Activating
LUAD-RT-S01711	p.S768I, p.LREAT747del	Activating
LUAD-NYU1142	p.T790M, p.L858R	Activating
LUAD-RT-S01813	p.H1129Y	Non-activating missense
LUAD-74TBW	p.O1020H	Non-activating missense
LUAD-F00368	p.R1100S	Non-activating missense
LUAD-NYU408	p.R574L	Non-activating missense
LUAD_E01319	p.R831H	Non-activating missense
LUAD-NYU608	p.S645C	Non-activating missense
LUAD-RT-S01810	p.V536M	Non-activating missense

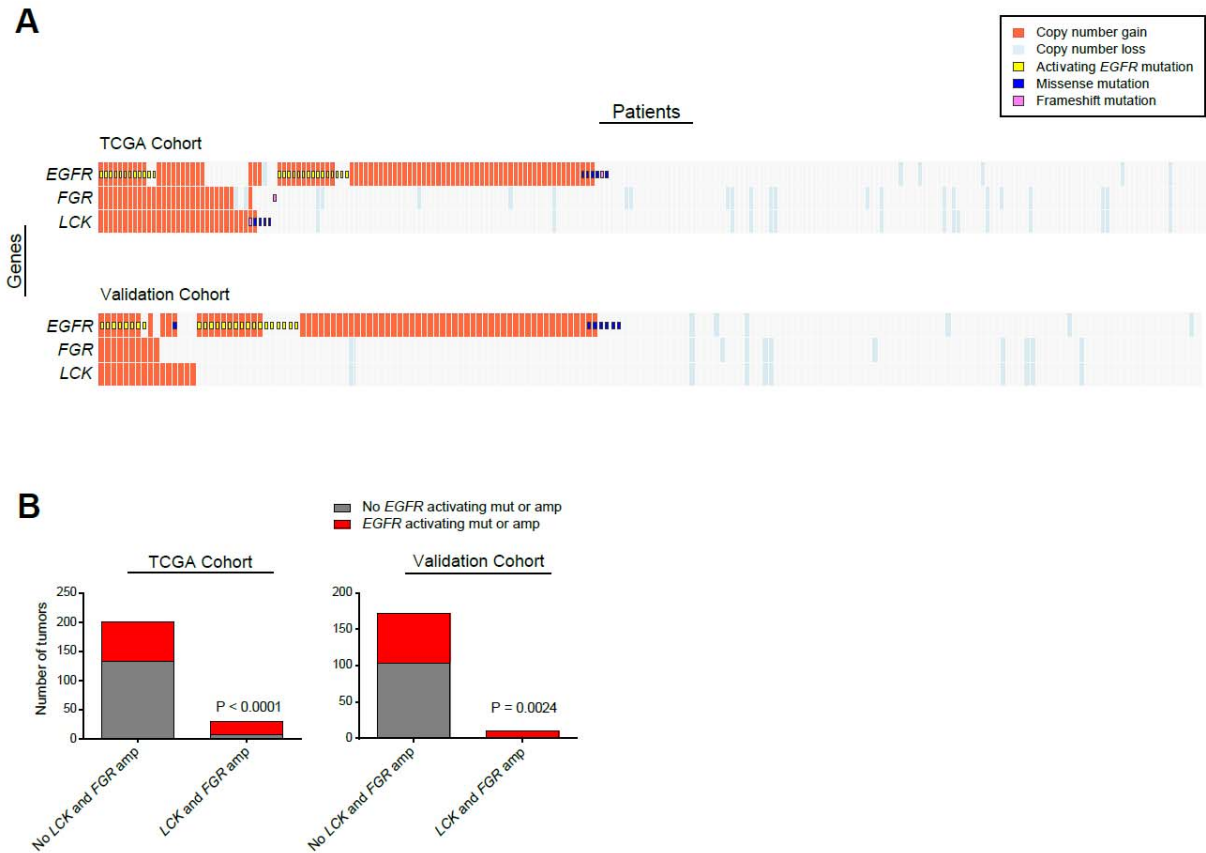


Figure 1-5. Lung adenocarcinomas harboring copy number gain in *FGR* and *LCK* are significantly more likely to possess concomitant *EGFR* mutations or amplifications. (A) Somatic copy number alterations and mutations in *EGFR* and resistance-promoting genes *FGR* and *LCK* across a cohort of 230 TCGA primary lung adenocarcinomas (top panel) and across a validation cohort of 182 primary lung adenocarcinomas (bottom panel). Each patient tumor is represented by a column. (B) Left panel: TCGA primary lung adenocarcinoma tumors harboring *FGR* and *LCK* amplifications possess *EGFR* activating mutations or *EGFR* amplifications at a significantly higher frequency than patients lacking copy number gain of *FGR* and *LCK*. Right panel: A validation cohort of 182 lung adenocarcinoma cases similarly reveals a significant enrichment for *EGFR* activating mutations or amplifications in tumors harboring copy number gain of *FGR* and *LCK*. P-values were calculated using a one-tailed Fisher's exact test.

apart on chromosome 1p and are frequently co-amplified in this sample set. Indeed, we found that patients harboring copy number gain in *FGR* and *LCK* were significantly more likely to have *EGFR* activating mutations (odds ratio [OR] = 8.8; 95% confidence interval [CI], 3.5 – 21.7; $P < 0.0001$), *EGFR* gene amplifications (OR = 5.5; 95% CI, 2.3 – 13.1; $P < 0.0001$) or either one or both alterations (OR = 7.5; 95% CI, 2.9 – 19.3; $P < 0.0001$) (Figure 1-6A; Figure 1-5B, left).

To validate these findings, we similarly surveyed somatic mutation and copy number alteration data from an independent sample set [35] of 182 lung adenocarcinoma patients for alterations in *EGFR* and resistance-promoting genes. As with the TCGA cohort, we observed significant enrichment of *EGFR* alterations in patients harboring *FGR* and *LCK* copy number gain (OR = 13.4; 95% CI, 1.7 – 108.5; $P = 0.0024$) (Figure 1-5A, bottom panel; Figure 1-5B, right). Consistent with the TCGA cohort, this enrichment remains significant when considering *EGFR* activating mutations and *EGFR* gene amplifications independently (OR = 36.5; 95% CI, 7.2 – 185.9; $P < 0.0001$) and (OR = 6.9; 95% CI, 1.4 – 33.6; $P = 0.0086$), respectively (Figure 1-6B). Collectively, these findings provide strong evidence for a non-random relationship between alterations in *FGR/LCK* and *EGFR* dependency in lung adenocarcinoma, and raise the possibility that alterations in these genes could modify *EGFR* dependence in the clinical setting.

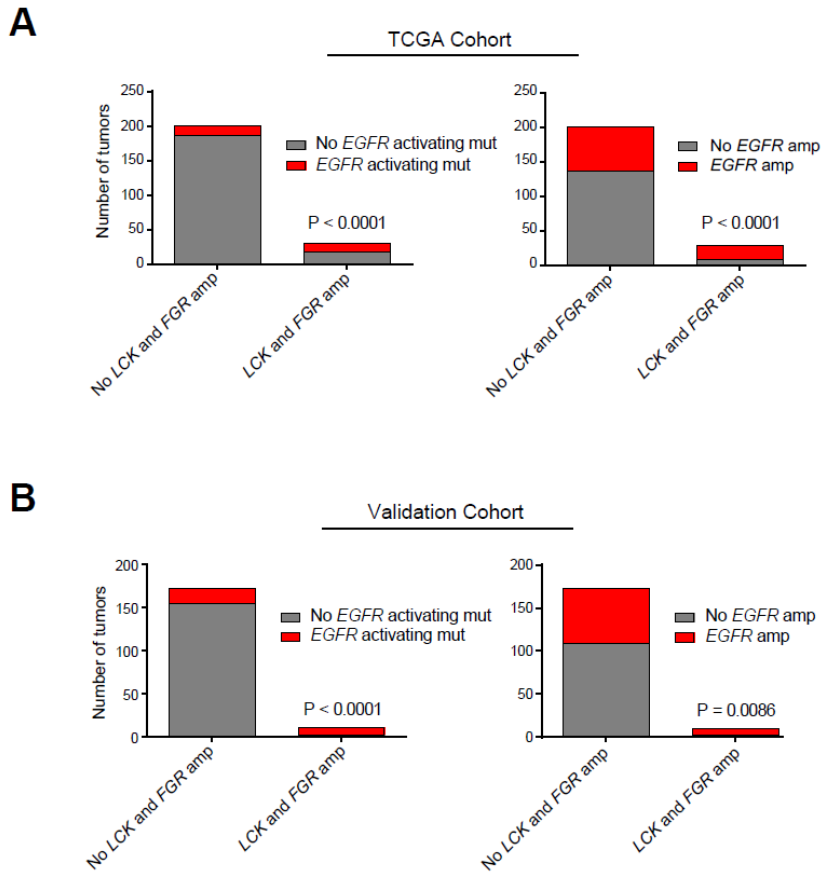


Figure 1-6. Lung adenocarcinoma patients harboring copy number gain of *FGR* and *LCK* have a significantly higher frequency of co-occurring *EGFR* activating mutations and a significantly higher frequency of co-occurring *EGFR* amplifications. (A) TCGA lung adenocarcinoma patients with copy number gain of *FGR* and *LCK* are significantly more likely to harbor *EGFR* activating mutations (left panel) and significantly more likely to harbor *EGFR* amplifications (right panel) than patients lacking amplification of *FGR* and *LCK*. A similar pattern can be observed in (B) a validation cohort of 182 lung adenocarcinoma cases. P-values were calculated using a one-tailed Fisher's exact test.

DISCUSSION

We report the use of a systematic ORF-based screening approach to identify the spectrum of human kinases capable of bypassing reliance on EGFR in *EGFR*-mutant lung cancer cells. To our knowledge, this represents the first instance of a cDNA-based genetic complementation screen for loss of EGFR in an *EGFR*-mutant model. This ORF-based screening approach has simultaneously recovered known, clinically-validated mechanisms of erlotinib resistance as well as identified novel mediators of EGFR bypass in *EGFR*-mutant NSCLC. More generally, it has revealed the breadth and diversity of kinase and kinase-related genes capable of replacing EGFR in the setting of EGFR oncogene-addiction: 18 genes reported herein, including several tyrosine kinase subfamilies of both receptor and non-receptor kinase classes, as well as serine/threonine kinases.

Identifying the spectrum of kinases capable of EGFR bypass is of considerable clinical interest given that patients with *EGFR*-mutant NSCLCs almost invariably acquire resistance to EGFR-directed therapy [10]; a large fraction (30%) of acquired resistance cases are driven by unknown mechanism(s) [14]; and because mounting evidence suggests that activation of alternative driver kinases, such as MET, represents a more general route by which kinase-driven cancers acquire resistance to therapy [16,17,21,29]. Importantly, in the case of MET activation in *EGFR*-mutant cells, combined EGFR and MET inhibition effectively overcomes MET-mediated EGFR-TKI resistance *in vitro* and *in vivo* [16,36]. These findings suggest that elucidating novel mechanisms of EGFR bypass could in principle inform the development of rational combination therapies to delay or treat the development of acquired resistance.

As previously mentioned, the known and clinically-validated drivers of erlotinib resistance recovered by our screen include the RTK gene *AXL*, the ErbB-family member *ERBB2* (*HER2*), and the gene encoding the adaptor protein CRKL [19,20,21]. Additionally, this ORF-based strategy has enabled the discovery of several novel drivers of EGFR bypass in *EGFR*-mutant NSCLC. Among these, we were struck by the high representation of Src-family

kinases, constituting greater than one-third of screening hits and including seven of the nine members of this family. The finding that Src-family kinases are sufficient to drive resistance to EGFR inhibition in an *EGFR*-mutant setting is in line with considerable evidence linking EGFR activity with this family: c-Src itself has been characterized extensively with respect to its cooperative relationship with EGFR [37], and Src-family kinase activation has been observed in and proposed to be a contributor to cetuximab-resistant colorectal adenocarcinoma and NSCLC squamous cell carcinoma *in vitro* models [38,39], as well as EGFR-TKI-resistant lung adenocarcinoma models [40]. Moreover, recent work suggests that the introduction of dominant-active c-Src can reduce the inhibitory effects of erlotinib in head and neck squamous cell carcinoma models [41].

Similarly, we noted that the fibroblast growth factor receptor (FGFR)-family kinase genes *FGFR1* and *FGFR2*, while not previously recognized as sufficient for EGFR bypass, nonetheless align with previous work describing upregulation of FGFR1 as well as the ligand FGF2 in gefitinib-resistant NSCLC models, with concomitant dependency on the FGFR pathway [42,43], as well as other studies implicating FGF ligands in TKI resistance [24,44]. In one recent study, a growth factor-based screen for drug resistance identified FGF, the ligand for these receptors, as sufficient to partially or completely rescue sensitivity to erlotinib in several *EGFR*-mutant cell line models, including PC9 [24]. Incidentally, this concordance also supports the notion that cDNA-based screens, which primarily interrogate cell-autonomous mechanisms of resistance, can nevertheless complement ligand- or growth factor-based rescue screens in nominating pathways important for non-cell-autonomous, microenvironment-driven resistance.

Other resistance-inducing genes, including the neurotrophic tyrosine kinase receptor (NTRK)-family kinases *NTRK1* and *NTRK2*, *MST1R*, and the serine/threonine kinases *MOS* and *RAF1*, have not previously been appreciated to drive TKI resistance in *EGFR*-mutant lung cancer cells, and thus underscore the power of this screening approach in identifying novel mediators of bypass for a given dependency.

When viewed collectively, our screening findings reveal a surprisingly extensive list of kinase and kinase-related genes with compensatory potential in the setting of oncogene-addicted EGFR. The finding that a large number of kinase inputs are capable of redundantly driving cancer cell growth is consistent with recent reports describing broad compensatory potential for growth factor-mediated inhibitor resistance in several tumor dependency models [23,24], and with the observation that coactivation of multiple RTKs in glioblastoma cells overcomes reliance on any single RTK for downstream signaling activation [25].

Finally, in querying somatic mutation and copy number alteration data from over 400 lung adenocarcinoma cases, we identified significantly co-occurring alterations in *EGFR* and resistance-mediating genes *FGR* and *LCK*. The consequence of this co-occurring pattern remains to be determined, but based on our *in vitro* data, one may hypothesize that these patients may be more likely to fail or experience a worse outcome on erlotinib treatment than *EGFR*-mutant patients lacking concurrent amplification of *FGR* and *LCK*. Analysis of pre- and post-relapse biopsy specimens will likely be critical in deducing the exact role this co-occurring pattern may play in TKI resistance.

Together, our findings uncover the spectrum of kinases sufficient to bypass dependence on EGFR in *EGFR*-mutant lung cancer cells and nominate two genes with potentially immediate clinical relevance.

MATERIALS AND METHODS

Cell culture and reagents

The *EGFR*-mutant NSCLC cell line PC9 (del E746_A750) has been described previously [45]. Cells were maintained in RPMI (Cellgro) supplemented with 10% FBS (Gemini Bioproducts). Erlotinib, dasatinib, and lestaurtinib were purchased from LC Laboratories; XL880, NVP-BGJ398, and AZ628 were purchased from Selleck Chemicals. Lapatinib was purified from patient-discarded tablets by James Bradner.

Kinase ORF screen

Screening was performed using a kinase ORF library of 589 ORFs encoding 584 genes (Center for Cancer Systems Biology (CCSB)/Broad Institute Kinase Open Reading Frame Collection) [28,29], along with the positive and negative controls described in the main text and displayed in Figure 1-1. PC9 cells were seeded overnight in 384-well microtiter plates at a density of 400 cells per well. The following day, cells were incubated with lentivirus corresponding to the kinase ORF library and controls in the presence of 4 µg/mL polybrene, spin-infected at 2200 rpm for 30min at 30°C, then incubated at 37°C for an additional 4.5h before replacing media with standard growth media. At 24h post-infection, standard growth media (6 replicates) or media containing 2 µg/mL blasticidin (1 replicate) was spiked into wells. At 72h post-infection, media was replaced with media containing 3 µM erlotinib (2 replicates), 300 nM erlotinib (2 replicates), DMSO (2 replicates), or DMSO + 2 µg/mL blasticidin (1 blasticidin-treated replicate). Cell viability was assayed 3d after the addition of erlotinib/DMSO using the CellTiter-Glo reagent (Promega).

Identification of candidate resistance-mediating genes

Raw luminescence values representing cell viability were averaged between replicates, following exclusion of wells failing detection or other quality control criteria (0.5% of wells). For each of the two drug dose screening arms, for a given ORF or control, viability under erlotinib treatment was normalized to that under DMSO treatment. Candidate resistance-inducing genes were defined as those having relative viability values of at least 39% in 300 nM erlotinib and at least 31% in 3 μ M erlotinib. Luminescence values corresponding to DMSO + blasticidin-treated cells were also compared to those of (unselected) DMSO-treated cells to assess each ORF's infection efficiency (data not shown).

Screen validation and drug sensitivity assays

PC9 cells were seeded overnight in 384-well microtiter plates at a density of 400 cells per well. The following day, cells were incubated with lentivirus (virus production methods described below) corresponding to candidate resistance-mediating ORFs as well as controls in the presence of 4 μ g/mL polybrene, spin-infected at 2200 rpm for 30min at 30°C, then incubated at 37°C for an additional 4.5h before replacing media with standard growth media. At 24h post-infection, additional standard growth media was spiked into wells. At 72h post-infection, media was replaced with media containing inhibitor(s) at their final concentrations or DMSO (1:1,000 dilution). For dose-response curves, inhibitor(s) were tested at each of the following concentrations: 10, 1, 0.1, 0.01, 0.001, and 0.0001 μ M. Cell viability was assayed 3d after the addition of inhibitor(s) or DMSO using CellTiter-Glo (Promega). Drug-treated cells were normalized to DMSO-treated cells to calculate relative percent viability. Relative percent viability values and dose-response curves were plotted using GraphPad Prism software (GraphPad); area under curve values were generated using GraphPad Prism software and displayed using GENE-E software. Absolute IC₅₀ values were calculated using GraphPad Prism software.

Virus production

Lentivirus was produced by transfection of 293T packaging cells with plasmids corresponding to pLX-Blast-V5-ORF, Δ 8.9 (*gag*, *pol*), and VSV-G; and FuGene6 transfection reagent (Roche) as described previously [29].

Viral transduction and culture of ORF-expressing cells for protein analysis

Cells were seeded in 6-well plates at a density of 54,000 cells per well. The next day, cells were incubated with lentivirus in the presence of 4 μ g/mL polybrene for 6-7h, after which media was replaced with standard growth media. At 24h post-infection, media was replaced with selective media containing 1-1.3 μ g/mL blasticidin, and blasticidin-containing media was replenished after another 72h. At 6d post-infection, cells were treated with media containing 0.5% FBS overnight. The following day, cells were treated with inhibitor(s) or DMSO at their final concentrations in media containing 0.5% FBS for 6h, then harvested for immunoblotting.

Immunoblotting

Cell pellets were resuspended in lysis buffer (50 mM Tris pH 7.4, 2.5 mM EDTA pH 8, 150 mM NaCl, 1% Triton X-100, 0.25% IGEPAL CA-630), supplemented with protease inhibitors (Roche) and Phosphatase Inhibitor Cocktails I and II (Calbiochem), incubated on ice for 2 min, then centrifuged for 2 min at 13,000 rpm. The protein concentrations of supernatants were determined using a BCA Protein Assay Kit (Pierce) and normalized. Lysates were reduced and denatured, then separated using Tris-Glycine gels (Novex) and transferred to iBlot Transfer Stack nitrocellulose membranes (Novex). Membranes were incubated with primary antibodies overnight at 4°C. Antibodies against phospho-EGFR (Y1068) (1:1,000) and V5 (1:5,000) were purchased from Invitrogen. The antibody recognizing total EGFR (1:1,000) was purchased from BD Biosciences. Antibodies against total AKT (1:1,000), phospho-AKT (S473 and T308), total

ERK1/2 (1:750), phospho-ERK1/2 (T202/Y204) (1:500), and cofilin (1:10,000) were purchased from Cell Signaling Technology. Phospho-AKT immunoblotting was performed with the S473-directed antibody (1:750) unless otherwise indicated. Incubation with IRDye secondary antibodies (1:10,000) (LI-COR Biosciences) and subsequent detection (Odyssey Imaging System, LI-COR Biosciences) were performed according to manufacturer recommendations.

Analysis of lung adenocarcinoma patient tumors

TCGA copy number and mutation data were obtained from the Broad Institute TCGA Firehose pipeline ([doi:10.7908/C1Z60M5H](https://doi.org/10.7908/C1Z60M5H)); and somatic alteration data for the validation cohort were obtained from [35]. The sample set and somatic variant calling pipelines used to generate both datasets are described in (TCGA paper, submitted, and [35]). Briefly, somatic mutations (substitutions, insertions, deletions) were identified through comparison of matched tumor and normal whole-exome sequencing alignments [46]; and somatic copy number alterations were derived through GISTIC 2.0 pre-processing of Affymetrix SNP 6.0 profiles of tumor and matched normal DNA [47]. For heatmap and statistical analyses, a gene was designated as harboring a copy gain or loss in a tumor sample if the average relative copy number change across its length was >0.3 or < -0.3 , respectively; and mutation data represent non-silent somatic mutations. Mutations in *EGFR* were further designated as “activating” if they met one of the following criteria: any deletion in exon 19; any insertion in exon 20; any mutations at residues 858, 719, 861, 768, 769, or 709 [31]; or any substitution within exons 18-21 that generated a PolyPhen-2 score of >0.9 [48]. For a list of non-silent somatic *EGFR* mutations present in these datasets and their characterization as activating or (non-activating) missense, see Table 1-2.

Statistical tests

One-tailed Fisher's exact tests, odds ratios, and 95% confidence intervals were calculated using GraphPad Prism software. P-values less than 0.01 were considered significant.

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CHAPTER 2

A Gene-Expression-Based Approach to Identify Chemical Modifiers of EGFR Dependence

Attributions:

Portions of this chapter are reprinted from a manuscript in preparation.

All experiments and analyses were performed by Tanaz Sharifnia except as follows:

LINCS queries, and analyses represented in Figures 2-1A and 2-1C were designed and performed by Victor Rusu and Aravind Subramanian. Data visualization for Figures 2-1A, 2-2, 2-3, and 2-6A was done by Bang Wong and Tanaz Sharifnia.

ABSTRACT

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are effective therapies in the subset of non-small cell lung cancers with *EGFR* mutations, but clinical benefit is limited by the development of drug resistance. Previous work has identified 18 kinase and kinase-related genes whose overexpression can induce resistance to EGFR inhibition in *EGFR*-mutant NSCLC cells, but whether these EGFR bypass genes have shared functional effects is not known. In this study, we applied an unbiased gene-expression-based connectivity approach (Library of Integrated Network-based Cellular Signatures resource) to determine whether resistance-mediating genes act through common or divergent pathways, and to identify compounds that may reverse erlotinib resistance. Gene-expression analysis of cells overexpressing resistance-mediating genes, together with targeted validation studies, implicates the phosphoinositide 3-kinase (PI3K)-AKT and MEK-ERK pathways as active in these cells under erlotinib treatment. Combined treatment with the PI3K-mTOR inhibitor NVP-BEZ235 and MEK inhibitor AZD6244 restores erlotinib sensitivity of cells expressing all 18 resistance-inducing genes. Using this connectivity approach, we also identified two chemical compounds, the cardiac glycoside helveticoside and the NEDD8-activating enzyme inhibitor MLN4924, that can reduce EGFR dependence in EGFR-TKI-sensitive cells. Together, these data suggest that resistance-mediating genes commonly induce similar transcriptional effects, and that combined MEK and PI3K-mTOR inhibition may be an effective and universal strategy for overcoming kinase-driven resistance to EGFR-TKIs.

INTRODUCTION

Activating mutations in the epidermal growth factor receptor (*EGFR*) are a common driver alteration in non-small cell lung cancer [1,2,3,4,5] and can predict sensitivity to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib [6,7,8,9], but all patients eventually

develop resistance to therapy [10]. We have previously described an open reading frame (ORF)-based screen to identify the spectrum of kinases whose overexpression can induce erlotinib resistance in *EGFR*-mutant NSCLC cells (Chapter 1). An unexpected finding of this work was the breadth and diversity of kinase and kinase-related genes capable of substituting for EGFR in the setting of EGFR dependence: 18 kinase and kinase-related genes were identified in this screen, and this set of genes included several tyrosine kinase subfamilies of both receptor and non-receptor kinase classes, as well as serine/threonine kinases.

Given the broad subset of kinase genes that could induce erlotinib resistance in these cells, we were interested in identifying signaling pathways that were potentially common to overexpression of these genes; in principle, such pathways might be targets for a generalized strategy to overcome erlotinib resistance. A hypothesis-driven analysis described in Chapter 1 indicated a role for activation of the AKT pathway (Chapter 1, Figure 1-4B-G); however, an unbiased systematic approach might more clearly address whether resistance-inducing genes act through common or divergent pathways.

We reasoned that a high-dimensional transcriptional readout would provide an unbiased approach to address this question. Gene-expression profiling methods have been used extensively over the last 15 years to classify disease subtypes [11,12,13,14,15,16], identify new drug targets [12,17], and predict clinical outcome or response to therapy [18,19,20,21]. In recent years, large gene-expression catalogs, such as the resource created by the Library of Integrated Network-based Cellular Signatures (LINCS) Connectivity Map project (lincscloud.org), have allowed investigators to identify functional relationships between genes, drugs, or diseases on the basis of their shared transcriptional effects [22,23]. In this study, we have applied a gene expression-based approach using the Connectivity Map resource to detect commonalities between erlotinib resistance-mediating genes and to identify chemical compounds that can modify the resistant state.

RESULTS

Resistance-mediating genes commonly induce similar transcriptional effects

We assembled reagents for the 18 resistance-mediating genes (positive-phenotype genes) as well as 19 kinase genes that had failed to rescue erlotinib sensitivity (negative-phenotype genes) in the previously-described ORF screen (Chapter 1). PC9 cells were transduced with positive-phenotype, negative-phenotype, and control vectors for 72h, after which they were subjected to 24h erlotinib treatment. Gene-expression profiles for each ORF were generated in quadruplicate and used to compute signatures as described in Materials and Methods.

Unsupervised hierarchical clustering of these signatures yielded two distinct clusters (Figure 2-1A). Intriguingly, profiles showed a pattern of segregation largely in accordance with resistant versus sensitive phenotypes; in particular, we observed one subcluster comprised solely of 12 resistance-associated profiles and the two *EGFR* double-mutant positive controls included in the assay, labeled red in Figure 2-1A. Given that this cluster contained almost all resistance-associated gene profiles and none of the negative-phenotype profiles, we chose to focus our subsequent analyses on the profiles represented therein, henceforth referred to as the ‘positive-phenotype cluster.’

We next asked if the expression profiles of cells transduced with resistance-associated ORFs could yield insights into their biological effects. To investigate this, we turned to an expression-profiling resource created by the Library of Integrated Network-based Cellular Signatures (LINCS) program (lincscloud.org). The LINCS database is a large catalog of gene-expression profiles generated from diverse human cell lines (A549, MCF7, PC3, A375, HepG2, VCaP, HCC515, HT29) treated with a large number of genetic and chemical perturbagens (lincscloud.org). Gene-expression signatures from cells expressing each of the 12 genes represented in the positive-phenotype cluster (excluding controls) were independently used to query LINCS.

We first sought to identify ORFs in the LINCS dataset whose transcriptional effects most positively-correlate with query ORFs. We hypothesized that identifying LINCS ORFs whose signatures positively-correlate with query ORFs could lend insight into whether resistance-mediating ORFs act through shared or distinct pathways. When considering the top ~3% of positively-correlated ORFs (Figure 2-1B), we observed that our query ORF signatures commonly correlate with other signatures generated using these same ORFs or from other positive-phenotype cluster members. These findings were particularly notable as none of the cell lines profiled in the LINCS dataset is *EGFR*-mutant, and most are not of a lung lineage. Together with the hierarchical clustering, these data suggest that a major subset of resistance-mediating genes induce similar transcriptional effects, and these effects do not appear to be restricted to an *EGFR*-mutant cellular context.

In order to identify genes that were differentially expressed between positive-phenotype cluster genes and genes unable to confer erlotinib resistance, a two-class comparison was performed on these two groups (Figure 2-1C). We observed that a large number of genes are significantly differentially expressed between these two classes (signal-to-noise ratios of > 2 or < -2). Genes that were expressed more highly in the positive-phenotype (erlotinib-resistant) class included *DPH2*, *FOSL1*, *MYC*, *CDC25A*, *PNP*, *PSMG1*, and *MCM3*, among others. Genes with lower expression in the positive-phenotype class included *ERBB3*, *TRAPPC6A*, *FOXO4*, *IGFBP3*, and *CBLB*, among others. Together, these genes help to define the transcriptional signature commonly associated with resistance-mediating ORFs.

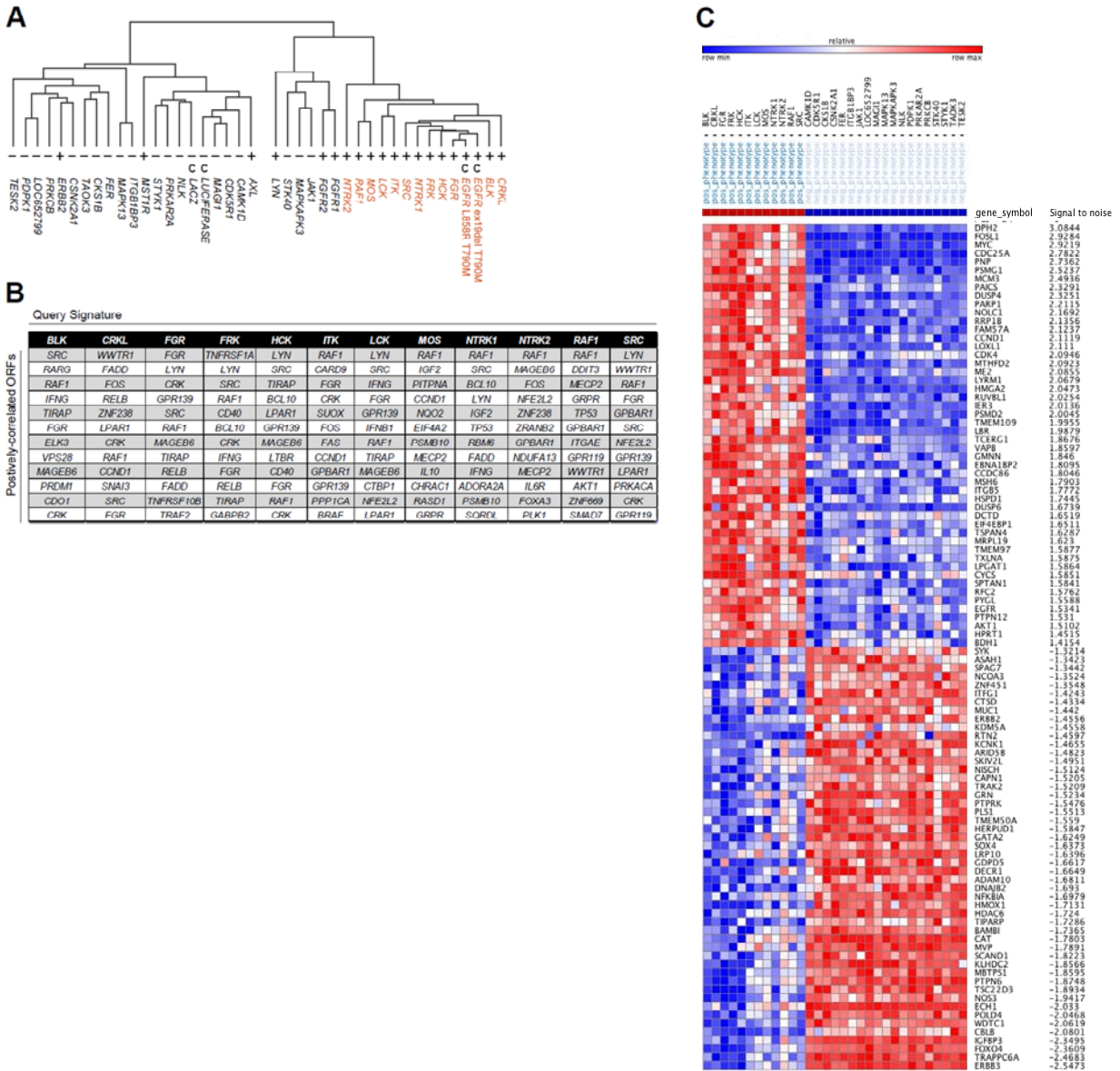


Figure 2-1. Resistance-mediating genes induce similar transcriptional effects. (A)

Unsupervised hierarchical clustering of PC9 cells overexpressing resistance-mediating ORFs (“+”); kinase ORFs unable to confer erlotinib resistance (“-”); and *EGFR* double-mutant positive controls and inert gene negative controls (“C”). Twelve resistance-mediating ORFs displaying membership in a single cluster (‘positive-phenotype cluster,’ labeled red) were used to query the LINCS dataset. (B) LINCS ORFs whose signatures most positively-correlate with those of

Figure 2-1 (Continued)

positive-phenotype cluster genes. Each of the twelve positive-phenotype genes was used to independently query the LINCS dataset, and the top ~3% positively-correlated ORFs are listed. Query ORFs commonly 'connect' with themselves or other positive-phenotype cluster members.

(C) Two-class comparison of 'positive-phenotype cluster' members and kinase ORFs unable to confer erlotinib resistance. The top and bottom 50 differentially expressed genes are listed, along with their respective signal-to-noise ratios.

Transcriptional effects induced by resistance-promoting genes are anti-correlated to those induced by MEK and PI3K inhibitors

In addition to signatures generated from genetic perturbagens, the LINCS database contains over 34,000 gene-expression profiles collected from human cells treated with 3,103 chemical reagents. Using this resource, we next asked if the expression profiles of cells transduced with resistance-associated ORFs could be used to nominate chemical compounds with potential to reverse ORF-mediated resistance.

As described above, gene-expression signatures from cells expressing each of the 12 genes represented in the positive-phenotype cluster (excluding controls) were independently used to query LINCS. In this case, we looked for chemical perturbations whose transcriptional effects were anti-correlated with those of resistance-promoting genes, hypothesizing that such perturbations could pinpoint pathways or mechanistic nodes distinctly active in resistant cells, as well as nominate compound classes with potential to reverse ORF-mediated resistance. Among the top 0.7% anti-correlated compounds (Figure 2-2, blue portion of barcode plot), 13 out of the 21 compounds (>61%) could be classified as either MEK or PI3K inhibitors (Figure 2-2, expanded view). These two drug targets, along with the third-most abundant target, SRC, were the only recurrent drug targets represented at the top of this list (Figure 2-3). The enrichment of these two inhibitor classes indicated that the cell states induced by our panel of resistance-promoting query genes were opposed to those induced by PI3K or MEK inhibition, and conversely suggested that either or both of these pathways could be active in resistant cells. The similarity in profiles observed via unsupervised clustering, along with the observed correlations to a shared set of LINCS compound signatures, suggests that the erlotinib resistance-promoting genes identified in our screen could mechanistically converge on a small number of signaling nodes.

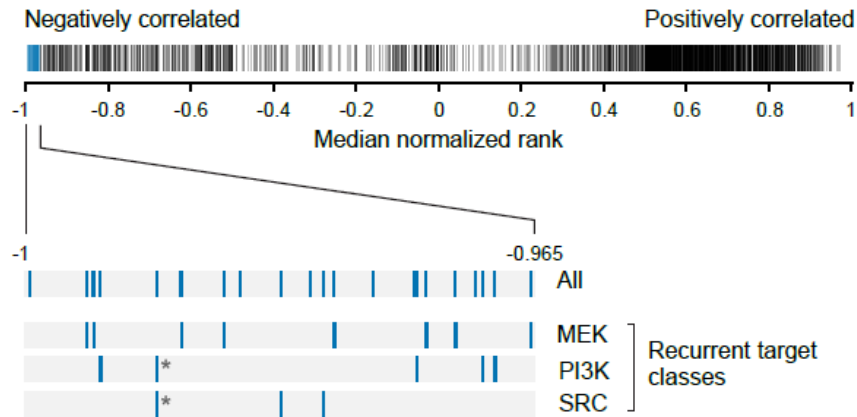


Figure 2-2. Transcriptional effects induced by resistance-mediating ORFs negatively-correlate with those induced by MEK and PI3K inhibitors. Barcode plot displaying LINCS compounds ranked by the correlations of their signatures to those of ‘positive-phenotype cluster’ ORFs. The most positively/negatively-correlated compounds approach ranks of 1 and -1 respectively. The 0.7% most negatively-correlated compounds (blue portion of barcode plot) include several inhibitors targeting MEK, PI3K, and SRC (Figure 2-3). Each bar represents a chemical reagent. A single reagent targeting both PI3K and SRC is denoted with an asterisk.

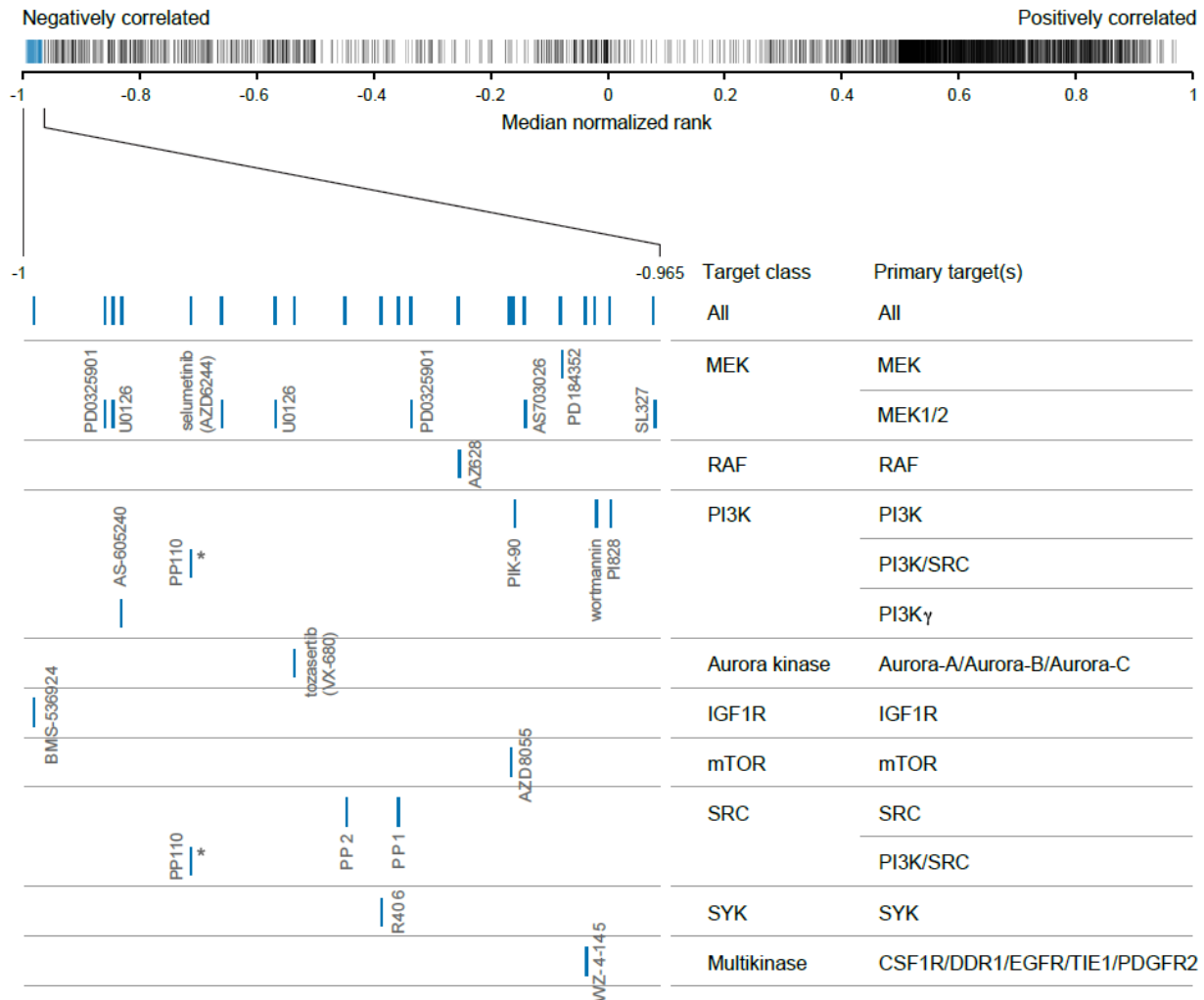


Figure 2-3. LINCX compounds whose transcriptional effects most negatively-correlate with those of resistance-mediating ORFs. Median normalized ranks of the top 0.7% negatively-correlated compounds and categorization by drug target class and primary target(s) (References [24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40]). Each bar represents a compound. A single compound targeting both PI3K and SRC is denoted with an asterisk.

Resistance-promoting ORFs commonly reactivate phospho-AKT and/or phospho-ERK1/2, and combined inhibition of PI3K-mTOR and MEK rescues ORF-mediated resistance to erlotinib

Given the identification of PI3K and MEK inhibitor treatment signatures as anti-correlated with our 'positive-phenotype' erlotinib resistance signatures (Figure 2-2), we sought to address whether resistance-inducing ORF genes confer erlotinib resistance via reactivation of the PI3K-AKT or MEK-ERK pathways. PC9 cells overexpressing resistance-inducing genes were treated with increasing doses of erlotinib for 6h and profiled for activation of EGFR, AKT, and ERK1/2 by immunoblot analysis (Figure 2-4A). Indeed, persistent activation of AKT and/or ERK1/2 under erlotinib treatment is a common feature of the majority of resistance-inducing ORFs, with sustained phospho-AKT most prominently associated with expression of several Src-family kinases (*BLK*, *FGR*, *FRK*, *HCK*, *LCK*, and *SRC* itself), *ITK*, and *CRKL*; and sustained phospho-ERK1/2 most prominently associated with *RAF1*, *BLK*, *ITK*, *MOS*, *AXL*, and *CRKL*. Other ORFs, such as those encoding FGFR-family genes, appear to display very modest activation of one or both of these pathways, which may also be reflective of their more modest rescue phenotype (Chapter 1, Figure 1-1). Interestingly, we also observed that cells expressing several kinase genes maintain phosphorylation of EGFR itself in the presence of erlotinib, and these include *BLK*, *LCK*, *FGR*, and *FRK*.

To determine whether reactivation of these signal transducers is required for ORF-associated resistance, cells transduced with resistance-promoting genes were treated with erlotinib; the dual PI3K-mTOR inhibitor NVP-BE235 (hereafter referred to as BE235); the MEK inhibitor AZD6244; or their combinations, and assayed for cell viability after 72h (Figure 2-4B). Drug doses of BE235 (500 nM) and AZD6244 (2.5 μ M) were determined empirically to be the lowest doses capable of maximally inhibiting AKT and ERK1/2 phosphorylation (Figure 2-5), and are in similar ranges to those used by other investigators in *EGFR*-mutant cell lines [41].

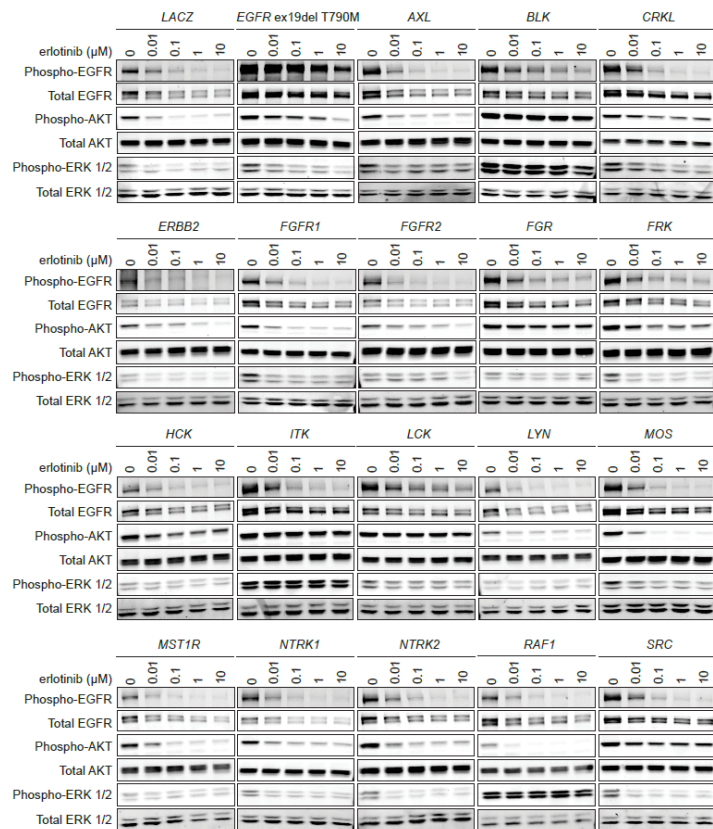
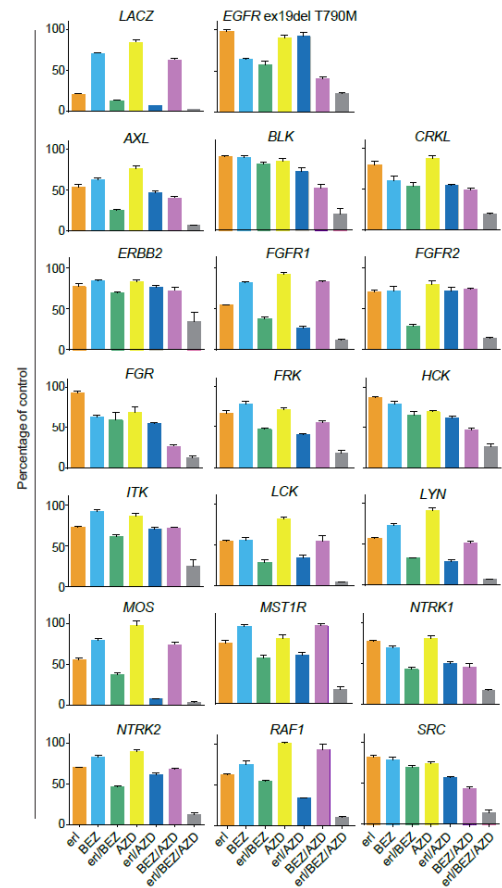
A**B**

Figure 2-4. Resistance-mediating ORFs commonly reactivate phospho-AKT and/or phospho-ERK1/2, and PI3K-mTOR and MEK co-inhibition restores sensitivity to erlotinib.

(A) Immunoblot analysis of PC9 cells overexpressing the indicated ORFs and treated with erlotinib for 6h. Cells were incubated with 0.5% serum media 18h before and during drug/DMSO treatment. Total cell lysates were immunoblotted for the indicated proteins. (B) Cell viability of PC9 cells overexpressing the indicated ORFs following treatment with 100nM erlotinib (erl), 500 nM of the PI3K-mTOR inhibitor BEZ235 (BEZ), 2.5 μ M of the MEK inhibitor AZD6244 (AZD), or their combinations for 72h. Cell viability was assayed with CellTiter-Glo. Data are expressed as percent viability relative to vehicle-treated cells and represent the mean \pm SD of 4 replicates.

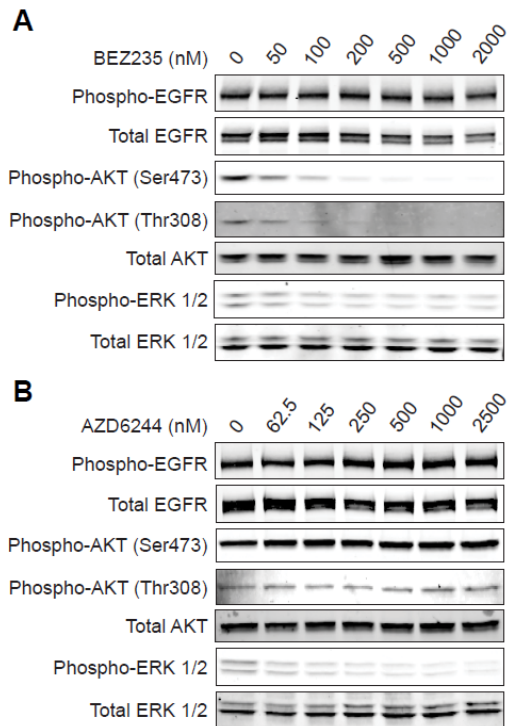


Figure 2-5. BEZ235 and AZD6244 treatment downregulate phospho-AKT and phospho-ERK1/2, respectively, in a dose-dependent fashion. (A) Immunoblot analysis of PC9 cells treated with indicated doses of the PI3K-mTOR inhibitor BEZ235 for 6h. Total cell lysates were immunoblotted for the indicated proteins. (B) Immunoblot analysis of PC9 cells treated with indicated doses of the MEK inhibitor AZD6244 for 4h. Total cell lysates were immunoblotted for the indicated proteins.

We observed that combined BEZ235 and AZD6244 treatment was sufficient to restore sensitivity to erlotinib across all ORF-expressing cell lines, with sensitivity often enhanced relative to erlotinib treatment in *LACZ*-expressing cells. We also noted that in cases where at least partial restoration of erlotinib sensitivity could be achieved using either BEZ235 or AZD6244, re-sensitization was always potentiated by combining these two agents. Together, these data demonstrate that reactivation of AKT and ERK1/2 signaling commonly underlies, and is required for, ORF-mediated resistance.

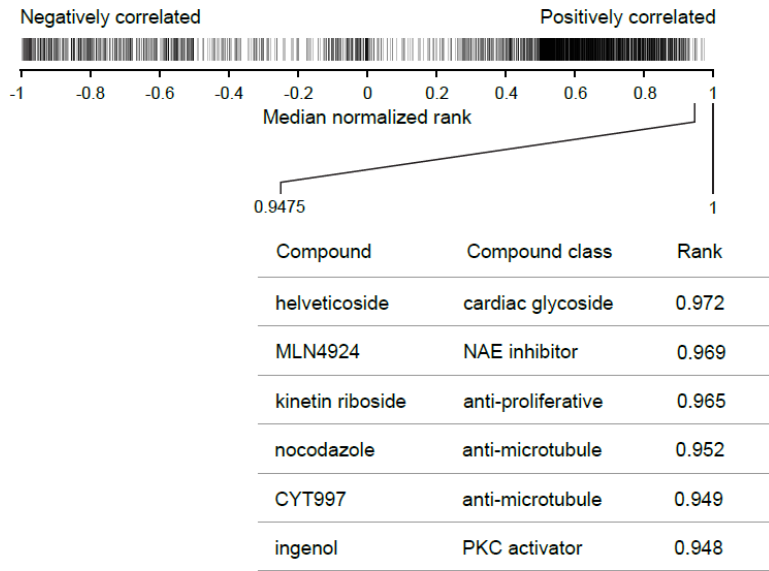
Compounds whose transcriptional effects positively-correlate with those of resistance-promoting genes can reduce dependence on EGFR

Intriguingly, the LINCS query described above also revealed the existence of chemical perturbations whose transcriptional effects were positively-correlated with those of resistance-promoting genes. We hypothesized that at least some subset of these chemical compounds could, as with resistance-mediating ORFs, reduce dependence on EGFR in *EGFR*-mutant cells. The most positively-correlated compounds (corresponding to the top 0.2% of all compounds) are displayed in Figure 2-6A (expanded view). They include the cardiac glycoside helveticoside [42], the NEDD8-activating enzyme inhibitor MLN4924 [43], the anti-proliferative agent kinetin riboside [44,45], the microtubule inhibitors nocodazole [46] and CYT997[47], and the PKC activator ingenol [48]. To determine whether positively-correlating compounds, like resistance-mediating ORFs, could reduce erlotinib sensitivity, PC9 cells were treated with increasing concentrations of these compounds, in combination with either erlotinib (500 nM or 1 μ M in parallel) or DMSO (Figure 2-6B). Remarkably, we observed that the highest-scoring compounds, helveticoside and MLN4924, indeed conferred a modest, dose-dependent increase in cell viability in the presence of erlotinib, relative to erlotinib-treatment alone. In contrast, these compounds did not increase cell viability in the presence of DMSO, suggesting that their effects

are not generally growth-enhancing. The ability of other positively-correlated compounds to increase cell viability in the presence of erlotinib was less apparent (Figure 2-6B).

These results indicate that in some cases chemical perturbagens whose transcriptional effects are shared with resistance-promoting genes also share the ability to reduce dependence on EGFR. As with the LINCS ORF query described in Figure 2-1, this is the case despite the diverse cell line backgrounds used to generate gene-expression signatures. Using this approach, we have identified two compounds with a previously unappreciated ability to modify dependence on EGFR.

A



B

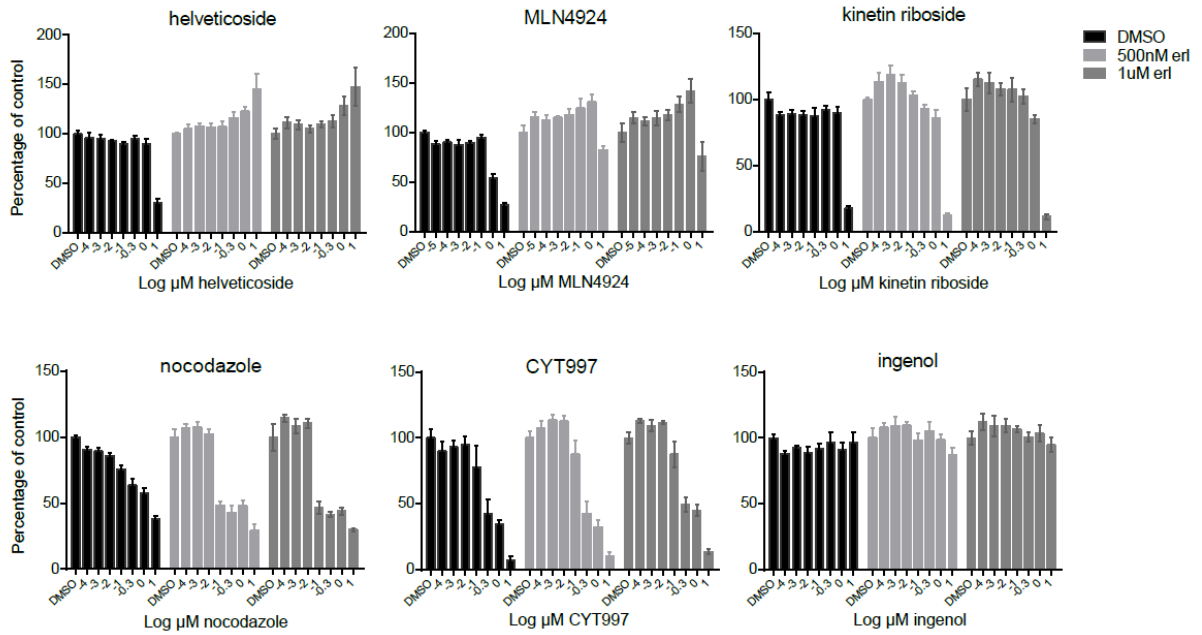


Figure 2-6. LINC compounds whose transcriptional effects most positively-correlate with those of resistance-mediating ORFs. (A) Barcode plot displaying LINC compounds

Figure 2-6 (Continued)

ranked by the correlations of their signatures to those of 'positive-phenotype cluster' ORFs, as described in Figure 2-2. The top 0.2% most positively-correlated compounds are displayed in the expanded view and are annotated by compound class (References [42,43,44,45,46,47,48]). Each bar represents a chemical reagent. NAE, NEDD8-activating enzyme. (B) Positively-correlated compounds' effects on EGFR dependence. PC9 cells were treated with DMSO, 500 nM erlotinib, or 1 μ M erlotinib, in combination with either vehicle or increasing concentrations of the query compound. Cell viability was assayed after 72h using CellTiter-Glo. Data are expressed as percent viability relative to DMSO- or erlotinib-treated cells in combination with vehicle. Data represent the mean \pm SD of 4 replicates.

DISCUSSION

This study has applied a gene-expression-based approach to functionally characterize a set of kinase and kinase-related genes whose overexpression can bypass dependence on EGFR (see Chapter 1). The finding that a broad set of kinase inputs can redundantly drive cancer cell growth has been observed in several tumor dependency models in addition to that of EGFR [49,50,51] and led us to ask whether these bypass genes act through distinct or shared signaling pathways in conferring the resistant phenotype. The latter case would imply that targeting a shared pathway might serve as a generalized strategy to overcome kinase-mediated erlotinib resistance, irrespective of the specific kinase responsible for EGFR bypass.

Using an unbiased gene-expression readout, we observed that resistance-mediating genes commonly induce similar gene-expression signatures, distinguishable from those of kinases unable to mediate erlotinib resistance. More strikingly, when queried against all ORFs in the LINCS dataset, resistance-mediating ORFs often ‘connect’ most strongly to each other, even across highly diverse genetic contexts.

Querying the LINCS dataset also allowed us, in an unbiased fashion, to nominate MEK and PI3K as selectively active in resistant versus sensitive cells, and this finding was corroborated with immunoblot analysis demonstrating that resistance ORF-expressing cells commonly reactivate one or both of these signaling pathways under erlotinib treatment. Importantly, the re-engagement of these pathways was required for erlotinib resistance, as evidenced by restoration of erlotinib sensitivity for all resistance-mediating ORFs with combined AZD6244 and BEZ235 inhibitor treatment. That is, we found that despite the breadth and diversity of kinases capable of overcoming inhibition of EGFR in this context, these genes are remarkably uniform in their convergence upon the PI3K-AKT and/or MEK-ERK pathways.

Finally, we have identified two compounds, the cardiac glycoside helveticoside and the NEDD8-activating enzyme inhibitor MLN4924, that can reduce sensitivity to EGFR inhibition in

erlotinib-sensitive cells. To our knowledge, this represents the first demonstration of chemical suppression of EGFR-TKI sensitivity. These findings warrant further study to dissect the mechanism by which these compounds can modify EGFR dependence.

Collectively, these data identify transcriptional similarities between resistance-mediating ORFs and underscore these genes' convergence on the PI3K-AKT and MEK-ERK signaling axes in sustaining EGFR-independent survival.

MATERIALS AND METHODS

Cell culture and reagents

The *EGFR*-mutant NSCLC cell line PC9 (del E746_A750) has been described previously [52]. Cells were maintained in RPMI (Cellgro) supplemented with 10% FBS (Gemini Bioproducts). Erlotinib was purchased from LC Laboratories; AZD6244, NVP-BEZ235, CYT997, nocodazole, and MLN4924 were purchased from Selleck Chemicals; kinetin riboside and ingenol were purchased from Santa Cruz Biotechnology; helveticoside was purchased from MolPort.

Virus production

Lentivirus was produced by transfection of 293T packaging cells with plasmids corresponding to pLX-Blast-V5-ORF, $\Delta 8.9$ (*gag*, *pol*), and VSV-G; and FuGene6 transfection reagent (Roche) as described previously [53].

Gene-expression profiling and LINCS analysis

ORFs selected for profiling included 18 validated resistance-promoting ORFs; 19 kinase ORFs unable to confer erlotinib resistance in the primary ORF screen (as measured by a z-

score less than 0.2 under both drug doses) (Chapter 1); as well as controls. PC9 cells were seeded overnight in 384-well microtiter plates at a density of 400 cells per well. The following day, cells were incubated with lentivirus corresponding to ORFs in the presence of 4 µg/mL polybrene, spin-infected at 2200 rpm for 30min at 30°C, then incubated at 37°C for an additional 4.5h before replacing media with standard growth media. At 24h post-infection, additional standard growth media was spiked into wells. At 72h post-infection, media was replaced with media containing 300 nM erlotinib. After 24h of drug treatment, media was aspirated and replaced with TCL Buffer (Qiagen) for cell lysis. Plates were incubated at 25°C for 25 min, then stored at -80°C until gene-expression profiling steps.

Gene-expression profiles consisted of 978 transcripts that were selected by the LINCS program (lincscloud.org) to represent an unbiased reduced representation of the transcriptome and measured using a Luminex bead-based system [54]. Each ORF was assayed and profiled in quadruplicate and all expression data were quantile-normalized. To quantify the magnitude of differential expression in our data we computed robust z-scores for each gene in each sample according to,

$$z_i = \frac{X_i - \text{median}(Y)}{\text{MAD}(Y) \times 1.4826}$$

where X_i is the scaled expression value of the sample of interest, Y is the vector of observed control expression values for the gene of interest, and MAD is the mean absolute deviation.

After computing a robust z-score vector for each replicate, we combined the robust z-scored replicate vectors into a single representative vector that we refer to as a signature. Unsupervised hierarchical clustering using the Spearman correlation metric was performed on signatures generated from PC9 cells expressing 18 resistance-inducing genes, 19 kinases unable to induce resistance, and controls. Hierarchical clustering revealed a tight cluster comprised of 12 resistance-promoting genes and the 2 *EGFR* double-mutant positive controls.

Querying the compound signatures in the LINCS dataset was performed as follows: signatures from each of the 12 resistance-promoting genes were used to independently query compound signatures in the LINCS dataset. Each compound–ORF query pair was assigned a connectivity score [23] computed using the weighted Kolmogorov-Smirnov statistic [55]. All compounds in the dataset were rank-ordered by their connectivity scores to a given ORF query. To identify compounds that were consistently correlated/anti-correlated to the query ORFs, we computed every compound's median normalized rank across all 12 ORFs. The resultant ranks are displayed in Figure 2-2. An analogous analysis was performed to query the ORF signatures in the LINCS dataset.

Comparative marker selection analysis was performed using GENE-E software.

Viral transduction and culture of ORF-expressing cells for protein analysis

Cells were seeded in 6-well plates at a density of 54,000 cells per well. The next day, cells were incubated with lentivirus in the presence of 4 µg/mL polybrene for 6-7h, after which media was replaced with standard growth media. At 24h post-infection, media was replaced with selective media containing 1-1.3 µg/mL blasticidin, and blasticidin-containing media was replenished after another 72h. At 6d post-infection, cells were treated with media containing 0.5% FBS overnight. The following day, cells were treated with inhibitor(s) or DMSO at their final concentrations in media containing 0.5% FBS for 6h, then harvested for immunoblotting.

Immunoblotting

Cell pellets were resuspended in lysis buffer (50 mM Tris pH 7.4, 2.5 mM EDTA pH 8, 150 mM NaCl, 1% Triton X-100, 0.25% IGEPAL CA-630), supplemented with protease inhibitors (Roche) and Phosphatase Inhibitor Cocktails I and II (Calbiochem), incubated on ice for 2 min, then centrifuged for 2 min at 13,000 rpm. The protein concentrations of supernatants were determined using a BCA Protein Assay Kit (Pierce) and normalized. Lysates were reduced and

denatured, then separated using Tris-Glycine gels (Novex) and transferred to iBlot Transfer Stack nitrocellulose membranes (Novex). Membranes were incubated with primary antibodies overnight at 4°C. The antibody against phospho-EGFR (Y1068) (1:1,000) was purchased from Invitrogen. The antibody recognizing total EGFR (1:1,000) was purchased from BD Biosciences. Antibodies against total AKT (1:1,000), phospho-AKT (S473, 1:750), total ERK1/2 (1:750), and phospho-ERK1/2 (T202/Y204) (1:500) were purchased from Cell Signaling Technology. Incubation with IRDye secondary antibodies (1:10,000) (LI-COR Biosciences) and subsequent detection (Odyssey Imaging System, LI-COR Biosciences) were performed according to manufacturer recommendations.

Drug sensitivity assays

For drug sensitivity assays of ORF-expressing cells: PC9 cells were seeded overnight in 384-well microtiter plates at a density of 400 cells per well. The following day, cells were incubated with lentivirus (virus production methods described above) corresponding to candidate resistance-mediating ORFs as well as controls in the presence of 4 µg/mL polybrene, spin-infected at 2200 rpm for 30min at 30°C, then incubated at 37°C for an additional 4.5h before replacing media with standard growth media. At 24h post-infection, additional standard growth media was spiked into wells. At 72h post-infection, media was replaced with media containing inhibitor(s) at their final concentrations or DMSO (1:1,000 dilution). For drug sensitivity assays of parental cells: PC9 cells were seeded overnight in 384-well microtiter plates at a density of 800 cells per well. The following day, cells were treated with media containing inhibitor(s) or DMSO.

Cell viability was assayed 3d after the addition of inhibitor(s) or DMSO using CellTiter-Glo (Promega). Drug-treated cells were normalized as described in the figure legends to

calculate relative percent viability. Relative percent viability values and dose-response curves were plotted using GraphPad Prism software (GraphPad).

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CHAPTER 3

A Genome-Scale shRNA Screen for Mediators of EGFR-Inhibitor Response

Attributions:

All experiments and analyses were performed by Tanaz Sharifnia except as follows:

Primary screening was designed and performed by Tanaz Sharifnia, with assistance from Glenn Cowley and Jessica Hsiao. Analyses represented in Figures 3-2B and 3-2C were performed by Marcin Imielinski.

LINCS queries were designed and performed by Victor Rusu and Aravind Subramanian.

ABSTRACT

Activating mutations in the epidermal growth factor receptor (*EGFR*) gene are the major genetic determinant underlying clinical response of non-small cell lung cancers (NSCLCs) to the EGFR-tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib. Despite the marked efficacy of EGFR-targeted therapies in NSCLC patients harboring these mutations, however, the development of acquired drug resistance in these patients limits the ability of EGFR-TKIs to serve as a long-term treatment strategy. We have sought to uncover mediators of EGFR-TKI response, and loss-of-function mechanisms of drug resistance, using an RNA interference screening approach, potentially providing insight into new therapeutic opportunities to overcome acquired resistance. Using this approach, we have identified numerous candidate genes for further investigation and present intersecting findings with gene-expression-based studies of EGFR-TKI resistance.

INTRODUCTION

The epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib are effective therapies for non-small cell lung cancers (NSCLCs) harboring activating mutations in *EGFR* [1,2,3,4,5,6,7,8,9], but all patients eventually develop resistance to therapy [10]. While mutations in *EGFR* provide a genetic determinant of sensitivity to EGFR-TKIs, this sensitivity cannot be invoked simply by expressing mutant *EGFR* in transfected cells [11]. Rather, *EGFR* 'oncogene addiction' seems to imply that dependence on the EGFR pathway relies on not only these mutations but the presence of a unique cellular context-- one that may potentially be exploited therapeutically in addition to targeting the receptor itself. We have sought to identify genes that mediate EGFR-TKI sensitivity and thus cooperate to maintain the 'oncogene-addicted' state in *EGFR*-mutant cells, potentially providing insight into alternative

'druggable' targets that could be used to develop long-term treatment strategies and overcome acquired resistance.

To this end, we have screened for genetic mediators of drug sensitivity in an *EGFR*-mutant cell line using an unbiased pooled RNAi screening approach. Genome-wide multiplex shRNA screening strategies aim to systematically interrogate gene function in a given biological system in an unbiased fashion. This approach involves infecting cultured cells with a pool of shRNAs, allowing the cells to proliferate for a period of time, selecting cells displaying a desired phenotype (often, survival), isolating the shRNA sequences from the selected cells by PCR amplification, and measuring the relative abundance of the shRNAs using either a microarray or next-generation sequencing [12,13,14,15]. This study describes a genome-wide RNAi approach to interrogate sensitivity to the EGFR-TKI erlotinib. Using this approach, we have identified numerous candidate genes for further investigation, a subset of which intersect with gene-expression-based studies of EGFR dependence.

RESULTS

A genome-scale pooled shRNA-based screen for mediators of erlotinib sensitivity

We have performed a genome-scale, multiplex shRNA screen to interrogate sensitivity to the EGFR tyrosine kinase inhibitor erlotinib. *EGFR*-mutant, erlotinib-sensitive PC9 NSCLC cells were infected with a genome-scale lentiviral pool of shRNAs (~90K unique shRNAs targeting approximately 17,000 genes; RNAi platform, Broad Institute) at an MOI of 0.3, and following selection for infected cells, treated with 70 nM or 250 nM erlotinib (corresponding to ~97% and ~99% cell killing over the course of the screen, respectively) or DMSO. Cells were subsequently cultured for approximately 1-2 weeks, and surviving (i.e. drug-resistant) cells at two time points were harvested and subjected to downstream DNA processing and shRNA deconvolution by next-generation sequencing (Figure 3-1).

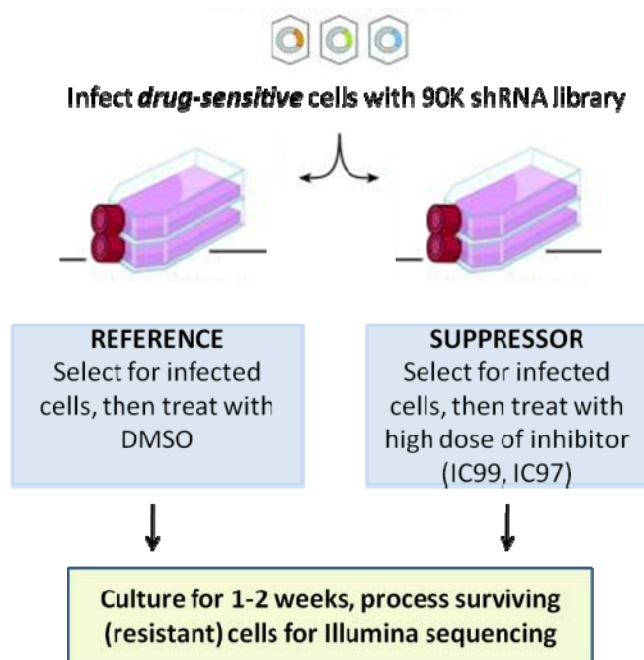


Figure 3-1. Loss-of-function pooled shRNA screening approach. Pooled shRNA screening workflow. *EGFR*-mutant, erlotinib sensitive PC9 cells were infected with a pooled shRNA library of approximately 90K shRNAs. Following selection for infected cells, cells were treated with 70 nM or 250 nM erlotinib (suppressor treatment arm) or DMSO (reference treatment arm). Surviving cells were harvested after 9 and 16 days of DMSO/drug treatment and processed for shRNA deconvolution using next-generation sequencing (Illumina). Figure adapted from [12].

In seeking to distinguish shRNAs reflecting biological ‘hits’ from those corresponding to background signal—a task potentially confounded by the presence of multiple screening conditions and the absence of a known positive control-- we have devised a new analytical approach to identify candidate erlotinib-sensitizing genes that allows us to integrate data generated from all screening conditions, without reliance on calibration to known reference genes or inference of on/off-target hairpin performance. This approach is based on 1) the observation, made by comparing the distributions of sequencing reads across the six screening conditions (early and late time points for each of three treatment conditions: DMSO, 70 nM erlotinib, and 250 nM erlotinib), that the proportion of depleted hairpins escalates coordinately with drug dose and time (Figure 3-2A), and 2) the premise that shRNAs conferring drug resistance will become successively enriched in a population as increasingly stringent selective conditions are imposed.

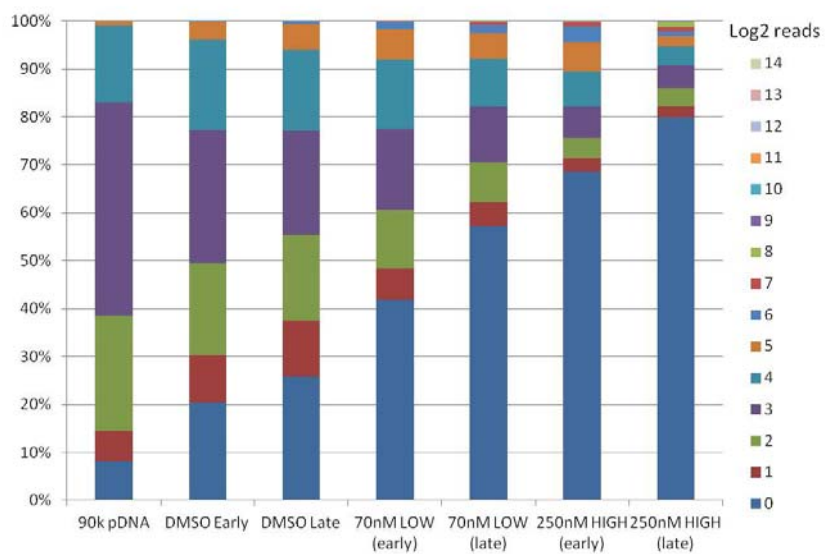
In this way, we identified shRNAs whose relative abundance increases monotonically as a function of selective pressure. The analysis displayed in Figure 3-2A suggested that screening conditions could be ordered by their increasing degree of stringency as follows: DMSO early, DMSO late, 70 nM erlotinib early, 70 nM erlotinib late, 250 nM erlotinib early, and finally, 250 nM erlotinib late. We next identified ~7000 shRNAs that were enriched relative to DMSO under the most stringent condition, late time point, 250 nM erlotinib-treatment (Figure 3-2B, left). These enriched shRNAs were then clustered by their relative abundance to DMSO across all screening conditions, with screening conditions plotted in increasing order of stringency (Figure 3-2C). Three of eight clusters were found to display the expected pattern of shRNA enrichment with increasing drug dose and treatment duration (Figure 3-2C, red boxes; Figure 3-2B, right). To further filter out off-target effects, candidate erlotinib-sensitizers were designated as genes that had at least two shRNAs represented among these three cluster groups. These resultant 566 genes (listed in Table 3-1) were carried forward for functional validation.

Figure 3-2. Analytical methods used to detect shRNAs enriched with drug treatment. (A)

The proportion of depleted shRNAs increases with drug dose and time. shRNAs across six screening conditions, along with the starting 90K plasmid pool, are binned according to their corresponding number of (log₂-normalized) sequencing reads. (B) Volcano plots illustrating p-value vs. fold- change of the 250 nM late condition relative to DMSO. Left, all points with a positive fold-change were subjected to the clustering analysis in (C). Right, points in red represent shRNAs belonging to clusters 1, 3, and 5 described in (C). (C) ~7000 shRNAs found to be enriched over DMSO in late time point, 250 nM drug-treated samples (see (B), left panel) were clustered by their relative abundance to DMSO across all screening conditions, plotted in increasing order of stringency. Clusters 1, 3, and 5 (red boxes) were selected as those exhibiting the expected pattern of enrichment.

Figure 3-2 (Continued)

A



B

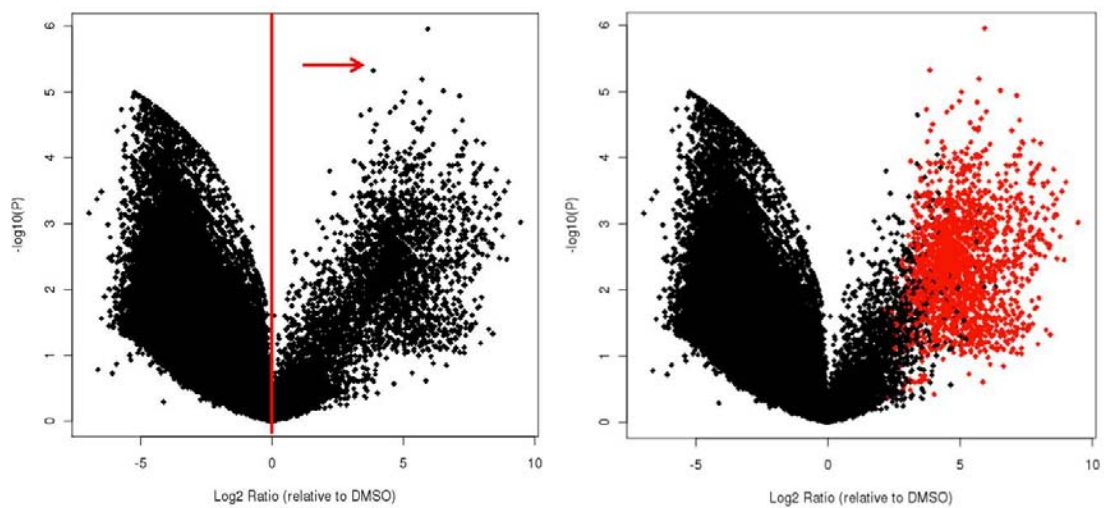


Figure 3-2 (Continued)

C

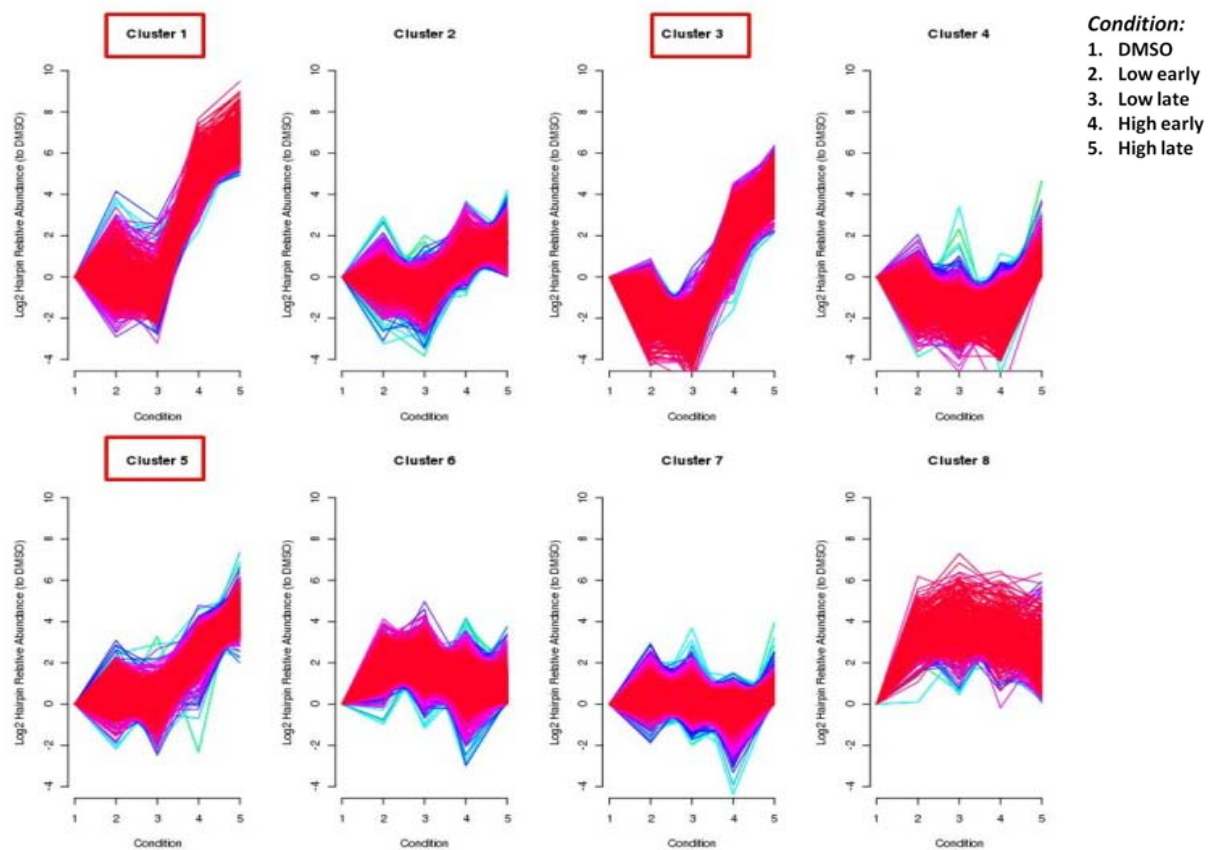


Table 3-1. Candidate erlotinib-sensitizing genes. 566 genes with 2 or more shRNAs represented among clusters 1, 3, and 5 in Figure 3-2C.

ABCF1	BCR	CDK9	DHCR24	GBAS	ITGB1BP1	MBTPS2	NOP10	POLR3K	RFC5	SFPQ	TES	UCHL1
ABHD15	BICC1	CDR2L	DKC1	GCC1	ITM2B	MCEE	NPR1	POLRMT	RFNG	SH2D4A	TFIP11	UFSP2
ABHD3	BMPR1B	CEP120	DNAJB12	GCLC	ITPK1	MCM3AP	NR2F2	PPA2	RGS5	SH3BGRL3	TGFB1	UHMK1
ABHD5	BNC2	CFL1	DOK4	GLMN	ITPR3	MDK	NR5A2	PPFIBP1	RIOK3	SHCBP1	THOC3	ULK1
ABHD6	BOLA3	CHD4	DUS4L	GLRX2	JTB	MEMO1	NRBP1	PPP1CA	RIPK1	SIRPA	THRA	UNC13B
ABL1	BRCA2	CHEK2	DYNC2L1	GLUD1	JTV1	MEST	NSBP1	PPP1CC	RNF6	SLC25A36	THUMPD1	UQCRB
ACAD8	BRD4	CHMP4C	E2F1	GNAQ	KCNN4	METTL11A	NSF	PPP1R11	ROCK2	SLC38A10	TIMM8B	USP1
ACCN2	BRMS1	CHMP7	EBP	GNS	KDELRL2	METTL13	NUBP1	PPP2CB	RP11-217H1.1	SLC7A5	TKT	USP12
ACO1	BSG	CHN1	ECD	GOLGA7	KIAA0528	MFS08	NUP205	PPP2R2C	RPL36AL	SLFN5	TM7SF3	USP25
ACSS3	BUD31	CIB1	EEF1A1	GOLPH3	KIAA0831	MGRN1	NXPH4	PPP3R1	RPL37	SMARCD1	TMBIM6	USP30
ACTB	C15orf24	CKAP2	EGLN2	GRPEL1	KIAA1303	MGST2	OAS1	PRICKLE1	RPL39	SMARCD2	TMEM120A	USP33
ACTR3	C16orf53	CLDND1	EIF2S1	GSTO1	KIAA1804	MIB1	OBFC2B	PRKAR2B	RPL39L	SMC5	TMEM14A	USP7
ADAR	C16orf62	CLIC1	EIF5A	GTF2F1	KIDINS220	MIF4GD	ODC1	PRKD2	RPL8	SNAP25	TMEM214	UTP11L
ADC	C17orf81	CLPP	EIF5B	GTF2H3	KIF3C	MKNK2	OLFML2A	PRKD3	RPL1	SNF1LK2	TMEM30A	VAPB
ADCK1	C19orf56	CLTC	ELAC2	GTPBP8	KRCC1	MLF2	OSBPL10	PROS1	RPP40	SNX11	TMEM38B	VHL
AFTPH	C1orf174	CMPK	EMG1	GUSB	KRT10	MLPH	OSBPL9	PRPS1L1	RPRD1B	SNX3	TMEM45A	VRK2
AIP	C21orf6	CTM16	EPHA7	H1FX	L3MBTL	MLX	P4HA2	PRPS2	RPS6KC1	SNX6	TMEM47	WASF3
AK1	C4orf27	COASY	ERCC1	HBS1L	LAMB1	MMAB	PAF1	PSEN1	RRM2B	SOD1	TMEM50B	WBP2
AK7	C6orf108	COBRA1	ERCC8	HD	LAMC1	MOSPD1	PAK1	PSMA4	RRP12	SOX9	TMEM70	WDR13
ALCAM	C9orf46	COG7	ESF1	HDAC11	LARP6	MOSPD2	PAPD4	PSMA6	RRP1B	SPAG5	TMEM85	WDR45
ALDH7A1	C9orf6	CORO2A	ESRRA	HDAC2	LARP7	MPP5	PAQR5	PSMA7	RSRC2	SPHK1	TNKS	WDR8
ALG8	C9orf80	COX4NB	ETV6	HEATR1	LBR	MRLC3	PARVB	PSMB8	SAMHD1	SPOP	TOMM34	XRCC6
AMD1	CA9	CPNE2	EXO1	HINT2	LEO1	MRLC2	PAWR	PSMC3IP	SAP130	SPR	TOMM7	XRN2
AMOT	CALM2	CPSF1	F2R	HN1	LEPREL4	MRPL22	PCCB	PSMC5	SARS	SRP14	TOX4	ZAK
ANKRD11	CALM3	CPSF3L	FAM136A	HNRPH2	LHPP	MRPL32	PCTK1	PSMD1	SAT2	SRPR	TP53	ZBTB80S
ANXA10	CALR	CRABP2	FAM162A	HRB	LPCAT4	MRPS22	PDCD6IP	PSMD6	SBF1	STAG2	TPD52L3	ZC3H14
AP1S2	CALU	CSGALNACT2	FAM172A	HSCB	LPIN2	MRPS31	PDIA3	PSMF1	SCAMP1	STAT3	TRIM16	ZCCHC10
AP2M1	CAMK1D	CSK	FAM175B	HSPA9B	LRBA	MSH2	PDK1	PSTPIP2	SCAP2	STK24	TRIM24	ZFAND1
APEX1	CAND1	CSNK1A1	FAM48A	HSPB1	LSM7	MTFR1	PEX11B	PTCD1	SCARB2	STK32B	TRIM36	ZFR
ARFIP1	CAPG	CSNK1G2	FAM50A	HSPB8	LTB4DH	MTHFD1	PEX3	PTDSS1	SCCPDH	STK32C	TSC22D1	ZHX3
ARHGAP18	CASK	CTSL	FAM65A	IDH1	LUM	MYLK	PFKFB1	PTGES	SCPEP1	STOM	TSPAN14	ZMYM4
ARL2BP	CASP9	CUL2	FASTKD2	IFI35	MAGI3	NACA	PFKFB2	PTK2	SCRN1	STUB1	TSPAN6	ZNF263
ARMC8	CCDC104	CUL4B	FAU	IFT81	MANEA	NARS	PFKL	PTTG1	SCYL1	STX12	TSPYL1	ZNF426
ARPC1B	CCDC91	CYB5B	FBXO17	IHPK1	MAP1B	NCKAP1	PGK1	PXMP4	SDCCAG10	STX18	TTC35	ZNF45
ARPC3	CCDC92	CYBA	FBXO5	IKBKB	MAP2K2	NDUF4F4	PHACTR2	RAB11FIP1	SEC14L4	SUB1	TTK	ZNF767
ATOH8	CCND3	CYCS	FEZ2	ILF2	MAP3K11	NDUF87	PHB	RAB25	SEC61A1	SUCLG1	TUBA6	ZNHIT1
ATP2B1	CCT3	CYP51A1	FGL1	ILF3	MAP3K6	NDUF54	PHKA1	RAD23B	SEC61A2	SULT2B1	TUBB2C	ZW10
ATP5G2	CCT4	DCAF6	FKBP10	INTS3	MAPK1	NEDD8	PIGV	RAD51	SEC61B	TAF11	TXNDC5	ZWINT
ATP6V0C	CCT6B	DCAF7	FKBP1B	IP6K1	MAPK15	NFU1	PLCB3	RAE1	SECTM1	TARBP2	UAP1	
ATP6V0D1	CDC2L5	DDX23	FKBP3	IPO7	MAPK1IP1L	NLK	PLEKHA1	RANGAP1	SELT	TARS	UBB	
AUP1	CDC42BPB	DDX46	FLII	IPP	MAPK8	NMD3	PLEKHM1	RBMX2	SEPT7	TBC1D1	UBC	
BCKDHA	CDIPT	DECR1	FLJ25476	IRAK1	MARK4	NME2	PLK1	RCAN3	SEPW1	TCEB3	UBE2Q1	
BCKDK	CDK5	DENR	FOSL2	ITFG1	MAST2	NME7	POLD1	RER1	SERINC1	TCP1	UBE3B	
BCL3	CDK5RAP3	DGKE	GAK	ITGAV	MBD4	NOL8	POLR2I	REXO2	SETD4	TCTN3	UBE3C	

Functional validation approaches of candidate erlotinib-sensitizing genes

The 566 genes described above were first used to perform a pathway analysis (Figure 3-3) (IPA, Ingenuity Systems) to identify biological functions that are significantly associated with candidate-erlotinib sensitizing genes. The most strongly-associated biological function was post-translational modification, followed by molecular transport, protein trafficking, and cell death.

We pursued a two-pronged approach to validate candidate erlotinib-sensitizing genes. The first approach was to perform low-throughput validation of a small and biased set of genes. Experimental approaches included assaying target gene expression using immunoblotting and quantitative PCR (qPCR), and measuring changes in drug sensitivity induced by these shRNAs using standard multipoint growth inhibition assays. The second approach was to pursue high-throughput and unbiased validation of all 566 candidate erlotinib-sensitizing genes. In this case, the experimental approach was to perform secondary screens using a targeted 'minipool' of 'hit' shRNAs and controls in additional *EGFR*-mutant, erlotinib-sensitive cell lines. Given the large set of 'hit' genes, secondary screens using a targeted minipool, which may be more readily scaled to test a large number of genes across multiple cell lines and conditions, were favored to an arrayed format.

Six 'hit' genes of particular biological interest were chosen for low-throughput validation (*RAD51*, *MBD4*, *ZBTB80S*, *IDH1*, *PTTG1*, and *BMP1B*). Each of these genes had at least 3 shRNAs enriched over DMSO in the primary screen. To assess the on-target effects of these genes' corresponding shRNAs in the primary screen, we compared the enrichment of these shRNAs in the primary screen with their ability confer gene knockdown, as measured by high-throughput qPCR data (previously generated to assess library performance by the RNAi Consortium, Broad Institute) (Figure 3-4). This analysis suggested a strong relationship between shRNAs that scored most significantly in the primary screen (as assessed by p-value; see Figure 3-2B, left) and the degree of target knockdown (Figure 3-4), suggesting that greater

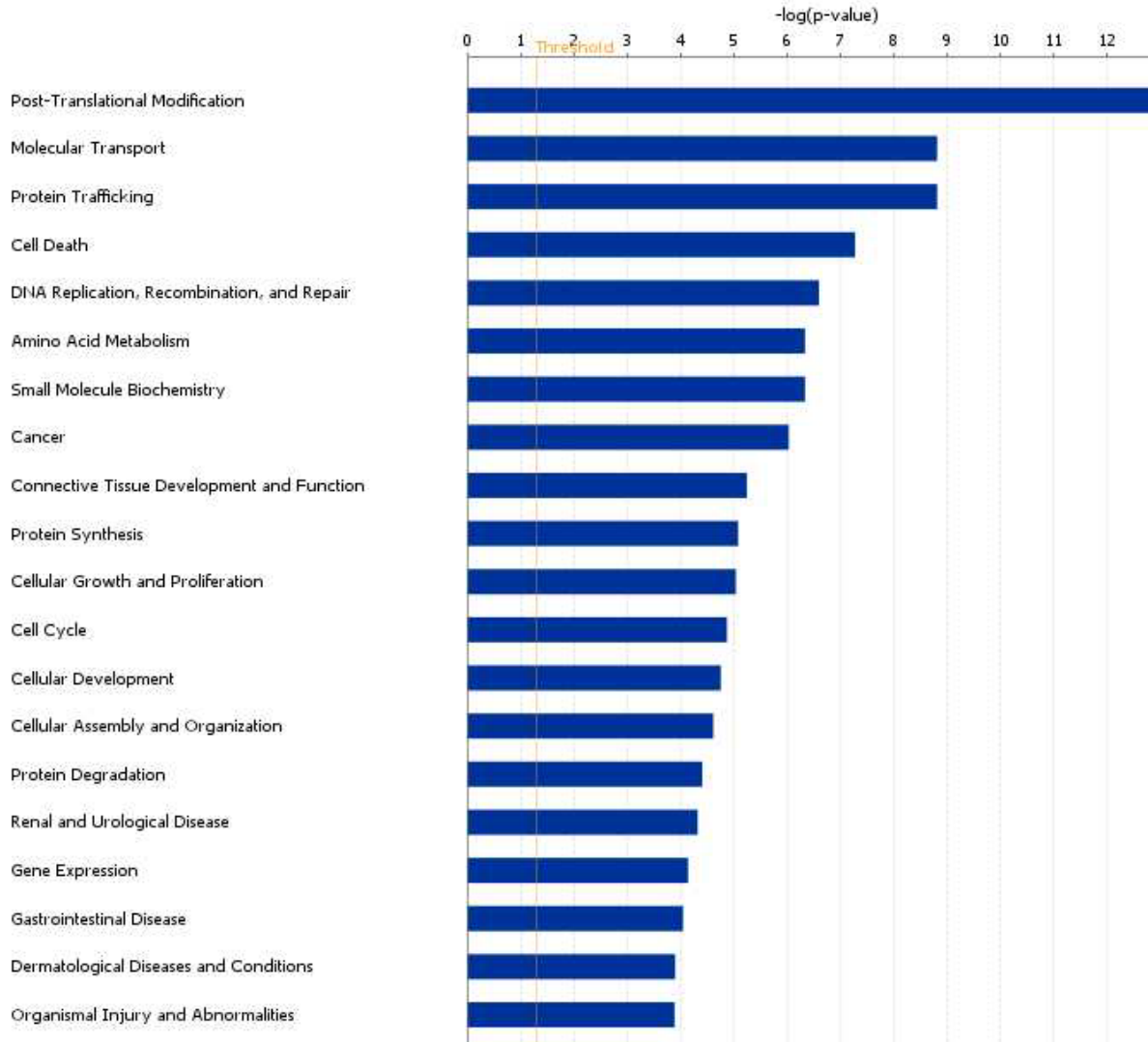


Figure 3-3. Biological functions associated with candidate erlotinib-sensitizing genes.

566 candidate erlotinib-sensitizing genes were used to perform IPA pathway analysis (Ingenuity Systems) to identify significantly associated biological functions.

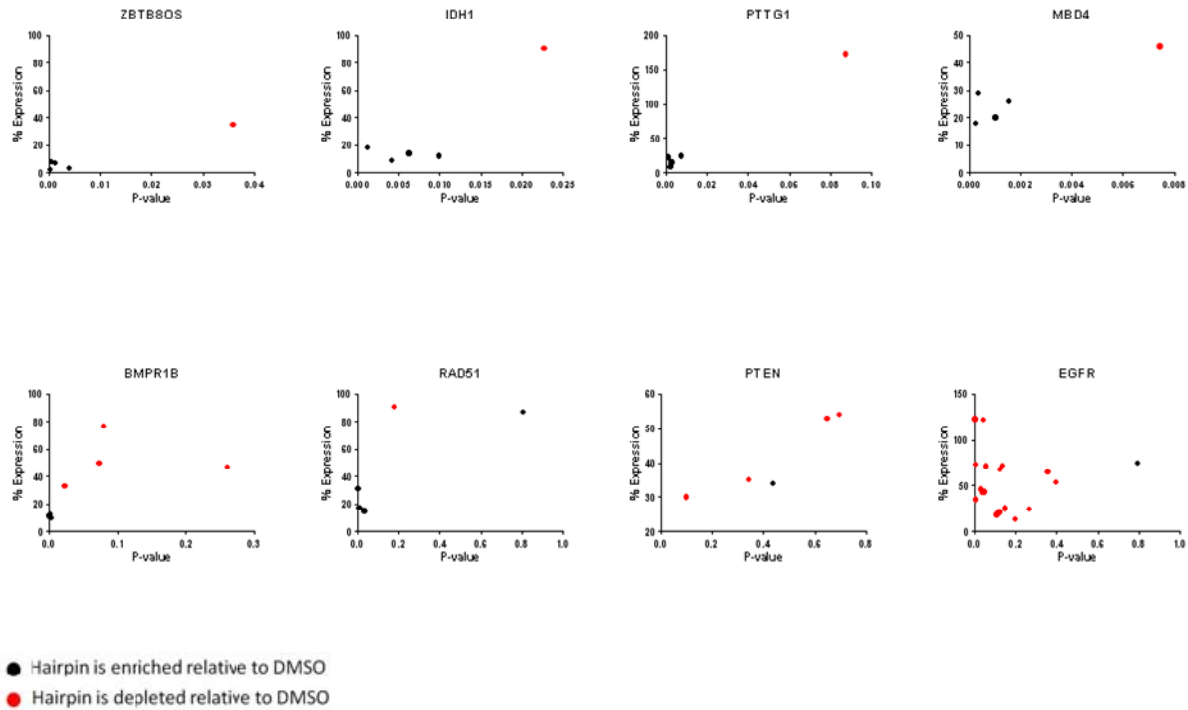


Figure 3-4. On-target effects of candidate resistance-mediating shRNAs. Selected ‘hit’ genes and the screening performance of their associated shRNAs versus degree of target knockdown. Enrichment in the primary screen is measured by a given shRNA’s p-value (see Figure 3-2B, left), and percent gene expression is measured by high-throughput qPCR. Black points denote shRNAs that are enriched relative to DMSO, and red points denote shRNAs that are depleted relative to DMSO. Clustering of black points near the origin suggests on-target shRNA effects. *PTEN* and *EGFR* genes are listed for reference.

knockdown is associated with more significant enrichment under erlotinib treatment.

To test whether shRNAs that are highly enriched in the primary screen and appear to be on-target can induce erlotinib resistance in validation assays, we performed multipoint drug sensitivity assays on PC9 cells expressing candidate resistance-mediating shRNAs. shRNAs were chosen based on enrichment in the primary screen (based on p-value) as well as evidence of knockdown as measured by high-throughput qPCR data. In several cases, knockdown was also confirmed by Western blot (data not shown). PC9 cells were transduced with two shRNAs for each of the six genes listed above, and cells stably expressing these shRNAs were subjected to 72-hour drug-sensitivity assays (Figure 3-5). While these 'hit' shRNAs were expected to shift drug sensitivity curves in a manner consistent with drug resistance, no appreciable shift was observed (Figure 3-5) relative to cells transduced with shRFP.

It is unclear what is underlying the discrepancy between the findings of Figure 3-4 and Figure 3-5. The inability of these selected genes to validate the primary screening results, despite exhibiting patterns of knockdown-dependent enrichment in the primary screen, suggests that knockdown of these genes may have provided some fitness advantage unrelated to EGFR-inhibitor resistance. These data also suggest that *bona fide* resistance-mediating shRNAs may represent a smaller fraction of candidate shRNAs than initially expected.

Thus, high-throughput validation approaches are being pursued using a targeted minipool of hit shRNAs (described in Materials and Methods). Next steps will be to screen additional cell lines using multiple drug doses and a longer time course than that of the primary screen, to elicit a higher level of selection. To avoid selection of potentially preexisting gatekeeper mutation-positive subpopulations during the course of this longer follow-up screen, single cell clones of the original screening cell line (PC9) were isolated and confirmed to be WT at the gatekeeper residue (T790) as well as positive for the primary *EGFR* mutation.

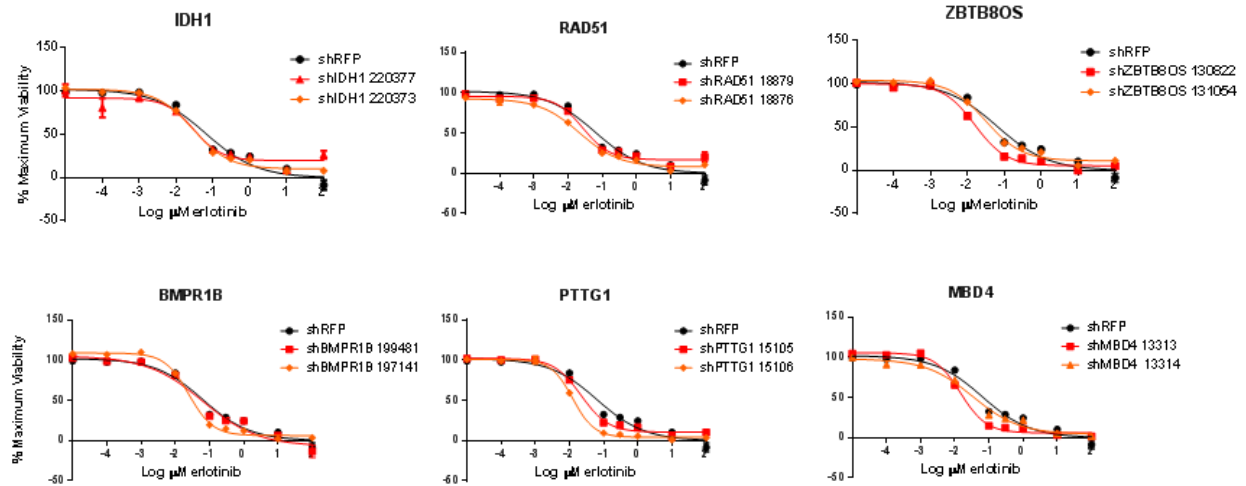


Figure 3-5. Selected candidate resistance-mediating shRNAs do not confer erlotinib resistance in validation assays. PC9 cells transduced with selected candidate resistance-mediating shRNAs were treated with increasing doses of erlotinib and assayed for cell viability after 72h using the WST-1 reagent. Data are expressed as percent viability relative to vehicle-treated cells and represent the mean \pm SD of 5 replicates. Graphs with identical control curves reflect experiments performed in parallel on the same day.

Intersection of screening findings with gene-expression-based studies of EGFR-TKI sensitivity

We have previously described a gene-expression-based strategy for identifying genetic and chemical perturbations that can induce or reverse EGFR-TKI resistance (Chapter 2). This approach entailed using an expression-profiling resource created by the Library of Integrated Network-based Cellular Signatures (LINCS) program (lincscloud.org) to identify genes and compounds whose transcriptional effects mimic those of erlotinib-resistance-mediating open reading frames (ORFs) (Chapter 2). As the LINCS database contains gene-expression profiles generated from human cells treated with shRNA reagents, we asked if an analogous query of LINCS shRNAs would yield target genes that intersect with our pooled screen findings.

Gene-expression signatures from cells expressing resistance-mediating genes were independently used to query LINCS, as described in Chapter 2. In this case, we looked for shRNA perturbations whose transcriptional effects were correlated with those of resistance-inducing genes. We hypothesized that, on the basis of their shared transcriptional effects, such shRNAs could potentially also induce erlotinib resistance and thus may overlap with pooled shRNA findings. We observed that, among the LINCS shRNA target genes that most positively-correlate with resistance-mediating ORFs (the top 5% of all shRNA target genes), 14 genes intersect with pooled screen candidates (Figure 3-6). We noted that one of these genes, *CSK* or C-Src kinase, is a known negative regulator of the Src-family of kinases [16,17] and thus is consistent with previous work implicating the Src-family of kinases in EGFR-inhibitor resistance (Chapter 1). On the basis of their identification using orthogonal approaches, these 14 genes warrant further study for their ability to mediate response to EGFR inhibition.

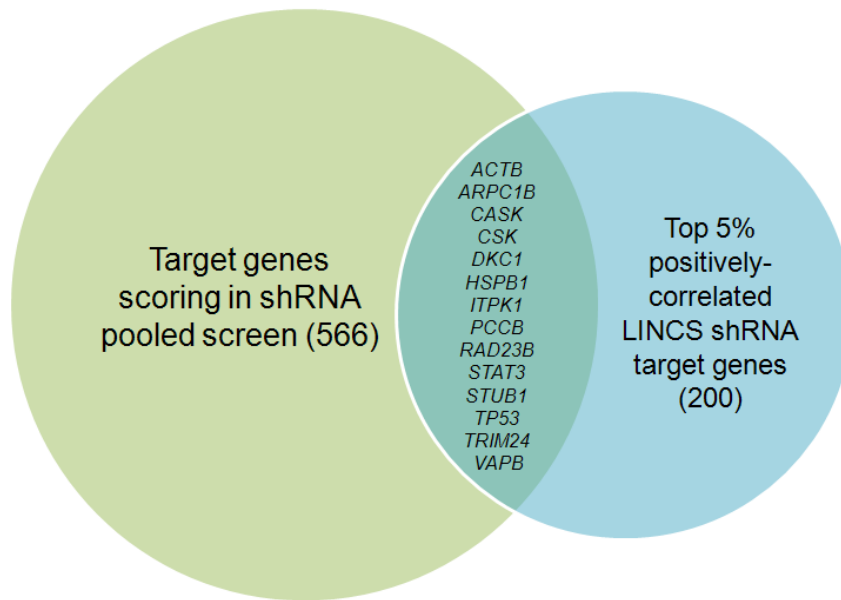


Figure 3-6. Intersection of candidate erlotinib-sensitizing genes nominated by pooled RNAi screening and LINCS analysis. The LINCS database was queried to identify shRNA target genes whose transcriptional effects correlate with those of resistance-inducing ORFs. The candidate erlotinib-sensitizing genes that are common to both the LINCS analysis and the pooled RNAi screening approach are listed.

DISCUSSION

In this study, we have aimed to screen for mediators of EGFR-TKI sensitivity, and loss-of-function mediators of EGFR-TKI resistance, in an EGFR-dependent model. While EGFR-TKI resistance is commonly attributable to gain-of-function alterations, such as amplification of the receptor tyrosine kinase *MET* [18], loss-of-function events have also been implicated in reduced EGFR-TKI sensitivity. Examples include loss of the phosphatase and tensin homolog (*PTEN*) gene, and lower expression of the pro-apoptotic BCL2 family member BIM, which are both associated with reduced response to EGFR-TKIs [19,20]. These observations suggest that certain genes mediate EGFR-TKI response, and thus lead to drug resistance when lost or downregulated. Here, we report a genome-scale shRNA-based screen to identify genes whose suppression can confer resistance to EGFR inhibition, thus representing mediators of erlotinib sensitivity in EGFR-dependent cells.

This approach yielded a large number of candidate erlotinib-sensitizing genes, though preliminary validation data (Figure 3-5 and data not shown) suggest that many of these genes are likely not erlotinib-sensitizing. Current efforts include a more systematic and unbiased validation approach using a targeted minipool of candidate resistance-mediating shRNAs. Intersection of screening findings with a LINCS-based query, however, has identified 14 genes that display evidence of being erlotinib-sensitizing using two independent approaches. Collectively, these data identify a large number of candidate erlotinib-sensitizing genes, and nominate a subset of genes for further investigation.

MATERIALS AND METHODS

Cell culture and reagents

The *EGFR*-mutant NSCLC cell line PC9 (del E746_A750) has been described previously [21]. Cells were maintained in RPMI (Cellgro) supplemented with 10% FBS (Gemini Bioproducts). Erlotinib was purchased from LC Laboratories.

Pooled shRNA screen

Screening was performed using a genome-scale library of 89,771 shRNAs (Broad Institute pooled 90K shRNA library). PC9 cells were incubated with lentivirus corresponding to the 90K pooled shRNA library in the presence of 4 µg/mL polybrene, dispensed in 12-well plates (24 plates with 3×10^6 cells per well), and spin-infected at 2000 rpm for 2h at 30°C. Lentivirus was titered to achieve a MOI of approximately 0.3. After spin-infection, virus-containing media was replaced with standard growth media, and cells were incubated at 37°C overnight. The following day, cells were trypsinized and pooled, then expanded for 4 days in selective media containing 2 µg/mL puromycin. Five days after infection, cells were divided across drug-treated (approximately 100×10^6 cells for each of 12 replicates) and DMSO-treated (30×10^6 cells for each of 3 DMSO replicates) arms; cells were treated with DMSO (3 replicates), 70 nM erlotinib (6 replicates), or 250 nM erlotinib (6 replicates), in 2 µg/mL puromycin-containing media. Media containing DMSO or erlotinib, plus 2 µg/mL puromycin, was replenished every three days, and cells were re-plated as needed until cell harvests at two timepoints. After 9 days of drug/DMSO treatment (early timepoint), all cells were collected for three replicates each of the two drug-treated arms, and $>30 \times 10^6$ cells were harvested for each of the three DMSO replicates. After 16 days of drug/DMSO treatment (late timepoint), all cells were collected for the remaining drug-treated replicates (3 replicates per dose), and 80×10^6 cells were harvested for each of the three DMSO replicates. Cells were stored at -20°C in PBS until genomic DNA isolation steps.

shRNA deconvolution by Illumina sequencing

Genomic DNA was purified from harvested cells (all 12 drug-treated replicates and 1 DMSO replicate from each timepoint) using the QIAamp DNA Blood Kit (Qiagen), and shRNA regions were amplified from purified genomic DNA using a nested PCR reaction. Conditions for the first round of amplification have been described previously [22]. Here, up to 6 µg of genomic DNA was used for each PCR reaction, and up to 29 parallel PCR reactions were performed for each sample. Thermal cycler conditions for the first round of amplification were 95°C for 5 min; 18 cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 20 sec; and 72°C for 10 min. Parallel PCR reactions for a given sample were pooled, then used to perform a second amplification round. Conditions for the second round of amplification were adapted from [22]. Thermal cycler conditions for the second round of amplification were 95°C for 1 min; 18 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 20 sec; and 72°C for 10 min. PCR-amplified DNA was pooled and processed for massively parallel sequencing (Illumina) as described previously [22].

Genomic DNA for each sample was also used to sequence *EGFR* for the presence of the resistance-associated T790M gatekeeper mutation [23,24] by Sanger sequencing. All samples were wild-type at position 790 (data not shown).

Identification of candidate resistance-mediating shRNAs

Sequencing reads were log₂-normalized as described previously [15]. A t-test was performed between 250 nM erlotinib-treated, late time point replicates and DMSO-treated replicates (early and late time points), and all shRNAs with a positive fold-change over DMSO were used for subsequent analyses. For each treatment condition, shRNA abundance was normalized to DMSO then subjected to clustering using Mfuzz soft clustering software [25]. Genes with 2 or more shRNAs represented among clusters 1, 3, or 5 (see Figure 3-2C) were designated as candidate erlotinib-sensitizing genes (listed in Table 3-1).

Quantitative real-time PCR

High-throughput quantitative real-time PCR data was previously generated by the Broad Institute's RNAi Platform.

Plasmids

The shRNA constructs used targeted: BMPR1B (TRCN0000197141 and TRCN0000199481); MBD4 (TRCN0000013313 and TRCN0000013314); IDH1 (TRCN0000220373 and TRCN0000220377); PTTG1 (TRCN0000015105 and TRCN0000015106); ZBTB8OS (TRCN0000131054 and TRCN0000130822); and RAD51 (TRCN0000018876 and TRCN0000018879).

Virus production

Lentivirus was produced by transfection of 293T packaging cells with plasmids corresponding to pLX-Blast-V5-ORF, Δ 8.9 (*gag, pol*), and VSV-G; and FuGene6 transfection reagent (Roche) as described previously [26].

Drug sensitivity assays

PC9 cells stably expressing candidate resistance-mediating shRNAs were seeded overnight in 96-well plates at a density of 4000 cells per well. The following day, were treated with erlotinib or DMSO (1:1,000 dilution). Erlotinib was tested at each of the following concentrations: 100, 10, 1, 0.3, 0.1, 0.01, 0.001, 0.0001 and 0.00001 μ M. Cell viability was assayed 3d after the addition of erlotinib or DMSO using WST-1Reagent (Roche). Drug-treated cells were normalized to DMSO-treated cells to calculate relative percent viability. Relative percent viability values and dose-response curves were plotted using GraphPad Prism software (GraphPad).

LINCS shRNA analysis

Gene-expression profiling and signature generation methods for resistance-mediating ORFs have been described previously (Chapter 2, Materials and Methods). Signatures from each of the 12 positive-phenotype genes (Chapter 2) were used to independently query consensus gene signatures (CGS) derived from multiple shRNAs in the LINCS dataset. Each CGS–ORF query pair was assigned a connectivity score [27] computed using the weighted Kolmogorov-Smirnov statistic [28]. All CGSs in the dataset were rank-ordered by their connectivity scores to a given ORF query. To identify CGSs that were consistently correlated to the query ORFs, we computed every CGS' median normalized rank across all 12 ORFs.

Curation of targeted minipool

A targeted minipool of candidate erlotinib-sensitizing genes was curated as follows. In an attempt to include biologically relevant shRNAs for each 'hit' gene while limiting the overall size of the minipool, three shRNAs targeting each of the 566 genes described above were manually curated based on two independent metrics: performance in the primary screen (shRNAs with most the significant q-value), and degree of knockdown as measured by high-throughput qPCR data (previously generated to assess library performance by the RNAi Consortium, Broad Institute). For controls, standard inert shRNAs (e.g. targeting GFP, RFP, luciferase); shRNAs targeting potentially biologically relevant genes (e.g. *EGFR*, *PTEN*); and an additional ~100 shRNAs that were deemed the most inert in DMSO early/late samples in the primary screen (but depleted with drug-treatment) were included. These ~100 shRNAs were included to help define the distribution of background signal.

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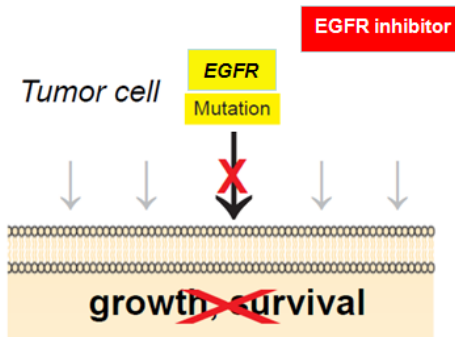
CONCLUSIONS

The phenomenon of ‘oncogene addiction’ has contributed to several paradigm shifts in the characterization and management of cancer. The understanding that a tumor cell can exhibit exquisite dependence on a single protein despite the accumulation of multiple genetic alterations has had important implications with respect to the diagnosis and classification of tumors, the patient selection criteria for a specific therapy, the types of therapeutic agents now used to treat cancers, and the way resistance to therapy develops and is treated. Drug resistance in particular is closely intertwined with the phenomenon of oncogene dependence, as the ‘addicted state’ presumably necessitates either retained activity of the primary oncogene in the presence of drug, or, in the face of successful oncogene inactivation, the enlistment of other genes with redundant functional effects. In this way, addiction and resistance represent two faces of a common coin in oncogene-driven cancers, related by their shared ability to sustain a specific cellular state.

The advent of large-scale functional screening libraries to perturb mammalian models has enabled unbiased and comprehensive interrogation of oncogene dependence and drug resistance. It is now possible to identify the full-range of genes that can enhance or suppress dependence on a given oncogene, using both gain- and loss-of-function approaches. Interrogating a wide-range of genes is useful not only in terms of broadening the scope of these studies, but because this may permit investigators to identify crucial commonalities among genes sharing a particular phenotype. This may be particularly relevant in the case of drug resistance, where the sufficiency to induce resistance in *in vitro* models does not necessarily translate to an endogenous resistance mechanism in the *in vivo* setting. Yet understanding shared features among various *in vitro* resistance mechanisms, even if many are not clinically relevant, may elucidate signaling effectors critical to oncogene bypass.

In our gain-of-function studies of EGFR-inhibitor resistance, for example, we identified a broad spectrum of kinase genes that could bypass dependence on EGFR (Figure C-1). In some respects, the kinases that were identified using this approach were relatively diverse: they

A



B

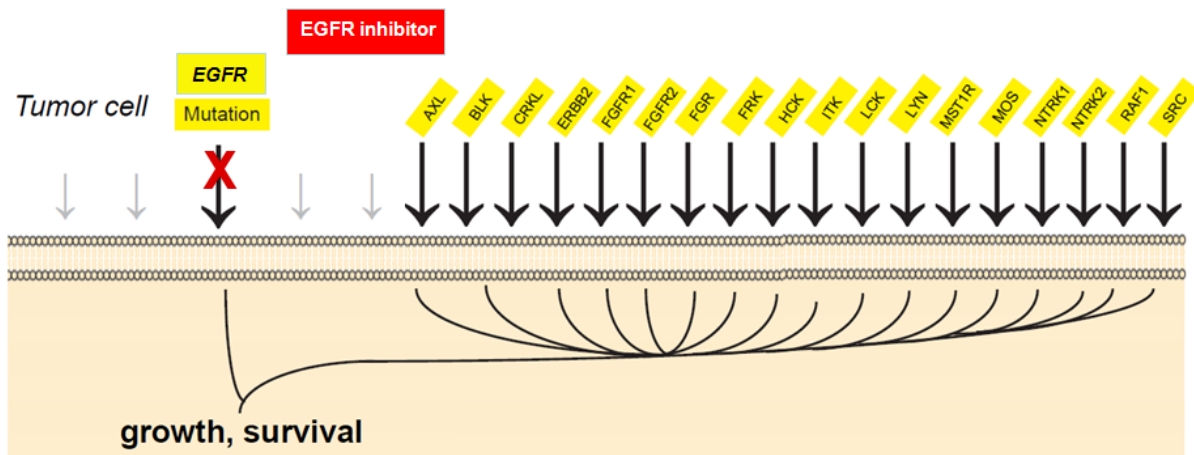


Figure C-1. Utilizing large-scale functional screening approaches to identify shared effectors of oncogene bypass. (A) *EGFR*-mutant cancers are singularly dependent on the *EGFR* signaling input (bold arrow) for activation of downstream signaling pathways, at the expense of other signaling inputs (gray arrows). These tumors are thus highly susceptible to *EGFR* inhibition with an *EGFR* inhibitor. (B) In Chapter 1, we identified a broad range of kinase genes with the ability to drive *EGFR*-dependent cells in the setting of *EGFR* inhibition. Identifying a spectrum of kinase genes with this function facilitated the unbiased identification of signaling effectors commonly required for *EGFR* bypass. A portion of this figure is reprinted from [1].

included several tyrosine kinase subfamilies of both receptor and non-receptor kinase classes, as well as serine/threonine kinases. Yet we determined that many of these resistance-mediating genes were remarkably similar with respect to the transcriptional changes they could effect. It was on the basis of their shared gene-expression signatures that we could, in an unbiased fashion, implicate the PI3K-AKT and MEK-ERK pathways as the critical and shared mediators of EGFR bypass for these genes (Figure C-1). One might imagine other scenarios in which several subclasses of resistance mechanisms could be defined using such an approach.

These studies also underscore the combined power of gene-expression profiling, the LINCS gene-expression resource, and pattern-matching software to identify functional relationships between genetic and chemical perturbations. Our gene-expression-based studies in Chapter 2 illustrate that a cell's gene-expression state can faithfully reflect its phenotypic behavior with respect to drug response and resistance. Remarkably, these gene-expression states were also highly context-independent. With this analysis, we were able to identify compounds that could reverse EGFR-inhibitor resistance and as well as compounds that could reduce EGFR-inhibitor sensitivity. We were also able to integrate findings from our ORF- and shRNA-based screens based on their shared transcriptional profiles. The genetic and chemical modifiers of EGFR dependence that were identified using these functional genetic and integrative approaches are summarized in Figure C-2.

Several questions emerge from these studies. For example, given the broad spectrum of kinases sufficient to drive EGFR-TKI-resistance, it is unclear why there appears to be preferential activation of specific bypass genes, such as *MET*, in the clinical setting. One possible explanation is that genes like *MET* provide additional fitness advantages or are more readily altered. Related to this, our finding that copy number gains of *FGR* and *LCK* frequently co-occur with *EGFR* alterations raises the question of if and how these alterations may influence EGFR dependence in the clinical setting. The ultimate goal of these studies remains how to better understand resistance as it relates to patients with cancer.

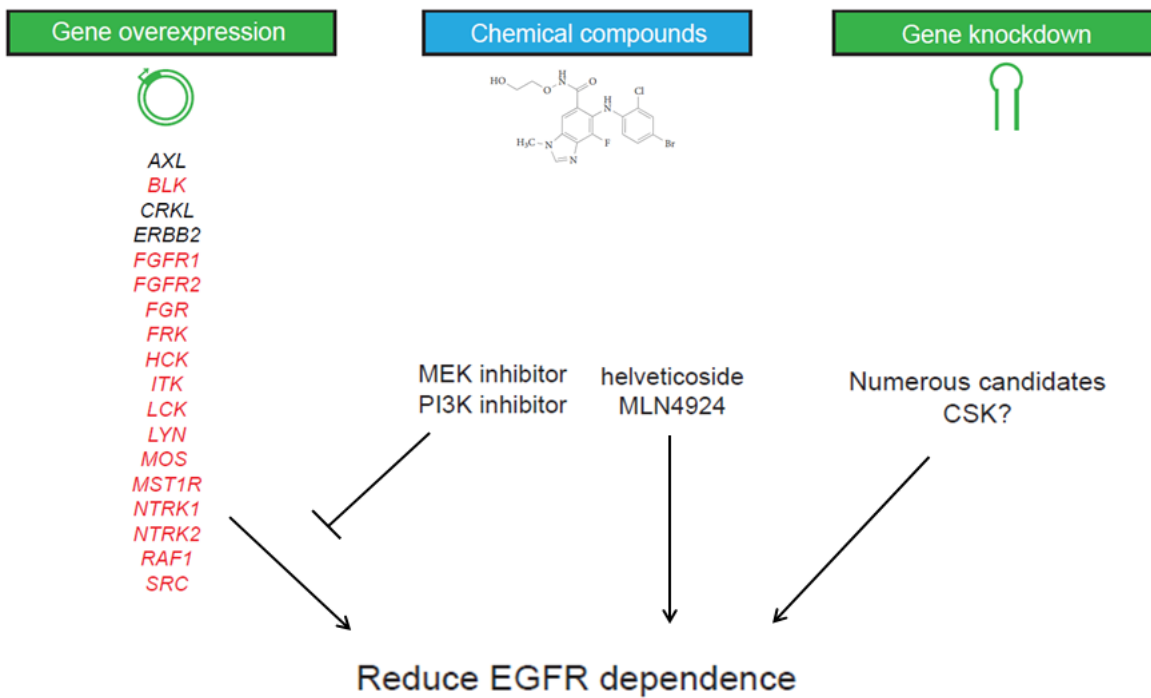


Figure C-2. Genetic and chemical modifiers of EGFR dependence in non-small cell lung cancer. Summary of genes whose overexpression can reduce EGFR dependence (left column); chemical compounds that can reverse ORF-mediated resistance or reduce EGFR-inhibitor sensitivity (middle column); and candidate genes whose knockdown can reduce EGFR dependence (right column). Newly-described resistance-mediating ORFs are labeled red. The chemical structure displayed is reprinted from [2].

In summary, we describe a methodology in which resistance-mediating genes were first identified via a functional genetic screening approach, then on the basis of their transcriptional effects, used to identify chemical compounds with the ability to reverse (or induce) resistance. As emerging evidence suggests that drug resistance is an inexorable outcome of kinase inhibitor therapy-- irrespective of cancer type, driver alteration, or drug target—similar experimental pipelines may permit investigators to anticipate resistance mechanisms and designate appropriate therapies in the preclinical, rather than post-relapse, phase. Moreover, while our studies focused on a drug whose target and downstream signaling effectors are well-characterized, this approach may be particularly useful for drugs with unknown or poorly characterized mechanisms of action. In this way, for a given therapy, functional screening tools may soon permit the simultaneous identification of resistance mechanisms and the inhibitors that can undo them.

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APPENDIX

Exploiting synthetic lethality in *EGFR*-mutant cancers

EGFR-targeted therapies for the treatment of *EGFR*-mutant NSCLC are modeled on the premise that one may achieve cancer-cell selectivity (and a high therapeutic index) by exploiting these cancer cells' unique addiction to the *EGFR* oncogene. In theory, an alternative approach to achieving cancer-cell selectivity could be to exploit non-oncogene addiction—that is, to inhibit not EGFR itself, but rather a target that is differentially required in an *EGFR*-mutant context versus a normal cellular context [1]. This principle of synthetic lethality as applied to anticancer therapy—referring to the situation wherein mutation of two genes, but not of either one alone, results in cell death—has proven to be powerful in practice, most notably in the case of PARP1 inhibition in the context of *BRCA1* or *BRCA2* mutant cells [2,3,4]. The challenge one faces in pursuing this 'context-driven' approach, however, lies in identifying the synthetic lethal interactions, which cannot be readily predicted *a priori*. Unbiased, high-throughput chemical and genetic screening approaches have proven to be effective strategies [5], but remain labor-intensive and costly.

An alternative strategy to identify candidate synthetic lethal partner(s) of mutant-*EGFR* is to identify genes that are anti-correlated with *EGFR* with respect to mutational status in NSCLC primary tumors. That is, under the premise that mutations in synthetic lethal genes are, by definition, never co-occurring in a viable (tumor) cell, one could use patterns of mutational mutual exclusivity to predict potential synthetic lethal partners. With the availability of large-scale and comprehensive gene resequencing data for lung adenocarcinoma [6], it is now possible to identify recurrent combinatorial patterns of somatic mutations in lung cancer. Multiple lung adenocarcinoma sequencing efforts have reported a negative correlation between mutations in *EGFR* and *STK11* (the gene encoding LKB1), in addition to well-known negative correlation between mutations in *EGFR* and *KRAS* [6,7,8].

LKB1 inactivation may alternately promote and suppress apoptosis.

LKB1 is a serine/threonine kinase originally identified as the causative mutation in Peutz-Jeghers syndrome (PJS) [9,10], an inherited autosomal dominant disorder characterized by benign polyps of the gastrointestinal tract and a predisposition to developing certain cancers [11,12]. LKB1, also known as STK11 and par-4, phosphorylates several conserved targets but is perhaps best known as the major upstream kinase to AMP-activated protein kinase (AMPK), a sensor of cellular energy levels and, by way of its activation of the tumor suppressor TSC2, an inhibitor of mammalian target of rapamycin (mTOR) signaling [13].

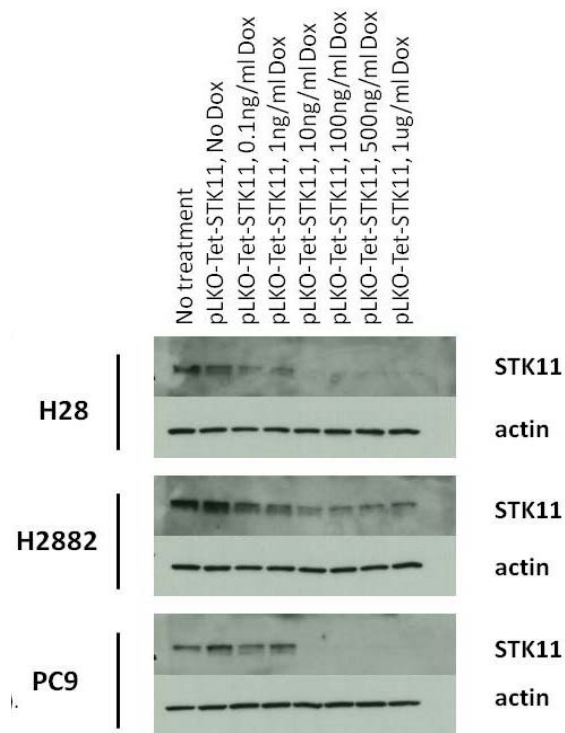
In addition to germline mutations in PJS, LKB1 has been found to be somatically inactivated in a large percentage (~30%) of sporadic non-small cell lung carcinoma (NSCLC) primary tumors and cell lines [14,15,16,17] and this, in combination with substantial functional and genetic data, has supported a role for *LKB1* as a human tumor suppressor gene [18]. Despite this, several reports have revealed a portrait of LKB1 deficiency that is more complex, and include findings that *LKB1*^{-/-} mouse embryonic fibroblasts are resistant to Ha-Ras-mediated transformation either alone or with immortalizing oncogenes [19]; LKB1 loss attenuates Wnt signaling in *Xenopus* and mouse models [20]; and that, in tumor cells with constitutively active Akt, LKB1 depletion reduces Akt-mediated phosphorylation and inactivation of several pro-apoptotic protein targets, including FOXO3a [21]. Together, these studies suggest that LKB1 may be required for the transformation or maintenance of cancer cells in specific cellular contexts.

Determining whether LKB1 is required for the EGFR-driven oncogenic state, thus representing a synthetic lethal partner to the *EGFR* oncogene

EGFR mutations in NSCLC primary tumors exhibit a pattern of mutual exclusivity with mutations in *LKB1* [6,22], raising the possibility that LKB1 function is required in the setting of oncogenic *EGFR*. While the function of LKB1 is classically characterized as tumor suppressive, recent evidence implicates LKB1 as a required factor in AKT-mediated inactivation of the

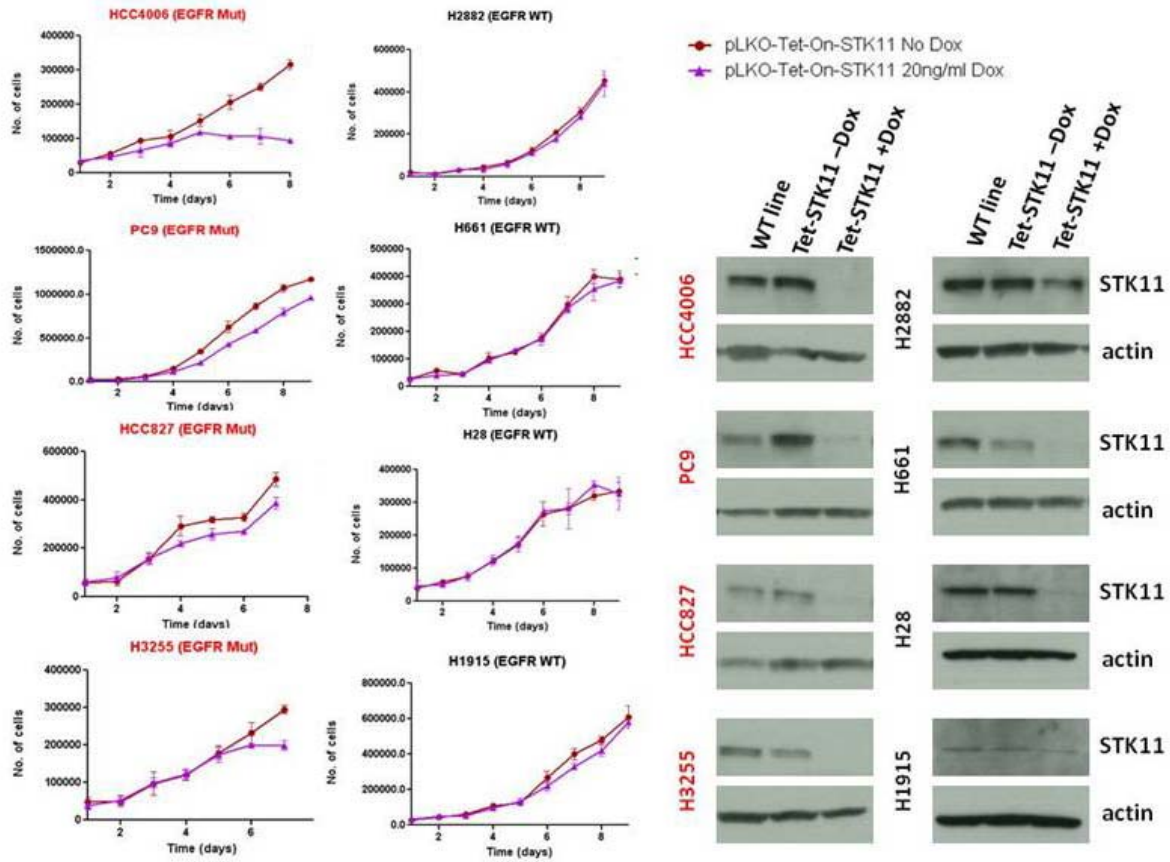
proapoptotic protein FOXO3a, thereby supporting a heretofore unappreciated antiapoptotic role for LKB1 in NSCLC [21]. While the requirement for LKB1 is claimed by Zhong et al. to exist in the context of aberrant AKT activation rather than that of mutant *EGFR per se*, these findings lend support to the notion that LKB1 may serve a pro-survival function specifically in the setting of oncogene-addicted cancers. The following set of experiments serves to test the hypothesis that LKB1 inactivation is synthetic lethal to *EGFR*-mutant cancers by testing whether LKB1 suppression selectively reduces the viability of cell lines harboring *EGFR* mutations versus non-mutant lines.

To assess the role of LKB1 in promoting cell survival specifically in the setting of oncogenic *EGFR*, a tetracycline/doxycycline- inducible short hairpin RNA (shRNA) lentiviral vector targeting LKB1 (Supplementary Figure 1) was used to infect NSCLC cells lines of two genotypic/phenotypic classes: cell lines that both harbor an activating *EGFR* mutation and demonstrate EGFR dependence, as demonstrated by exquisite sensitivity to EGFR inhibitors (PC9, HCC4006, H3255, HCC827); and as negative controls, lines that are wild-type for *EGFR* and are impervious to EGFR inhibition (H2882, H28, H1915, H661) [23]. Briefly, an inducible shRNA construct, generated by sequential PCR-based modification of the pLKO.1 lentiviral vector, pLKO-Tet-On-STK11[24,25] was used to transduce cells. Cell viability was measured by seeding equal numbers of stably-infected cells in the presence or absence of 20 ng/ml doxycycline and measuring cell number at fixed intervals over an 8-9 day time period (Supplementary Figure 2, left). Gene knockdown was confirmed by immunoblot analysis using affinity-purified antibody specific for LKB1 (Supplementary Figure 2, right). These data demonstrate a dramatic loss of viability in one *EGFR*-mutant cell line, HCC4006, and a modest reduction in viability in the three other *EGFR*-mutant cell lines.



Supplementary Figure 1. Generating a system for inducible STK11 (LKB1) shRNA

expression. An inducible shRNA construct, pLKO-Tet-On-STK11, is able to induce doxycycline-dependent knockdown of LKB1 in three NSCLC cell lines (H28, H2882, and PC9).

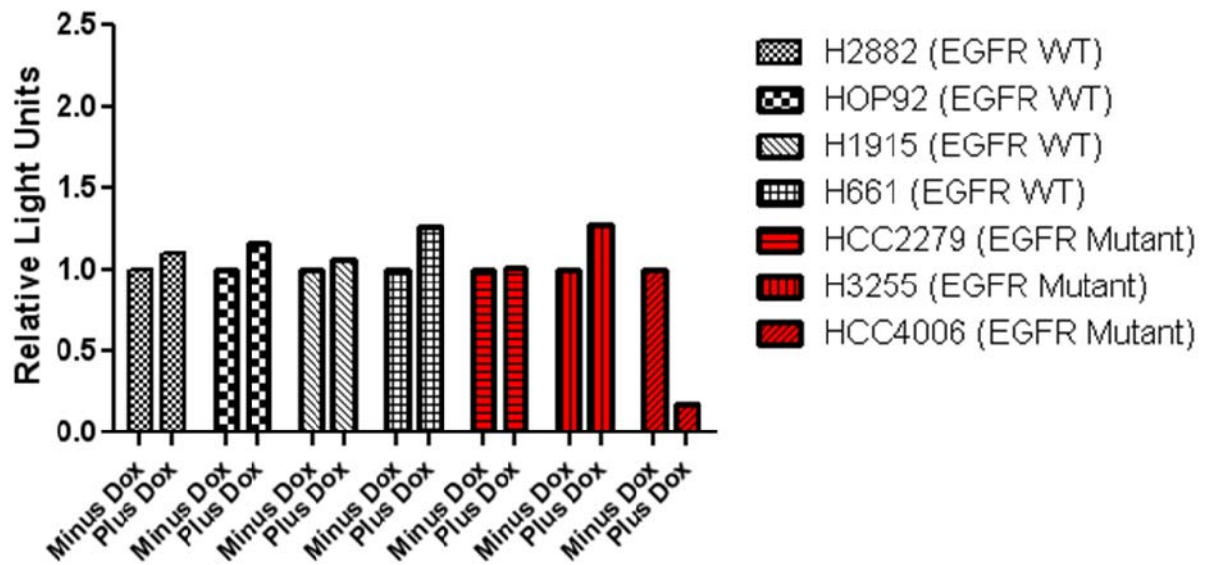


Supplementary Figure 2. Cell viability of NSCLC cell lines expressing pLKO-Tet-On-STK11. Left, *EGFR*-mutant (red titles) and *EGFR*-WT (black titles) NSCLC cell lines expressing pLKO-Tet-On-STK11 were grown in the presence or absence of 20 ng/ml doxycycline, and cell number was measured at fixed intervals. Data represent the mean \pm SD of 3 replicates. Right, conditional shSTK11 induction was confirmed by Western blot.

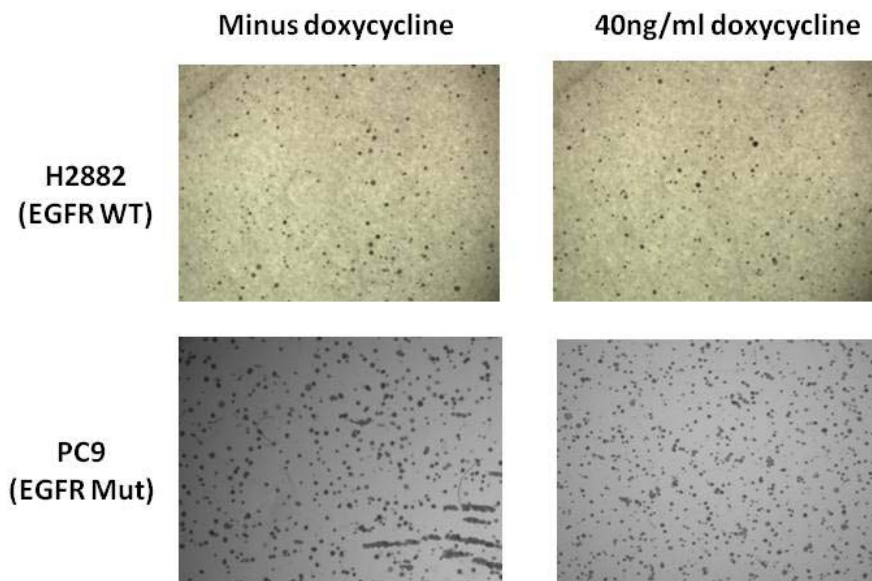
To determine whether the observed loss of viability in some *EGFR*-mutant, shLKB1-expressing cells is attributable to reduced proliferative potential of *EGFR*-mutant cells in the absence of LKB1, *EGFR*-mutant and wild-type NSCLC lines expressing shLKB1 were assayed to detect levels of proliferation using a BrdU incorporation assay. *EGFR*-mutant and wild-type lines expressing pLKO-Tet-On-STK11 were grown in the presence or absence of 20 ng/ml doxycycline for 8 days, thereby achieving conditional LKB1 knockdown, before equal numbers were reseeded into 96-well plates, labeled with BrdU for 4 hours, and stained according to the manufacturer's protocol (Roche) (Supplementary Figure 3). Consistent with the results of Supplementary Figure 1, these data revealed a dramatic reduction in the proliferation of one cell line, HCC4006.

The role of LKB1 knockdown in anchorage-independent growth, and sensitivity to EGFR-TKIs, was also assayed (Supplementary Figure 4 and Supplementary Figure 5, respectively). LKB1 knockdown did not alter either of these two phenotypes.

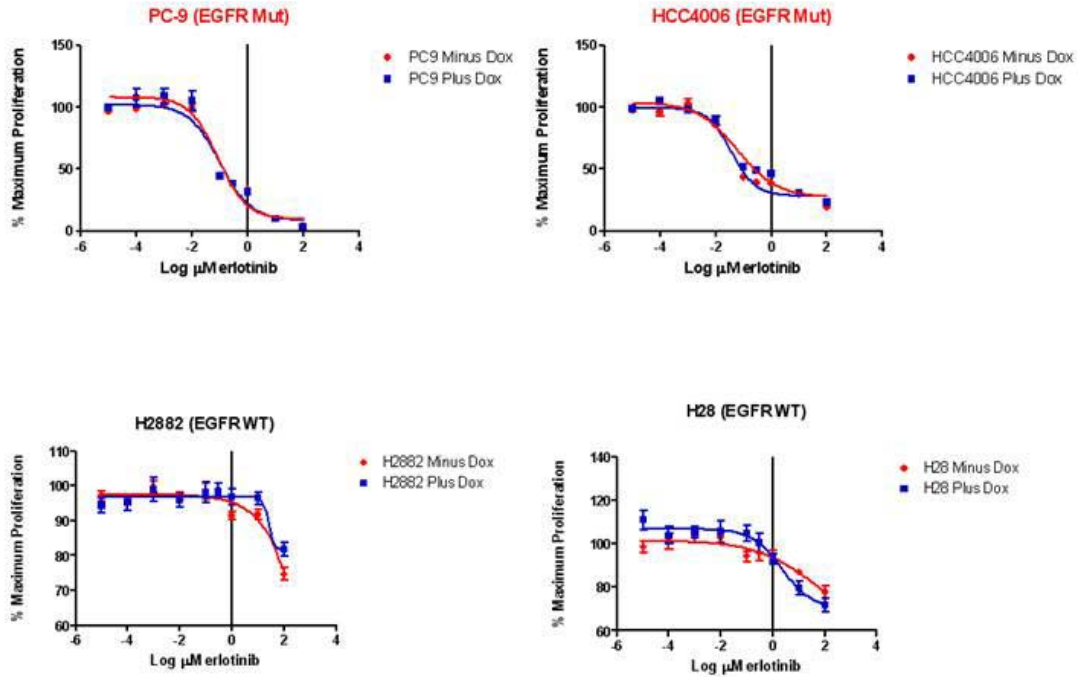
Together, these data do not suggest a synthetic lethal relationship between STK11 and EGFR, but do identify one cell line, HCC4006, that is highly susceptible to LKB1 knockdown.



Supplementary Figure 3. Cell proliferation NSCLC cell lines expressing pLKO-Tet-On-STK11. *EGFR*-mutant (red) and *EGFR*-WT (white) cell lines expressing pLKO-Tet-On-STK11 were grown in the presence or absence of 20 ng/ml doxycycline for 8 days, labeled with BrdU for 4 hours, then assayed for BrdU incorporation. Data represent the mean of 8 replicates.



Supplementary Figure 4. LKB1 knockdown does not impair soft agar colony formation in *EGFR*-mutant cells. *EGFR* WT (H2882) and mutant (PC9) cells expressing pLKO-Tet-On-STK11 were assayed for anchorage-independent growth in the presence or absence of 40 ng/ml doxycycline.



Supplementary Figure 5. LKB1 knockdown does not alter sensitivity of *EGFR*-mutant cells to erlotinib. *EGFR*-mutant and wild-type cells expressing pLKO-Tet-On-STK11 were grown in the presence or absence of 20 ng/ml doxycycline for 7 days, then treated with varying doses of erlotinib for 72 hours. Data represent the mean of 5 replicates.

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