ONCOGENIC FUSION KINASES IN LUNG CANCER

by

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Lung cancer is the global leader in cancer related deaths, but treatment outcomes have improved in recent years with the utilization of oncogene-targeted therapies. Here, we were able to identify a new class of actionable oncogenic fusion kinases in 3.3% of lung cancer patient samples harboring the kinase domain of the \textit{NTRK1} gene that encodes the high-affinity nerve growth factor receptor (TRKA protein). These fusions are oncogenic and result in constitutive TRKA kinase activity. Treatment of \textit{NTRK1}$^+$ cells with inhibitors of TRKA inhibited autophosphorylation of TRKA and cell growth.

The use of high-throughput next-generation sequencing techniques during the last few years has identified \textit{NTRK1}, 2, and 3 gene rearrangements encoding novel oncogenic fusions in 19 different tumor types to date. However, no drug has been approved by the FDA for cancers harboring this class of oncogenes since they were first identified 32 years ago. Our lab utilized preclinical models of LOXO-101 using TRK-fusion–bearing human-derived cancer cell lines to demonstrate inhibition of the fusion kinase and cellular proliferation \textit{in vitro}, and tumor growth \textit{in vivo}. Our work resulted in a phase I study of LOXO-101 (ClinicalTrials.gov no. NCT02122913). LOXO-101 is an orally administered inhibitor of the TRK kinase and is highly selective only for the TRK family of receptors. Recently, a metastatic soft-tissue sarcoma patient with an \textit{LMNA–NTRK1} fusion had rapid and substantial tumor regression with a novel, highly selective TRK inhibitor, LOXO-101, providing the first clinical evidence of benefit from inhibiting TRK fusions, and for the preclinical work presented in this dissertation.
*NTRK1*, *ALK*, *ROS1*, and *RET* are all oncogene fusions and driver mutations in lung and other cancers. Treatment of patients that harbor these fusions, such as those described above for *NTRK1*, with oncogene targeted therapy induces significant tumor responses, but results in residual tumor burden. This ultimately permits the emergence of drug resistant clones, limiting the long-term effectiveness of this monotherapy strategy. In order to determine the signaling mechanisms underlying incomplete tumor cell killing in oncogene-addicted cancer cells, we investigated the role of wild-type EGFR signaling in drug naïve cancer cells harboring these oncogene fusions. We observed three distinct roles for EGFR in the presence of oncogene-specific therapies: 1) direct transactivation of the oncogenic fusion kinase domains that resulted in disruption of the inhibitor binding to the kinase domain and restoration of fusion kinase signaling complexes by EGF-induced EGFR activation, 2) adaptor protein switching from the fusion to EGFR following fusion kinase inhibition in the absence of EGF stimulation and 3) EGF-induced by-pass signaling to critical downstream signaling pathways. Our study also demonstrated the commonality of EGFR expression and function in multiple fusion kinase positive models. This work also suggests that EGFR may control downstream signaling in these cells by regulating additional downstream effector proteins that control distinct cell fates. Collectively, the work presented here demonstrates that EGFR signaling can provide a critical, adaptive survival mechanism for cancer cells treated with a fusion oncogene-specific inhibitor, and provides a rationale for co-targeting EGFR with the oncogene fusion.

This form and its content are approved. I recommend its publication.

Approved: Robert C. Doebele.
I dedicate this work to my loving and supportive parents, Dr. and Mrs. Bihari and Rani Vaishnavi
ACKNOWLEDGEMENTS

I would like to thank my parents, Bihari and Rani Vaishnavi for their unconditional love and support. I would also like to thank my brother, Omar and his family for being such a strong source of support, friendship, and happiness for me. I would like to thank all of my friends, for the encouragement and support. I would not have come this far without all of their support and good times and providing me with the strength to never give up.

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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>FFPE</td>
<td>formalin-fixed paraffin embedded</td>
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<tr>
<td>FKI</td>
<td>fusion kinase inhibitor</td>
</tr>
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<td>FRET</td>
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<td>HiSeq</td>
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<td>HSAN</td>
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<td>IC50</td>
<td>half maximal inhibitory concentration</td>
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<td>IGF</td>
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CHAPTER I
FUSION ONCOGENE ADDICTION AND PERSONALIZED MEDICINE

Introduction

Lung cancer is a genetically heterogeneous disease

Lung cancer is the leading cause of cancer-related death globally (2). Traditionally, lung cancers have been classified by histological subtype, and empiric treatment was administered accordingly. Until recently, the standard of care for non-small cell lung cancers (NSCLC), which comprise approximately 80% of all lung cancers, was platinum-based chemotherapy (Fig. 1). Advances in the last decade have demonstrated that NSCLC are a heterogeneous disease at the genetic level, and therapies can be administered to clinically relevant subsets dictated by somatic genetic alterations. Within these subsets, treatment can be targeted to the specific molecular abnormality, known as “driver” oncogenic mutations. The reason this is of significance is that in 2002 Bernard Weinstein published the theory known as oncogene addiction, describing a dependency or addiction of many tumors to the signaling cascades driven by these oncogenes, and ultimately, targeting them can induce substantial growth arrest and/or apoptosis resulting in tumor shrinkage (3). This approach of identifying dominant oncogenic mutations, and our ability to specifically inhibit these genetic abnormalities with targeted inhibitors has altered the therapeutic approach for many cancer patients, but particularly those with lung adenocarcinomas. Activating point mutations, in-frame insertions/deletions, gene amplification, exon skipping, and gene rearrangements (instead of tumor histology) can serve as predictive biomarkers for oncogene-targeted therapies and thus help select patients that have a high likelihood of

1 Parts of this chapter were reprinted with permission from 1. Vaishnavi A, Le AT, Doebele RC. TRKing down an old oncogene in a new era of targeted therapy. Cancer Discov 2015;5:25-34
benefiting from a particular therapy. There are currently three well-established examples of this paradigm for the targeted therapy approach in NSCLC, all of which highlight the potential success of this strategy for other oncogene targets. The treatment of epidermal growth factor receptor (EGFR) mutation positive NSCLC patients (comprising approximately 10-15% of lung adenocarcinomas) and anaplastic lymphoma kinase, (ALK), or c-ros (ROS1) gene rearrangement positive NSCLC patients (encompassing approximately 3-9%, or 1-2% of lung adenocarcinomas), respectively, respond significantly better to the targeted therapies erlotinib and crizotinib, respectively, compared with the standard-of-care chemotherapy (4). EGFR mutation positive patients who are treated with a first-generation EGFR tyrosine kinase inhibitor (TKI) have an objective response rate (ORR) of about 50-74%, and progression free survival (PFS) time of approximately 9-13 months, both of which are superior to chemotherapy (5-7). ALK gene rearrangement positive patients showed an ORR of approximately 60-74%, and a PFS of approximately 7-10 months when treated with crizotinib, also superior to chemotherapy (8,9). ROS1 gene rearrangement positive patients showed similar trends, with an ORR of 72%, and PFS of 19.2 months (10). Since these first-generation therapies were initially approved, other EGFR and ALK inhibitors have also been sanctioned for these patients, and so have immune checkpoint inhibitors that have shown promise for some patient populations (11-18).

Substantial progress has been made in the movement towards successfully identifying more of these druggable molecular targets in lung cancer, and ultimately improved the way this disease is being treated. The paradigm of cancer treatment is currently shifting, towards precision oncology, when it is available (19). In this model, patients are selected for therapy using predictive biomarkers, such as the oncogenic mutations described in EGFR, ALK, and ROS1 above, rather than using empiric chemotherapy. This model may be the preferred choice, but it is not readily available for
patients that do not harbor certain established mutations. It may be similarly unavailable to those that harbor different cancer types where this approach is not as heavily utilized as it is in lung cancer. President Barack Obama has recently announced the instigation of a research initiative in the U.S. to make the vision of precision medicine a reality for as many people, as soon as can be successfully implemented (20). This initiative further validates the early successes and potential of this approach.

Many of the actionable or potentially actionable oncogenes that represent molecular subtypes in NSCLC involve genomic rearrangements with genes encoding receptor tyrosine kinases (RTKs) such as ALK, ROS1, REarranged during Transfection (RET), and most recently neurotrophic receptor kinase 1 (NTRK1) (21-24). Additional gene fusions include those involving FGFR, AXL, EGFR, MET, and NRG1 have been identified and can also result in the constitutive activation of kinase signaling cascades, but will not be studied in this dissertation (25-28). The improvement in patient outcomes with oncogene-targeted therapies suggest that even rare oncogenes, such as ROS1 gene rearrangements (which occur at a frequency of ~1-2%) should be investigated as therapeutic targets, as this NSCLC molecular subset still represents approximately 2,500 patients in the U.S. each year (29,30). Indeed, a recent study of crizotinib in ROS1+ NSCLC patients highlights the ability to effectively accrue rare oncogene subtypes (31). The study of these low frequency oncogenes not only applies to NSCLC, but is also relevant to the treatment of numerous other cancer types: ALK, ROS1, RET, and NTRK1 gene rearrangements have also been observed in other malignancies, expanding the relevance of the work presented in this dissertation to colorectal cancer, thyroid cancer, cholangiocarcinoma, glioblastoma, inflammatory myofibroblastic tumors (IMT), ovarian cancer, bladder cancer, sarcomas, and others (28,32-37). In support of this, reports show the success of targeting oncogenes across multiple tumor types (36,38-44).
It was estimated in 2007 that gene fusions were reported in approximately 20% of all cancers accounting for a significant proportion of cancer morbidity and mortality (45). The emergence of high-throughput genomics technologies and programmatic sequencing efforts such as the NCI/NHGRI Cancer Genome Atlas Network and the Sanger Cancer Genome Project have unveiled the genetic profiles of numerous cancers, and this emergent technology has enabled the identification of many additional gene fusions that are putative oncogenes and predicted to be conserved as drivers across breast, glioblastoma, lung, colorectal cancer, and others tumors (28,37,46-48). Subsequent sections in this thesis will describe in detail the emergence of an increasingly described class of oncogene targets in cancer, the TRK family of kinases. However, general oncogenic fusion kinase biology will be described first, and will expand from TRK fusions to provide an overview of the 4 best characterized and established fusion kinase oncogenes in lung cancer: ALK, ROS1, RET, and NTRK1. From there, further refinements of the oncogene addiction paradigm, and its implications for modifying the targeted monotherapy approach will be covered.

**Oncogenic fusion kinases: background and detection**

Many actionable genetic abnormalities described in lung cancer involve the creation of a “gene fusion”. The typical gene structure for an oncogenic fusion is that the 3’ region of a proto-oncogene (encoding the kinase-domain) is juxtaposed to 5’ sequences from an unrelated gene via an intra- or interchromosomal rearrangement or deletion. However, it is important to note that there are other structures (such as oncogenic fusions involving transcription factors or growth factor ligands, etc.) but they go beyond the scope of this work (27,49-52). The 5’ activating gene partner often provides a strong promoter, and a dimerization domain that enable the kinase domain to homodimerize in the absence of ligand (53,54). That resultant chimeric oncogene is both aberrantly expressed and has constitutive activation of the kinase domain. It should be noted that other variations of gene
fusions occur that retain the 5’ portion of the RTK, replacing the 3’ or c-terminal sequencing with a dimerization domain thereby retaining the original promoter and membrane localization and orientation of the RTK but providing ligand-independent dimerization via the presence of cytosolic dimerization-inducing domains (25). These variants will not be discussed further here as the cancer cell models utilized here all harbor the classical fusion variant in which the 5’ portion of the RTK-encoding gene is replaced. ALK, for example, is not normally expressed in adult lung epithelia, but the EML4 promoter enables expression of the oncogenic fusion protein, and its coiled-coiled domain promotes dimerization in the absence of a ligand and the 5’ ligand-binding domain of ALK (22,55). ALK fusions will mostly be described in the context of lung cancer in this thesis, but were first discovered in anaplastic large-cell lymphoma (ALCL) years before they were identified in lung cancer (56,57). In 1982, the BCR and ABL genes were implicated in the first oncogenic translocation also known as “the Philadelphia chromosome” in chronic myelogenous leukemia (CML) that became known as the stereotypical example of a fusion kinase oncogene (58).

Recurrent oncogenic kinase rearrangements are found in diverse organ microenvironments and cell lineages (22,50,51,58-63). Such widespread diversity in their origin suggests that the molecular mechanisms responsible for creating them may be universal at the cellular level, and not a cell-type specific process. Both intra- and inter-chromosomal translocations are known to include two double stranded breaks (64). This is followed by the apposition, and incorrect joining of the DNA ends, often by an error-prone DNA repair pathway like non-homologous end joining (NHEJ) (65). They can also be formed through interstitial deletions or chromosomal inversions, which have previously been observed with ROS1 and ALK gene rearrangements (22,62). Another key feature of both epithelial and hematologic malignancies that harbor such fusions is that they tend to follow
the successful paradigm of therapies targeted against cancers addicted to oncogenes, which continues to make them an appealing class of drug targets (9,66,67).

Cell-type context and differential subcellular localization of fusions might alter the signaling program of the oncogenic fusion kinases. For example, different adaptor proteins may be expressed at different levels or available at different compartments within the cell. ROS1 fusions in lung cancer have been predicted to be subcellularly localized, based on sequence analysis, to the membrane, cytoplasm, as well as the golgi apparatus (102,103). As one example, CD74-ROS1 is predicted, based on sequence analysis to be a two-pass transmembrane protein, as it retained both the signal sequence and transmembrane domain from CD74, and an additional transmembrane domain from ROS1. This fusion may have more success signaling through the MAPK pathway, because of its proximity to RAS lipid anchors at the membrane, compared to a cytoplasmic SLC34A2-ROS1 (102-104). Further functional studies of differentially localized fusions would be required to demonstrate this. Such required studies have not been conducted to date for TRK or ROS1 fusions in any tumor histology, but some work has been conducted on this with ALK fusions, and will be elaborated on in the subsequent ALK biology section.

Such oncogenic fusions have often been detected historically using break-apart fluorescent in-situ hybridization (FISH) assays, or when it can be, detected in a tissue specific manner by protein expression, through immunohistochemistry (IHC) (68,69). There has been an increase in the use of next-generation sequencing assays especially for the efforts focused on the discovery of novel gene rearrangements. Efforts geared towards the discovery of novel fusions are restricted by more antiquated techniques like FISH, which require preliminary knowledge of the kinase involved and breakpoints to create successful break-apart FISH probes. The efficient detection and diagnoses of oncogenic fusions in patients can be challenging, depending on the mechanism and genes involved. Evidence in
the literature, guided by the recent improvements in next-generation sequencing
technologies, has demonstrated the potential of other mechanisms of creating a fusion
kinase oncogene, distinct from the chromosomal translocations, deletions, or inversions
already described. *BCAN-NTRK1* was recently identified using RNA sequencing techniques,
but was not detected at the DNA level. This is of interest, because these genes are adjacent
on chromosome 1 and are oriented in the same direction, suggesting that long-range RNA
splicing, rather than the generation of a novel gene at the DNA level may account for the
expression of this novel oncofusion protein (70). The example used here may be relevant to
current clinical methodologies of detecting and ultimately treating patients that harbor this
class of oncogenes. It is also important to note that while TRK fusions were not detected
initially by the TCGA, revisiting these data in 20 different solid tumor types with a different
bioinformatics algorithm resulted in the identification of multiple new TRK oncogenic fusions,
in 8 separate tumor histologies (28,30). Collectively, this work validates the importance of
the method or assay used for fusion detection.

Interestingly, many activating 5’ gene fusion partners are promiscuous amongst
various kinase fusion classes, particularly within lung cancer (33,37). The tissue-specific
preference or association for one particular fusion partner with a kinase or of an entire
rearrangement has been observed consistently in certain malignancies, such as BCR-ABL
in CML. One could speculate several biological explanations for this, such as these chimeric
proteins are limited by certain cell lineage or chromatin constraints within a tissue (71). The
proximity of two fusion partners can be both cell-type and cell cycle stage specific. An
example of this is the proximity between *BCR* and *ABL* genes in CD34+ hematopoietic
precursor cells, indicating that the relative closeness of these genes measured in interphase
nuclei may promote the frequency of translocation between them (72). This suggests
potential importance of the cell of origin (established or relatively undifferentiated) imposing
on the rearrangement process or alternatively the fusion influencing the development of the cell lineage. Certain epigenetic modifications, such as histone methylation marks and the resulting protein interactions can increase the likelihood of certain types of rearrangements (73). One potential explanation for this is that it could be due in part to fragile sites of genomic regions that are more sensitive or prone to breakage and recombination (74). There is also structural evidence using three-dimensional microscopy for spatial contiguity of interphase nuclei being a contributing factor in certain cellular contexts, such as for the CCDC6 and RET genes (75). Another potential explanation is conserved “activating” features of those activating 5’ partners, such as the strong promoters and dimerization domains the drive oncogenic activity of the kinase (28). These functionally beneficial traits may provide a selection advantage such that they are more commonly observed in functional oncogenes. One example of a promiscuous 5’ gene partner is TFG, which has been fused to create functional oncogenic fusions with NTRK1, ALK, ROS1, and MET (28,36,76,77). In spite of the diversity in fusion partners, the kinase fusions described in this thesis tend to carry a conserved mechanism of oncogenic action, which is that they all result in a constitutively activated kinase domain.

Many of the in-frame NTRK1, NTRK2, and NTRK3 fusions identified thus far fit the paradigm described above and contain a 5’ gene partner with a dimerization domain (Fig. 2). The dimerization domains of the 5’ gene partners contain are often the stereotypical coiled-coil domain(s), and the corresponding constitutive tyrosine kinase activity that occurs results in uninterrupted downstream signaling messages for the cell to proliferate abnormally and survive (33). The different dimerization or interaction domains retained in the fusion product may result in different protein-protein interactions and oncogenic behavior, such has been observed with RET and its interactions with Enigma through the LIM domain in papillary thyroid cancer (PTC) (78,79). Another example of this is the increased metastatic
abilities of ALK observed when fused to TPM3 over other partners like NPM (80). Importantly, different BCR-ABL fusion variants have different transforming activities, some that only manage weak or no transforming ability, further validating the importance of the functional domains retained in the fusion product and their role in transformation of NIH-3T3 cells (81,82). Studies have also shown that different 5’ fusion partners can alter the signaling capacity of ALK, ultimately resulting in changes in transforming/tumorigenic properties of the oncogenes when those are also expressed in NIH-3T3 cells (83). Oncogenic gene rearrangements involving ROS1 may be an exception to this fusion dimerization paradigm, as many of the 5’ gene partners have no known dimerization domains, but have still been shown to possess transforming properties (37). One can speculate based on this that activation of ROS1 may only require loss of 5’ sequences that act as an autoinhibitory signal in the full-length RTK. A similar mechanism may be worth investigating in TRK family fusions. Many of these TRK fusions that have been identified by NGS have not been studied extensively using functional analyses for transforming properties. However, one can speculate by sequence analysis that they may be activated without a 5’ dimerization domain through the loss of a regulatory domain or in a different unknown mechanism, similar to certain ROS1 fusions.

**Biology of the TRKA/B/C family**

The NTRK1 gene encodes the TRKA receptor tyrosine kinase, the TRK proto-oncogene, which is a member of the TRK (tropomyosin-receptor kinase) family of RTKs that includes TRKB (encoded by NTRK2) and TRKC (encoded by NTRK3) proteins (33,84). TRKA, B, and C play important roles in nervous system development through their regulation of cell proliferation, differentiation, apoptosis, and survival of neurons in both the central and peripheral nervous systems. The TRK receptors are expressed abundantly in the nervous system, as well as many other non-neuronal cell types and tissues, including...
monocytes, the lung, bone, and pancreatic beta cells (85). TRKA, B, and C are most frequently activated by their primary ligands nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 (NT-3), respectively (86,87). Overexpression studies in cell lines suggest the possibility of promiscuity among these neurotrophin ligands and all 3 of the TRK receptors, but that expression of each receptor may be cell-type and neurotrophin concentration dependent (84). Additional studies have indicated NT-3 may activate TRKA and TRKB, and NT-4/5 may activate TRKB (88).

The binding of each of these ligands to its cognate receptor, such as binding of NGF to TRKA, induces receptor homodimerization (Fig. 3a). The dimer formed by the nerve growth factor ligand mediates the homodimerization interface of two TRKA receptors without the direct interaction of the extracellular domains of the two receptors with each other (89). Prior to dimerization in its inactive conformation, the TRKA receptor utilizes protrusion of the activation loop tyrosine 591 to autoinhibit activation of the tyrosine kinase domain (90). Conformational changes from ligand binding result in transautophosphorylation of five critical tyrosine (Y) residues (Y496, Y676, Y680, Y681, and Y791) and an active kinase conformation, enabling its intrinsic catalytic activity (91). Y496 and Y791 serve as phosphorylation-dependent binding sites for various adaptor proteins that contain SH2 or PTB domains, primarily SHC1, PLCγ, and GAB1, but others include FRS2, GRB2, IRS-1, IRS-2, SH2B (33,91-93) (Fig. 3b). Evidence from several studies points to activation of the PI3K signaling pathway from RAS or GAB1, although it may also be activated from other mechanisms (92,94-96). Once activated, the three wild-type TRK family members most frequently signal through several downstream signaling pathways including SHC/RAS/MAPK, PI3K/AKT, or PLC-γ/PKC, depending on which docking protein(s) bind to the critical phosphorylated tyrosines Y496 or Y791 (97). Activation of these signaling cascades results in transcriptional and other cell programs that mediate cellular proliferation,
synaptic plasticity, neurite outgrowth and repair, prevention or repair of neurodegeneration, sensory neuron maintenance, or apoptosis (33,97-100).

It is expected most TRK fusions would employ many or all of the same downstream signaling cascades as the full-length receptors given the preservation of the kinase domain and the critical tyrosine docking sites. However, this is a relatively unexplored area compared to studies on the signaling mechanisms employed by the full-length receptors, particularly in the rat pheochromocytoma cell line PC12. The ETV6-NTRK3 fusion might be an exception to fusions that maintain the ability to signal like canonical full-length TRK receptors. It lacks the critical Y845 docking site for the preferential adaptor SHC1 due to the location of the breakpoint in the fusion and evidence points to the use of an alternate adaptor, IRS-1 (101).

**TRK family in cancer**

Mutations in TRK family members have been reported in numerous malignancies, including ovarian cancer, colorectal cancer, melanoma, and lung cancer (115-119). Among these reported TRKA alterations, an in-frame deletion of NTRK1 (ΔTRKA) in acute myeloid leukemia (AML) and a splice variant of NTRK1 (TRKAIII) in neuroblastoma have been functionally characterized as oncogenic to date (120,121) (Fig. 4). The deletion in ΔTRKA results in the loss of 75 amino acids in the extra-cellular domain of TRKA, removes four glycosylation sites adjacent to the transmembrane domain, and enables this variant of TRKA to transform both fibroblasts and epithelial cells (121). The TRKAIII splice variant, which was identified in a neuroblastoma cell line, results in the loss of exons 6, 7, and 9, and the corresponding loss of the extracellular Ig-like C2-type 1 (IG-C2) domain as well as multiple glycosylation sites (120). TRKAIII is constitutively activated (ligand-independent), and the expression of this splice variant is promoted by hypoxic conditions (120). Although not yet identified in human tumors samples, mutations in the extra-cellular domain of TRKA,
P203A and C345S, have both been characterized as transforming in vitro (122,123). These studies may point to regions of interest or hot spots where mutations will be identified in human tumors; for example, a relative cluster of mutations occur in NTRK1 at the R342 position in close proximity to the C345 mutation site (124).

Autocrine and paracrine signaling by TRK receptors have been implicated to be pro-tumorigenic in several different tumor types. An autocrine loop involving TRKA and NGF is associated with pro-tumorigenic activity in both breast and prostate carcinoma; similarly, TRKB and BDNF have been shown to play a pro-tumorigenic role in several malignancies, also including both breast and prostate cancers (125,126). TRKB signaling has also been shown to promote anoikis resistance and induce metastatic programs in numerous cancers (127). Expression of TRKA and TRKC wild-type receptors are associated with a positive prognosis in neuroblastoma patients (excluding expression of the splice variant TRKAIII), while TRKB expression is correlated with a poorer prognosis (128,129). These two prognostic associations may suggest that the expression of TRKB, compared with TRKA or TRKC, in neuroblastoma may be activating cellular programs resulting in more invasive or metastatic behavior and ultimately a more aggressive disease.

**TRK family oncogenic fusions**

The most common mechanism of oncogenic activation of TRKA is through genomic rearrangement, and the creation of a gene fusion (33). Interestingly, all of these different mechanisms of oncogenic activation of TRKA (gene rearrangements, deletion, and splice variant) contain the loss of some of the extracellular domain of TRKA. The loss of these common sequences suggests the possible presence of critical regulatory domain(s) in the extracellular domain of TRKA (and potentially B and C), which when lost, results in constitutive activation of the kinase domain and thus its oncogenic capacity and is supported.
by mutagenesis studies where Ig-like domains in the extracellular region of TRKA were deleted (130).

Studies of TRKA fusions in thyroid cancer have revealed the Trk oncogenes (Trk, Trk-T1-T3) are capable of binding through a number of different signaling adaptor molecules, similar to full-length TRKA, but engage in signaling predominantly through the RAS/RAF/MAPK pathway with the adaptors described to date (105-107) (Fig. 3b). The STAT3 signaling pathway was implicated for in NIH-3T3 transformation by Trk oncogenes (108). Interestingly, the constitutive signaling induced by Trk oncogenes has also been shown to result in neuronal differentiation of PC12 cells (109). It was also elegantly demonstrated that the Trk oncogenes are capable of transforming the more relevant in vivo model of cellular transformation, thyroid epithelial cells, not just NIH-3T3 fibroblasts, even though the 3T3 cells are the more commonly utilized model system for studies of oncogenic transformation (110). Similarly, in vivo transformation of mammary epithelia was shown using the ETV6-NTRK3 fusion (111). These two studies collectively demonstrate the potency of this family of oncogenes in transforming multiple model cell systems in vivo. Studies of the ETV6-NTRK3 fusion have demonstrated oncogenic signaling is engaged through IRS-1, but due to the limited availability of cell lines endogenously expressing ETV6-NTRK3, most studies were conducted using a cDNA of the fusion expressed in various cell lines, such as fibroblasts, resulting primarily in activation of the RAS-MAPK signaling pathway, but also PI3K/AKT, sometimes simultaneously (101) (Fig. 3b). This fusion required simultaneous, dual activation of multiple downstream pathways for cellular transformation of fibroblasts. The activation of both mitogenic signaling, through Mitogen Activated Protein Kinase ERK Kinase 1 (MEK1), and survival signaling, through Phosphoinositide 3-kinase (PI3K), pathways was necessary for the activation of CyclinD1 to properly execute cellular transformation (112). This dual activation was synergistic, and
could be blocked with downstream signaling pathway inhibitors against either pathway.

Studies by the same group have also demonstrated a contributing role for upstream RTK signaling, through the insulin-like growth factor (IGF) signaling axis in *ETV6-NTRK3* fusion driven tumorigenesis (101). Importantly, interactions between *ETV6-NTRK3* and the IRS-1 adaptor were shown to mediate the complex formation and signaling contribution of the insulin-like growth factor receptor, IGF1R (101). This work is important because it introduces a potential role for other RTK signaling axes in oncogenic fusion signaling. At the time these studies were conducted, no TRK specific therapies were available clinically, and both the critical downstream signaling mediators as well as the IGF signaling axis represent a potential therapeutic approach for patients that harbor these cancers. Studies in mice in select *NTRK1* and *NTRK3* fusions have suggested these fusions likely play an important, early role in tumor progression (111,113). Although the low-affinity nerve growth factor receptor (P75<sup>NTR</sup>) can modulate the activity and signaling of the full length TRK receptors, its interaction with oncogenic TRK fusions have not been studied to date (84). Interactions between full-length TRK receptors and p75NTR have generally been restricted to their localization at the plasma membrane and the extra-cellular region of Trk played a contributing role (114). The loss of that previously implicated 5’ region of Trk in the fusion product suggests any interaction between the fusion and p75<sup>NTR</sup> would be unlikely.

In 1982, the same year as the landmark identification of BCR-ABL, the initial steps toward the identification of the first *NTRK1* gene fusion were initiated in a colon cancer sample and contained sequences from *TPM3* (non-muscle tropomyosin), a finding that was characterized molecularly further in 1986 (32,58,131,132). The incidence and therapeutic potential of *TPM3-NTRK1* fusions in colorectal cancer was revisited recently, after 32 years. Isacchi and colleagues reaffirmed that this *NTRK1* fusion is indeed a recurrent, albeit infrequent, oncogene in colon cancer (48). The majority of the identified colorectal cases
harboring NTRK1 fusions identified thus far express the TPM3-NTRK1 oncogene, suggesting a preference for TPM3 as the partner gene in this particular tissue, similar to EML4 with ALK in lung cancer (21,32,37,41,48,131). Additionally, TRKC and very recently TRKB have also been shown to form oncogenic chimeras in multiple tumor types (133,134) (Fig. 2). The ETV6-NTRK3 fusion has been identified as the oncogene in several malignancies, including secretory breast carcinoma, mammary analogue secretory carcinoma (MASC) of the salivary gland, congenital fibrosarcoma, congenital mesoblastic nephroma, acute myeloid leukemia, and more frequently in radiation-associated papillary thyroid cancer compared to non-radiation associated papillary thyroid cancer (111,134-141). ETV6-NTRK3 fusions can vary slightly with different exon usage in the breakpoint across different cancer types, but do retain the SAM dimerization domain from ETV6 and the kinase domain of TRKC (111,142). Chromosomal rearrangements have been observed between NTRK1 and TFG, TPM3, or TPR in PTC, the most common malignancy of the thyroid gland (33). Interestingly, many of these activating 5’ gene fusion partners are promiscuous amongst various kinase fusion classes and tissues (33,37). While most of the in-frame NTRK1, NTRK2, and NTRK3 fusions identified thus far fit the paradigm and contain a 5’ gene partner with a dimerization domain, at least two for each NTRK family member do not (Fig. 2). (33).

NGS identification of NTRK1, 2, and 3 fusions

Many next generation sequencing (NGS) efforts, including programmatic, disease-oriented whole genome and/or transcriptome projects and also targeted clinical NGS platforms have resulted in the identification of NTRK family fusions in numerous tumor types. NTRK1 fusions were recently identified in intrahepatic cholangiocarcinoma, spitzoid neoplasms, glioblastoma, and pontine glioma (21,70,143-146). Additional fusions were reported in lung adenocarcinoma, as well as the discovery of novel fusions in papillary
thyroid cancer (PTC), and glioblastoma using a novel, targeted technique known as anchored multiplex PCR (147). The first evidence of gene fusions involving the NTRK2 gene came in pilocytic astrocytoma and very soon afterwards in pontine glioma (133,141,143). New tumor types with NTRK3 fusions were also identified, including PTC, pontine glioma, and Philadelphia chromosome like acute lymphoblastic leukemia (Ph-ALL) (135,143,148). Each of the three NTRK family genes can rearrange with multiple 5’ gene partners (Fig. 2). A unique biological aspect of the ETV6-NTRK3 fusion is that it was the first oncogenic gene fusion to be identified in numerous different cancer tumor tissues, including tissue from epithelial, mesenchymal, and hematological cell lineages. This is important, because it helped reinforce the need to search for such actionable fusions in malignant tissues of all origins. In each of those different tumor types, all of which are relatively rare malignancies, ETV6-NTRK3 is the dominant oncogene. For example, 100% of mammary analogous secretory carcinoma of salivary glands (MASC) and 93% of secretory breast cancers harbor ETV6-NTRK3 fusions (111,137). This observation is similar to CML where BCR-ABL is found in the vast majority of cases. However, in most tumors where TRK fusions are identified, they represent only a small proportion of patients (Table 1). Collectively, the TRK family represents a large number of cases distributed across multiple tumor types.

TRK in disease

In spite of the long history of oncogenic TRK alterations, it has taken decades to develop drugs for this target in cancer. Several reasons likely contribute to slow the pharmaceutical development of this target, including the lack of selective inhibitors and the relative difficulty in screening large tumor cohorts when this oncogene was first identified in the early 1980s. Neurotrophins and TRK receptors, particularly TRKA, have been pursued in the past as drug targets for the treatment of chronic pain (98), and a few studies have pursued the TRK family as a therapeutic target in cancer, (126,149,150). High levels of
homology between TRKA, B, and even higher homology and sequence identity within the ATP or drug-binding pocket, have resulted in the synthesis of small molecule inhibitors that target all three TRK family members (pan-TRK inhibitors) (151).

Loss of normal regulation of TRKA, B or C receptor activity can result in numerous human diseases. TRK receptors are known for mediating pain sensation and can play a role in chronic pain (98,152). TRKA loss-of-function mutations are seen in class IV hereditary sensory and autonomic neuropathies (HSAN), such as the genetic disorder congenital insensitivity to pain with anhidrosis (CIPA) (99,152). Loss-of-function mutations in TRKB result in energy imbalances, the loss of appetite control and obesity, and neuronal defects such as memory impairment (100). Similarly, loss of BDNF expression in the cerebellum of the mutant stargazer mouse is associated with a severe ataxia phenotype (153) and TRKB homozygous mutant mice have severe neurologic deficits (100), suggesting a critical role for the BDNF/TRKB signaling axis in normal neurologic development. However, it remains unclear if inhibition of the full-length TRK receptors will produce symptoms that mimic developmental loss of signaling in this receptor family. A more detailed evaluation of TRK receptors in non-cancer related diseases is beyond the scope of this thesis, but can be found in several reviews (154,155). Interestingly, one of the potential beneficial side effects of targeting TRK receptors in cancer might be a decrease in pain sensation or increased weight gain, frequent symptoms among advanced stage cancer patients. Monoclonal antibodies against TRKA or NGF have been developed for the treatment of pain (98); however, antibodies to TRKA or other TRK family members would not be effective against TRK fusions as the extracellular domains for these fusions are routinely lost in the gene rearrangement.

In summary of this section, oncogenic Trk is one of the first oncogenes identified more than 3 decades ago. The Trk oncogenes occur across a broad array of tumor types.
Oncogenic fusions involving \textit{NTRK1}, \textit{NTRK2}, and \textit{NTRK3} and in-frame deletions or splice variants of \textit{NTRK1} are likely to be actionable oncogenes based on pre-clinical data. The first clinical evidence of tumor responses in patients with TRK fusions suggests that this family of oncogenes represents a new valid drug target in cancer. This newly revisited family of fusion oncogenes will complement studies regarding the more established fusion kinase drug targets including \textit{ALK}, \textit{ROS1}, and \textit{RET} in lung cancer.

\textit{ALK, ROS1, and RET} \textbf{fusion kinase oncogenes}

The first and largest genetic subtype of oncogenic fusions was identified in lung cancer in 2007, involving the \textit{ALK} RTK (22). The \textit{ALK} gene is most frequently fused to the echinoderm microtubule-like-protein 4 (\textit{EML4}) gene in lung cancer, but other 5' fusion partners have been observed in other tumor types, as well as less frequently in lung cancer (156-161). This particular fusion is known to occur through a common type of intrachromosomal rearrangement: a paracentric inversion on the short arm of chromosome 2—this does not include the centromere—but fuses the two unrelated genes in-frame (22).

The \textit{ROS1} gene encodes another RTK and is known for being one of the only two remaining “orphan receptors” of the 58 RTKs (162). It was first identified to form oncogenic fusions in glioblastoma in 1989, and also in lung cancer shortly after \textit{ALK} (23,163,164). \textit{ROS1} has demonstrated strong diversity of fusion partners—with over twenty different partner genes identified to date, and limited preference for one over the others, with the marginal exception of \textit{CD74} (10,165). Interestingly, sequence homology between ALK and ROS1 at the kinase domain and the ATP binding site (49% and 77%, respectively) has resulted in promiscuity for several different ALK TKIs that demonstrate clinically relevant affinity for ROS1 as well (166,167).

The \textit{RET} proto-oncogene, also encodes an RTK. The first RET fusion was identified in 3T3 cells transfected with human lymphoma DNA, and was not identified in lung cancer.
until 2012 (24,37,59,168). Similar to \textit{NTRK1} fusions, \textit{RET} fusions have a documented increased incidence in papillary thyroid cancers (PTC) that are associated with either ionizing radiation exposure or environmental radiation exposures, such as the Chernobyl nuclear reactor disaster, but have still been found in cases without substantial radiation exposure(169). Current estimates suggest that the oncogenic fusions involving \textit{ALK} (~5-8%) (170,171), \textit{ROS1} (~1-2%) (102), \textit{RET} (~1-2%) (168), and \textit{NTRK1} (~1-2%) (172) account for approximately 10% of lung adenocarcinoma, or ~100,000 patients globally annually (2). While the percentage that each fusion occurs within lung cancer is low, it would be misleading to deduce the actual patient population numbers are correspondingly low. The numbers propelling global lung cancer incidence make this disease by far the highest provider of \textit{ALK}+ patient populations. The incidence \textit{ALK}+ lung cancer patients is comparable to total numbers of \textit{BCR-ABL}+ CML patients each year, roughly 50,000 people for each (173).

\textit{ALK}, \textit{ROS1}, and \textit{RET} oncogenic fusions have all been shown to signal in cancer similarly to the \textit{NTRK} fusion oncogenes already described above and shown in Figure 3b. More specifically, \textit{ALK} fusion oncogenes have been shown to signal through RAS-MAPK, PI3K-mTOR, PLCγ, RAP1, JAK-STAT, or JUN pathways (174-181). This can result in uniform tumorigenic hallmarks such as proliferation and survival, but also more specialized ones such as cytoskeletal or cell shape phenotypic changes that may result in more invasive phenotypes (80). While many different adaptor proteins can bind to \textit{ALK} fusions, one particular of note, which will be relevant in later chapters, is GRB2 (156). Recent work has highlighted a preference, and particular dependence in lung cancer, of \textit{ALK} fusion signaling through the MAPK pathway (182). In addition to the more canonical cancer signaling pathways described above for \textit{ALK}, \textit{ROS1} has also been shown to engage the phosphatases SHP1 and SHP2, as well as the ESYT1, IRS-1 and VAV3 adaptor mediated
signaling pathways, which may result in increased invasive and metastatic phenotypes (162,183). Importantly, oncogenic ROS1 can also signal through the GRB2 adaptor protein (184). Signaling studies of full-length ROS1 receptor have been limited by the lack of a known ligand, but studies using chimeric receptors and stimulation with the known ligand of the ectodomain from the insulin receptor (IR), EGFR, and TRKA have enabled some downstream signaling activation to be delineated (185-188). RET has been shown to signal principally through the same canonical signaling pathways already described above (189-191). A unique biological aspect of RET, is that it has 3 different protein isoforms (RET9, RET43, and RET51), with the corresponding amino acid variations occurring at the carboxyl-(c) terminus (192). Modifications like this are particularly critical for a kinase, because they can result in unique protein interactions, and changes in the preferred binding of different adaptor molecules that ultimately result in distinct differences in signaling output. The primary effects of these isoform differences are centered structurally around Y1096 of RET, which is critical for GRB2 binding. Importantly, RET can also signal through the GRB7 and SHC1 adaptors (191,193).

**Subcellular localization and biology**

The most extensive studies regarding differences in subcellular localization for gene fusion based on fusion partner and break-point variants have been conducted with ALK, with various brief reports and speculation on the other 3 fusions. More recent work in lung cancer cells from work on ALK fusion signaling has demonstrated the critical importance of both the break point variant and the protein domains retained from the 5’ fusion partner in determining both the subcellular localization and signaling pathway preference of oncogenic ALK fusion kinases (182,194). It was shown that the hydrophobic HELP domain in EML4 may be responsible for driving interactions between the fusion protein and the membrane, providing proximity to the crucial signaling effector RAS (182), instead of a more diffuse
cytoplasmic localization. Indeed, fusion variants that did not retain the HELP domain exhibited an altered localization, further reaffirming its importance (194). It is essential to point out that these studies were only conducted with EML4-ALK fusions retaining exon 20. Exon 19 fusions have also been reported, and would contain the transmembrane domain of ALK, which may alter the subcellular localization of this variant (195).

It has also been revealed for a number of full-length kinases and fusions kinases that the engagement of differential signaling pathways, and the kinetics of such pathway activities can ultimately result in different cell fate decisions (196). These cell-fate decisions will have a strong impact on the potency of the overall oncogenic program for each oncogenic fusion. For example, despite the redundant downstream signaling pathway, EGFR and TRKA activation trigger different cellular fates: proliferation and differentiation, respectively, within the same cell-type, depending on the length of time the MAPK pathway is sustained in PC-12 cells (197-200). Additional evidence of this importance has been observed with ALK. Earlier studies with the kinase domain of ALK in different cell types have demonstrated membrane attachment of the ALK kinase domain is required for neurite outgrowth and activation of the MAPK pathway, but cytosolic ALK promotes DNA synthesis through PI3K signaling, also in PC12 cells (201). In the context of cancer, it has been reported that the cytoplasmic, not nuclear localization of NPM-ALK is required for its transforming activity (202). Previous studies of RET fusions found in thyroid cancers demonstrated that the protein-protein interactions of the fusion with other membrane anchored proteins enabled it to be partially localized at the periphery of the cellular membrane compartment (78). In one particular example, the PDZ domain of Enigma both allowed RET to be anchored to the membrane, and mediated Enigma’s interaction with Y1062 of the RET-PTC2 oncogenic fusion (203). These studies also indicated changes in subcellular localization of the fusion depending on the 5’ fusion partner’s dimerization
domain. Membrane attachment of many different kinases is thought to be crucial for regulation, control, and specificity; demonstrating why their mis-localization, in an already dysregulated disease state, may further contribute to their enhanced activity (204-206). Understanding these pathways and the effects of their cell fates on pro-tumorigenic properties may also provide a rationale for targeting additional, vulnerabilities in signaling networks (207).

This past work highlights many of the potential biological differences between fusion proteins utilizing the same activated kinase domains, but with different 5’ genes or break point variants. Subtle differences in the protein domains retained in the final fusion product can result in structural variations in the fusion protein, as well as changes in dimerization abilities, protein-protein interactions and subcellular localization. Each of these factors can heavily influence the signaling capacity of the kinase domain in different cellular contexts and ultimately the output of the oncogenic program. Each of these signaling cascades can result in dramatic cell fate changes in growth, proliferation, apoptosis, etc. of the cancer cells.

**The failures of cancer monotherapy: drug resistance**

The personalized medicine approach has led to dramatic improvements for cancer patients, which has been highlighted so far with oncogenic rearrangements involving TRK family members, ALK, ROS1, and RET. While that progress is important, it also has its limitations. The successes achieved thus far with targeted monotherapy are ultimately constrained by the inevitable occurrence of drug resistance and disease progression. This would suggest our understanding of oncogene addiction is incomplete or at least that it is not a cell autonomous process. It could be argued that cellular drug resistance occurs, because complete radiologic responses to targeted therapies are extremely rare—even in the most impressive patient response groups (208). That suggests that a population of
tumor cells display intrinsic drug resistance to prevent a complete response and elimination of persisting tumors cells (209,210). It is from that original, incomplete ablation of tumor cells that the resistant cells are able to grow out. The core approach of most drug resistance studies has been studying tumor samples from progressing patients' tumor biopsies or through the analysis of established cancer cell lines that have endured long-term selective drug pressure (182,195,211-215). These strategies have been valuable in predicting resistance mechanisms that arise from the outgrowth of resistant cancer cell clones (acquired resistance), but do not yield insight into how to improve initial treatment responses with a combination therapy approach. The success of this combination therapy approach is highlighted in revamped rationale combination therapeutic strategies against certain infectious diseases (216).

Early adaptive signaling mechanisms could permit survival of a substantial number of cancer cells following the initial insult of a kinase inhibitor, thereby accelerating the occurrence of tumor progression (217-219). Early adaptive signaling in this thesis refers to any mitogenic or other cellular signaling that occurs in response to therapeutic stress that may enable tumor cell survival. Importantly, from those initial surviving cells a diverse array of acquired resistance mechanisms (i.e. genetically-driven) can emerge, increasing the critical need for improving initial tumor responses (210,220,221). A great example of the diverse mechanisms of acquired resistance that can present is with ALK fusion patients treated with an ALK inhibitor in lung cancer (195). Patients can develop secondary kinase domain mutations, copy number gain (CNG) of the drug target, and by-pass signaling from another RTK or signaling protein (182,195,215). One of the most common mechanisms of by-pass signaling for ALK, as well as ROS1, has been observed by EGFR (211,222-227).
**EGFR and kinase biology**

EGFR performs essential roles during development and adult homeostasis, as well as its numerous aberrant roles in diseases, including cancer. All RTKs, which includes EGFR, as well as full-length TRKA/B/C, ALK, ROS1, and RET, have a similar structural architecture. Each RTK is evolutionarily conserved, and contains a ligand-binding region in its extracellular domain, a single-pass transmembrane domain, and a cytoplasmic region containing the tyrosine kinase domain, flanked by an adjacent juxtamembrane region and a C-terminal domain (228). Each tyrosine kinase domain contains an N-lobe and a C-lobe. One mechanism of ligand-binding activation, for the TRK family that is entirely ligand-mediated, was already discussed in detail above in the TRK family biology section. The ERBB family of RTKs, which includes EGFR, has been characterized to dimerize uniquely compared to other kinase families. Its ligand-dependent dimerization is entirely receptor mediated and occurs asymmetrically. Meaning that the C-terminal portion of an "activator" interacts with and allosterically destabilizes the autoinhibitory interactions created by the activation loop at the N-terminus of a "receiver" EGFR (229) (Fig. 5). Each of the 4 members of the ERBB family can heterodimerize with each other (230-234). The structures of activated kinase domains from most tyrosine kinases carry significant homology, but there are significant differences in their inactive states (235). These variations reflect on the different mechanisms of regulation that exist for each. It is important to note that heterodimers and various mechanisms of transactivation have been observed in numerous cancers between EGFR and other members of the RTK super family, even outside the ERBB family (236-241). The homology documented among the activated kinase domains may be particularly relevant when evaluating constitutively active gene fusion kinase domains, because of the conserved loss of the 5' portion of the gene among this class of oncogenes. Importantly, measurable autophosphorylation of EGFR is not always associated
with its kinase activity or signaling output, which may explain reports in the literature of non-canonical mechanisms of activation associated with EGFR, addressed below, that have not been observed with other kinases to date (229). EGFR has 12 intracellular tyrosines, which can be autophosphorylated, and have been shown to be activated by different ligands, including EGF, TGFα, amphiregulin, β-cellulin, HB-EGF, epigen, and epiregulin (Fig. 5) (242-248).

EGFR is an established cancer related gene, as it is expressed at high levels or mutated in various epithelial malignancies including lung, glioma, HCC, breast, colorectal, head and neck squamous cell carcinomas (HNSCC), and ovarian (among others) (249). It is known for its activating, drug-sensitive mutations in lung cancer, but previous work has demonstrated an EGFR signaling dependence in cancers devoid of a clear genetically-mediated activation mechanism, as it is also an FDA approved drug target for the monoclonal antibody cetuximab in both colorectal and HNSCC (250-252). In addition to mutations that result in constitutive EGFR kinase activity, EGFR can also be activated in cancer by overexpression, copy number gain or amplification, chromosomal translocations, autocrine/paracrine signaling loops, kinase domain duplications, and other non-canonical mechanisms—all of which have not been fully elucidated to date (144,253,254). Recently, it was shown that one potential mechanism of tumorigenic activation of wild-type (WT) EGFR is the formation of ionic bonds between the juxtamembrane domain of EGFR with the lipid PIP₂ in the membrane (255). Other ligand-independent mechanisms of EGFR activation have been reported, such as enhanced dimerization activity (256).

Personalized medicine in lung cancer began with EGFR. The first-generation EGFR TKIs gefitinib and erlotinib were originally designed, developed, and approved to target WT EGFR in NSCLC. The drug sensitizing mutations in EGFR were identified retroactively shortly after approval in 2004 in patients who demonstrated clinically significant responses
Historically, there was clinical evidence of a survival advantage in unselected patients, suggesting a modest benefit of EGFR inhibition in patients with WT EGFR (259,260). EGFR is often expressed at high levels in cancers where it is not necessarily playing a detectable driver role, suggesting its presence may be contributing in a subtler way, such as a global signaling component. EGFR and its ligands are expressed at very high levels in lung cancer (261). Another example of a subtler EGFR component comes from reports in the literature that have also described a previously unappreciated accessory role for WT EGFR in KRAS mutant and EGFR mutant lung cancer (262-266). Collectively, this suggests evidence for a pro-tumorigenic role for the WT receptor even when a constitutively active oncogene is present in a cancer cell.

EGFR has previously been demonstrated to play an important role in the context of several fusion kinase oncogenes as well. EGFR has been shown to mediate both intrinsic and acquired resistance for ROS1 fusions in lung cancer, as well as acquired resistance and neoplastic maintenance for ALK fusions also in lung cancer (102,211,223,224). Molecular interactions between RET fusions and EGFR was observed in thyroid cancer, where contributions from EGFR to the oncogenic program primarily driven by RET were also characterized (239). These interactions are particularly of interest from a clinical perspective: while evidence from pre-clinical models suggested a lot of potential, clinical trials using several different RET inhibitors as a monotherapy have been lacking and unable to replicate the response rates observed with other oncogene directed therapies (267,268). This clinical evidence suggests that RET oncogenic fusions may be an extreme example of a fusion oncogene requiring assistance from other kinases in the cell.

In this dissertation, first a detailed description and characterization regarding the identification of the NTRK1 fusions in lung cancer will be provided. The identification of the primary drug target in specific molecular subsets in lung cancer is the first step for providing
each patient with the most efficacious treatment options. Following that, the research presented will expand into the importance of understanding why the use of a monotherapy is not sufficient for long term tumor control, based on our breakdown of a more cooperative oncogenic signaling network. In order to improve up-front responses, we must use a combination of targeted therapies. Specifically, this is necessary to improve targeted inhibition of fusion kinase oncogenes and initial, incomplete patient responses. This idea will be further expanded on, and focused in on some of the molecular mechanisms that may be associated with these fusion kinase oncogenes both “upstream” and “downstream” of their role in signaling pathways of cells that harbor such oncogenes. Finally, the implications of all the work presented in this dissertation and the impact it will have both pre-clinically and clinically in the future will be discussed.
Figure 1: Defining lung cancer subsets: Then and now. Pie chart breakdowns of lung cancer subsets and how they have changed over the last two decades. (a) 20 years ago lung cancers were categorized and treated based on histology. (b) 10 years ago we started to identify two of the main molecular subsets of oncogenic drivers: KRAS and EGFR. (c) Dramatic progress in recent years as identified a heterogeneous breakdown of molecular events driving lung adenocarcinomas.
Figure 2: TRK gene fusion partners. Schematic showing the known NTRK1 (blue), NTRK2 (red), and NTRK3 (green) fusions and the tumor types in which they have been identified. It is important to note that not all of these gene fusions have yet been characterized functionally, but each one occurred in-frame with an intact TRK kinase domain and are thus potentially oncogenic. Known 5’ dimerization domains are shown in grey, and 3’ domains shown in blue (NTRK1), red (NTRK2), or green (NTRK3). No protein domains are shown for fusions that lack a reported breakpoint. Fusion proteins are not drawn to scale. CCD is coiled coil domain. TM is transmembrane domain. KD is kinase domain. OD is OB-fold domain. KH is K homology domain. BTB is BR-C, ttk and bab domain. Zf-B is zinc finger B domain. ETS is E26 transformation-specific domain.
**Figure 3: Normal TRK receptor tyrosine kinase and TRK fusion signaling.** (a) Normal TRK RTK signaling schematic. Nerve growth factor (NT) stimulation induces receptor dimerization, autophosphorylation, and downstream signaling through SH2 and PTB domain containing adaptors. Receptors are localized to the plasma membrane. (b) Schematic showing common signaling mechanisms for an example of a cytoplasmic (non-membrane bound) chimeric TRK gene fusion are shown. Gene fusions are constitutively activated, or phosphorylated, often as a result of dimerization mediated by sequences in the 5’ gene. SH2 and PTB domain containing adaptors compete for binding at specific tyrosine residues, which most frequently results in propagation of the downstream signaling pathways shown.
Figure 4: TRKA oncogenic variants. Schematic of TRKA isoforms, deletions, and mutations are shown. Mutations are shown in the TRKAI (wild-type) isoform. Amino acid position numbers are shown in black.
Figure 5: EGFR: structure and signaling activation mechanisms. A cartoon schematic of the asymmetric homo-dimers formed between 2 EGFR proteins. At the top following the blue circle are the different ligands that can be used to activate EGFR. A breakdown of the 12 major autophosphorylation sites of EGFR in the intracellular portion including the juxtamembrane domain, the kinase domain and C-terminal domains. The red box lists the most common adaptor proteins known to dock onto EGFR and the tyrosines/domain location on EGFR that they bind to.
Table 1: Oncogenic TRK fusions are found across multiple tumor types. The frequency of NTRK1 (blue), NTRK2 (red), and NTRK3 (green) gene fusions indicating the tumor type and the detection method that was employed in each study. Only positive studies are listed and thus the actual prevalence may be lower than reported.

<table>
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<tr>
<th>Oncogene</th>
<th>Cancer</th>
<th>Frequency</th>
<th>Detection method(s)</th>
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* post-Chernobyl
+ sporadic

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CHAPTER II
ONCOGENIC AND DRUG SENSITIVE NTRK1 REARRANGEMENTS IN LUNG CANCER

Introduction

Orally active kinase inhibitors crizotinib and erlotinib or gefitinib are superior to standard chemotherapy with respect to both tumor response and progression free survival in lung cancer patients with ALK fusions or EGFR mutations, respectively (269,270). Additional oncogenes such as ROS1 and RET fusions have recently been identified in lung cancer and demonstrate great potential for therapeutic intervention (271-273). Many of these oncogenes also occur in several other common malignancies including, but not limited to, colorectal cancer, thyroid cancer, cholangiocarcinoma, and ovarian cancer potentially expanding the relevance of this therapeutic approach to other tumor types (274-276). Based on these clinical successes, the identification of additional actionable oncogenes in patients that do not harbor any known alterations is currently an area of interest. In this study, we set out to identify other potential driver mutations by sequencing lung cancer patient samples that initially tested negative for any known oncogenic drivers.

Methods

Patients

Local IRB approval was obtained for all patients in this study. FoundationOne testing and FISH analyses were performed in CLIA-certified laboratories. The index patient who underwent treatment with crizotinib consented to treatment outside of a clinical trial.

Next generation DNA sequencing

DNA was extracted from 40 µm of FFPE or frozen tissue using the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega) and quantified using the PicoGreen fluorescence

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assay (Invitrogen). Library Construction was performed as previously described using 50-200ng of DNA sheared by sonication to ~100-400bp prior to end-repair, dA addition and ligation of indexed, Illumina sequencing adaptors (277). Enrichment of target sequences (3,320 exons of 182 cancer-related genes and 37 introns from 14 genes recurrently rearranged in cancer representing ~1.1 Mb of the human genome) was achieved by solution-based hybrid capture with a custom Agilent SureSelect biotinylated RNA baitset (277). The libraries were sequenced on an Illumina HiSeq 2000 platform using 49x49 paired-end reads. Sequence data from genomic DNA was mapped to the reference human genome (hg19) using the Burrows-Wheeler Aligner and were processed using the publicly available SAMtools, Picard, and Genome Analysis Toolkit (278,279). Genomic rearrangements were detected by clustering chimeric reads mapped to targeted introns.

**RNA extraction from FFPE and frozen tissues**

RNA was isolated from FFPE or frozen tumor samples as described previously (271). Briefly, FFPE samples were processed using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion) following deparaffinization in xylene and washed with 100% ethanol prior to Protease K digest. Extraction of RNA from frozen tissue was accomplished using TriReagent (Ambion). Alternatively, tumors from NSCLC patients obtained at surgery were snap-frozen in liquid nitrogen, embedded in OCT and sectioned. RNA was prepared using Trizol (Invitrogen) followed by RNeasy MinElute cleanup kit (Qiagen).

**RT-PCR and sequencing of MPRIP- and CD74-NTRK1**

RT-PCR of MPRIP-NTRK1 was carried out using the SuperScript® III First-Strand Synthesis System (SSIII RT) from Invitrogen with a NTRK1 primer located in exon 15 (‘NTRK1 Y490R1’) for reverse transcription by PCR using the same reverse primer, ‘NTRK1 Y490R1’, and a primer to MPRIP located in its 3rd coil-coiled domain (‘MPRIP CC3F1’). PCR products were resolved on an agarose gel and the fragments were excised and treated with
ExoSapIT (Affymetrix) prior to sequencing by the University of Colorado Cancer Center DNA Sequencing and Analysis Core using the BigDye Terminator Cycle Sequencing Ready Reaction kit version 1.1 (Applied Biosystems) using the same forward and reverse primer in the RT-PCR reaction. For *CD74-NTRK1*, reverse transcription was carried out using the QuanTitec Reverse Transcription kit (Qiagen). PCR of the resulting cDNA was performed using the primers ‘CD74 Exon 3 FOR’ and ‘NTRK1 Exon 15 REV’. Primers used for RT-PCR and sequencing are available in (Table 2). The reference sequences used for exon alignment are NCBI Reference Sequences: NM_002529.3 (*NTRK1*), NM_015134.3 (*MPRIP*), and NM_001025159.2 (*CD74*).

**Cloning full length *MPRIP*, *CD74*, and *TPM3-NTRK1***

cDNA was generated from the patient using the SSIII RT kit describe above along with a primer located at the end of *NTRK1* (NTRK1stopR2). This cDNA was used to amplify two separate overlapping fragments that were used to generate full length *MPRIP-NTRK1* by overlap extension PCR using the two fragments alone for 10 cycles and then adding the MPRIPStart and NTRK1stopR1 primers for an additional 30 cycles of PCR amplification. The resulting 4kb PCR product was gel isolated and confirmed by Sanger sequencing. A 3’ hemagglutinin (HA) tag was added to *MPRIP-NTRK1* using PCR amplification with primers harboring the HA encoding sequence. The amplified product was subsequently cloned into the pCDH-CMV-MSC1-EF1-Puro lentiviral plasmid (System Biosciences). The K544N kinase dead variant was created using site-directed mutagenesis (Agilent, QuikChange II XL 200524) from the full-length MPRIP-NTRK1 fusion. Full length *TPM3-NTRK1* was amplified from KM12 cDNA using TPM3Start RI and NTRKStopNotI primers and cloned into the lentiviral plasmid as described above. The NCBI Reference Sequence used for *TPM3* is NM_153649.3. For the *CD74-NTRK1* construct, cDNA was transcribed with Quantiscript Reverse Transcriptase (Qiagen). Full length *CD74-NTRK1* was amplified using the primers
‘CD74 FOR’ and ‘NTRK1 REV’ using AccuPrime™ Taq DNA Polymerase (Invitrogen) and cloned into the pDNR-Dual vector (BD Biosciences) and recombined into JP1520 retroviral vector as previously described (280). The full-length cDNA of each gene was confirmed by sequencing. Primers used for cloning are available in (Table 2).

**Quantitative PCR of NTRK1**

Relative Quantification Polymerase Chain Reaction (RQ-PCR) assay of the NTRK1 tyrosine-kinase domain (Hs01021011_m1; Applied Biosystems) was used to evaluate its level of mRNA expression. The relative quantification method (ΔΔCT) in the StepOnePlus Real-time PCR system (Applied Biosystems) was used with GUSB (Applied Biosystems) as an endogenous control. All samples were evaluated in triplicate.

**RNA sequencing**

Paired-end RNA sequencing was performed as previously described (281). RNA FASTQ files were aligned and splice junctions mapped using TopHat (282) and analyzed for fusion reads using the Broad Institute Cancer Genome Analysis Tools Suite ([www.broadinstitute.org/cancer/cga](http://www.broadinstitute.org/cancer/cga)) and ([www.broadinstitute.org/cancer/software/genepattern/modules/RNA-seq/](http://www.broadinstitute.org/cancer/software/genepattern/modules/RNA-seq/)).

**Cell lines and reagents**

NIH-3T3 murine fibroblast cells, A549 lung adenocarcinoma cells, and human embryonic kidney (HEK)-293T cells were purchased from ATCC. Ba/F3 murine pro-B cells were a kind gift from Dan Theodorescu. H3122, H1650, H1299, and HCC78 lung adenocarcinoma cell lines were a kind gift from John D. Minna. KM12, HCT116, HCT15, HT29, and SW837 colorectal cancer cell lines were a kind gift from S. Gail Eckhardt. The lymphoblastoid cell line, GM09948 (Coriell Cell Repository), was used for genomic mapping in FISH studies.
All cancer cell lines were maintained in RPMI media with 10% calf serum. NIH-3T3 and Ba/F3 cells transduced with full length NTRK1 were supplemented with 100 ng/ml and 200 ng/ml β-NGF (R& D Systems), respectively. Crizotinib and gefitinib were purchased from Selleck Chemicals, CEP-701 from Sigma Aldrich or Santa Cruz Biotechnology, K252a from Tocris, and ARRY-470 was supplied by Array BioPharma. Total AKT (40D4), AKT pSer473 (D9E), total ERK (L34F12), ERK pThr202/Tyr204 (D13.14.4E,), total STAT3 (124H6,), STAT3 pY705 (D3A7), PARP(46D11), TRK pY490 (9141), pY674/675 (C50F3) (corresponding to Y496, Y680, and Y681 in TRKA, respectively), and HA (C29F4 or 6E2), antibodies were purchased from Cell-Signaling Technologies. Total TRKA (C-14), and α-tubulin (TU-02) were purchased from Santa Cruz Biotechnologies Inc. and GAPDH (MAB374) from Millipore.

Lentivirus or retrovirus production and cell transduction

MPRIP-NTRK1 or the kinase dead variant K544N was introduced into cells via lentivirus as previously described (283). Briefly, HEK-293T cells were transfected using a 3-vector lentiviral system (5 μg pCMV-VSV-G, 7.5 μg pH8.2 ΔR, and 10 μg of PCDH lentiviral vector). Virus was incubated for 48-72 hours, filtered, and concentrated prior to transduction. NIH-3T3 cells transduced with lentivirus were cultured in DMEM medium with 5% calf serum and 0.75 μg/ml puromycin. Ba/F3 cells transduced with lentivirus were cultured as above with 2 μg/ml puromycin, and with or without 1 ng/ml IL-3 (R&D Systems). Alternatively, CD74-NTRK1 was introduced into cells using retrovirus as previously described by the Janne Lab (280). Polyclonal cell lines were established by puromycin selection.

Mouse xenograft studies

NIH-3T3 cells (10^6) harboring the indicated expression vectors were resuspended in Matrigel (BD Biosciences) and injected subcutaneously into athymic nude mice (kind gift of...
James DeGregori). Mice were monitored three times weekly for tumor formation and sacrificed when tumors reached approximately 2 cm x 2 cm. Approval for the use of animals in this study was granted by the University of Colorado Institutional Animal Care and Use Committee.

**Immunoblotting**

Immunoblotting was performed as previously described (283). Briefly, cells were lysed in cold RIPA buffer (20 mM Tris-HCl pH 7.4, 137 mM NaCl, 5mM EDTA, 1% Triton-X 100, 0.1% SDS, 1% Sodium deoxycholate, H2O to volume) with Halt protease and phosphatase inhibitor cocktail (Thermo-Scientific) on ice. Following a 13,000 rotations per minute (RPM) spin at 4°C for 10-20 minutes, the supernatant was transferred to a fresh tube and diluted in PBS and 4x protein loading buffer (928-40004, LI-COR Biosciences) prior to boiling. Denatured lysates were run on 8-10% SDS-PAGE gels and proteins were separated through electrophoresis. Gels were transferred with either the Trans Blot SD semi-dry discontinuous buffer electrophoretic transfer cell system (BIO-RAD®) or the Pierce™ Power blot cassette (ThermoFisher Scientific) onto nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature (RT) (927-50100, LI-COR) and incubated with the indicated antibodies at 4°C rotating overnight. Membranes were washed, incubated with appropriate anti-mouse or anti-rabbit fluorescent secondary antibodies (925-32210, and 925-32211, LI-COR) for 1 hour, and washed again all at RT prior to scanning. Membranes were scanned and analyzed using the Odyssey Imaging System and software (LI-COR). Alternatively, immunoblotting was performed according to the antibody manufacturer's recommendations using chemiluminescent detection (Perkin Elmer). All western blot images are representative of at least 3 independent experiments.
**Proliferation assays**

All assays were performed as previously described by seeding 1000 cells/well into a 96-well plate, drug treatments were performed 24 hours after seeding, and Cell Titer 96 MTS (Promega) was added 72 hours later or as described previously (280,283,284). IL-3 was removed from Ba/F3 cells 48 hours prior to seeding. Plates were read using a Molecular Devices kinetic microplate reader at the wavelength 490 nm.

**Soft agar assays**

Anchorage-independent growth was measured by seeding 100,000 cells per well of 1% and 0.8% agar (Invitrogen) and 2x media in layers in 6-well plates as previously described (283). Media was changed every 4 days for 2 weeks. Quantification was performed with Metamorph Offline Version 7.5.0.0 (Molecular Devices).

**Fluorescence in-situ hybridization**

Formalin-fixed, paraffin-embedded (FFPE) tissue sections were submitted to a dual-color FISH assay using the laboratory developed NTRK1 break-apart probe (3' NTRK1 [SpectrumRed] and 5' NTRK1 [SpectrumGreen]) or the fusion MPRIP [SpectrumGreen]-NTRK1 [SpectrumRed] probe. The pre-hybridization treatment was performed using the reagents from the Vysis Paraffin Kit IV (Abbott Molecular). Hybridization and analysis was performed as previously described (271,283). Samples were deemed positive for NTRK1 rearrangement if ≥15% of tumor cells demonstrated an isolated 3’ signal or a separation of 5’ and 3’ signals that was greater than one signal diameter.

**siRNA transfection**

KM12 cells were transfected with 30nM NTRK1 Silencer Select siRNAs (Life Technologies) using siPORT NeoFX transfection reagent (Life Technologies) at 4µL/mL.
Flow cytometry

Cell cycle analysis of KM12 cells was performed as previously described (271). Briefly, cells were washed following treatment and then permeabilized with 70% ethanol. Cells were then stained with propidium iodide (BD Pharmingen) and analyzed on a Gallios flow cytometer (Beckman Coulter). Cell-cycle distribution analysis was performed using ModFit software (Verity Software House). Apoptosis was measured in KM12 cells using the Vybrant apoptosis YO-PRO/PI kit (Invitrogen). Briefly, KM12 cells were seeded 24 hours prior to treatment at 500,000 cells/well prior to trypsinization and staining.

Immunohistochemistry

Immunohistochemical studies for TTF-1 and thyroglobulin were performed using standard procedures. Antibody against TTF-1 (Cell Marque, Cat#CMC-573) was applied at 1:100 dilution and thyroglobulin (Signet, Cat#228-13) was applied at 1:25 dilution and incubated at 37°C for 32 min. Detection for TTF-1 was performed using Ventana multiview (UltraView) and detection for thyroglobulin using Ventana Avidin-Biotin (iView).

Results

In an effort to identify additional potential oncogenes in lung cancer we performed a targeted next generation sequencing (NGS) assay for ~200 cancer-related genes on tumor samples from 36 patients with lung adenocarcinoma (274). These patient tumors tested negative for activating genetic alterations in EGFR, KRAS, ALK, and ROS1 using standard clinical assays to detect activating mutations or chromosomal breaks with FISH. Patient characteristics are provided (Table 3).

In tumors from two patients, this NGS assay detected evidence of an in-frame gene fusion event involving the kinase domain of the NTRK1 gene, which encodes the high affinity nerve growth factor receptor, also known as the TRKA receptor tyrosine kinase (Fig. 6a, Fig. 7a, Fig. 7b). In the index case, the 5’ end of the myosin phosphatase Rho
interacting protein (MPRIP) gene is joined with the 3’ end of NTRK1 (Fig. 8a). MPRIP is involved in regulation of the actin cytoskeleton and has recently been implicated as a gene fusion partner with TP53 in small cell lung cancer, putatively causing early termination of TP53 (285). A second case harbored a CD74-NTRK1 gene fusion (Fig. 8b). CD74, which encodes the MHC class II invariant chain, has previously been identified in ROS1 gene fusions (23,271,286). In order to confirm the gene fusion exon junctions and demonstrate expression of the fusion transcript we performed RT-PCR using primers homologous to sequences on each side of the predicted rearrangement (Fig. 9a, 9b).

FISH is commonly used to evaluate patient tumor samples for the presence of chromosomal aberrations that result in gene fusions (271,287). We therefore developed a break-apart FISH assay to detect chromosomal rearrangements within the NTRK1 gene, regardless of the identity of the 5’ fusion partner (Fig. 10). Hybridization of these probes showed clear separation of the 5’ and 3’ probes in the tumor samples containing the MPRIP- or CD74-NTRK1 gene fusions, but not in a control sample (Fig. 6b, Fig. 10). Chromosomal rearrangements in which the 5’ region of TPM3, TFG, or TPR is fused to the 3’ end of the NTRK1 gene have previously been identified in colorectal and thyroid cancers (32,275). Although the TPM3 (1q22-23) and TPR (1q25) genes lie in close proximity to NTRK1 (1q21-22), FISH could also detect a separation in signals in the KM12 colorectal cell line that harbors a TPM3-NTRK1 fusion (Fig. 10, 11) (288). Using this FISH assay, 56 additional lung adenocarcinoma samples without detectable EGFR, KRAS, ALK, ROS1, or RET oncogenic mutations were screened for NTRK1 rearrangements (Table 4). One case was identified with a clear separation of the signals (Fig. 10). Quantitative PCR (qPCR) demonstrated high NTRK1 kinase domain expression only in the tumors with the known NTRK1 rearrangements or in the KM12 cell line (Fig. 12).
To demonstrate expression of the fusion protein derived from *MPRIP-NTRK1*, we performed immunoblot analysis on cells from a frozen pleural fluid sample or early passage cells growing in culture (CUTO-3) from the index patient (Fig. 13). We detected expression of the fusion protein, RIP-TRKA (encoded by *MPRIP-NTRK1*) in both pleural fluid and cells. These cells also demonstrated autophosphorylation of this novel protein at critical TRKA tyrosine residues (91). MPRIP harbors three coiled-coil domains, a type of domain that is known to mediate dimerization (and hence autophosphorylation) in EML4-ALK and other fusion proteins (Fig. 14) (22). CD74 is a known activating fusion partner of ROS1 and the CD74-TRKA protein is predicted to be localized in the plasma membrane (Fig. 14) (289). This predicted subcellular localization is based on the breakpoint of the two genes involved in the fusion: the signal peptide sequence from the CD74 gene was retained, as were 2 transmembrane domains (one from each gene).

To prove that these novel fusion proteins are oncogenic, *MPRIP-* and *CD74-NTRK1* cDNA constructs were expressed in HEK-293T cells, NIH-3T3 fibroblasts and Ba/F3 cells. Similar to the CUTO-3 cells, introduction of these genes led to expression of the appropriate-sized chimeric protein and autophosphorylation (Fig. 6c, Fig. 15, Fig. 18, Fig. 21). Introduction of the kinase-dead mutant variants, *MPRIP-NTRK1* (K544N) or *CD74-NTRK1* (K544M) yielded protein expression but not autophosphorylation (Fig. 6c, Fig. 15, Fig. 18, Fig. 21) (91). Introduction of the gene fusions, but not the kinase dead variants, increased ERK1/2 and AKT phosphorylation (Fig. 6c, Fig. 18, Fig. 21). Similar autophosphorylation results were obtained in NIH-3T3 cells expressing these constructs (Fig. 15). To measure the ability of these genes to sustain cellular proliferation, IL-3 was removed from the medium of Ba/F3 cells and proliferation was assayed (Fig. 6d). *MPRIP-* and *CD74-NTRK1*, but not their kinase-dead counterparts, induced IL-3 independent proliferation of Ba/F3 cells. Similarly, *MPRIP-* and *CD74-NTRK1*, but not the kinase-dead
variants, supported anchorage-independent growth of NIH3T3 cells and CD74-NTRK1 but not the kinase dead version induced a refractory appearance (an oncogenic trait) of NIH-3T3 cells (Fig. 16, 17). NIH-3T3 cells expressing CD74- or MPRIP-NTRK1, but not the kinase dead version also formed tumors in nude mice (Fig. 6e). Knockdown of TPM3-NTRK1 by siRNA specific for NTRK1 in KM12 cells decreased protein expression of TPM3-TRKA and reduced proliferation, further supporting the role of NTRK1 fusions as oncogenes (Fig. 18a, Fig. 19).

Given the prior success of treating ALK and ROS1 fusion positive patients with targeted kinase inhibitors, we asked whether NTRK1 fusions might provide a similar oncogene target in patients with lung cancer or other malignancies. We therefore tested several candidate inhibitors with reported activity against TRKA. ARRY-470 is a selective kinase inhibitor with nanomolar activity against TRKA, TRKB, and TRKC but no other significant kinase inhibition below 1000nM (Fig. 20, Table 5). CEP-701 and crizotinib also have activity against TRKA as well as other kinases (290,291). Treatment of Ba/F3 cells with ARRY-470, CEP-701 and, to a lesser extent, crizotinib inhibited autophosphorylation of RIP-TRKA and CD74-TRKA (Fig. 18b, Fig. 21). Activation of the MAPK and AKT pathways was also inhibited in Ba/F3 cells expressing TRKA fusion proteins (Fig. 18b, Fig. 21). Similar results were obtained in NIH-3T3 cells expressing TRKA fusion proteins following treatment with the drugs (Fig. 15). Phosphorylation of TPM3-TRKA in KM12 cells and RIP-TRKA in CUTO-3 is similarly inhibited by all three drugs (Fig. 18c, Fig. 22).

In order to test whether these inhibitors would be a potentially effective treatment for patients harboring NTRK1 gene fusions, Ba/F3 cells expressing NTRK1 gene fusions were treated with ARRY-470, CEP-701 or crizotinib in the absence of IL-3 (Fig. 18d, Fig. 21). Inhibition of proliferation was greatest with CEP-701 and ARRY-470. Crizotinib was a less potent inhibitor of Ba/F3 cells harboring both fusion genes, although in a similar range seen
for inhibition of EML4-ALK or SDC4-ROS1 (Fig. 23) (271). The less potent effects of crizotinib on cell proliferation are consistent with decreased inhibition of pTRKA and downstream pERK1/2 (Fig. 18b, Fig. 21). ARRY-470 did not inhibit proliferation of Ba/F3 cells expressing other oncogene targets such as EGFR, ALK, or ROS1 (Fig. 24). All three drugs also inhibited colony formation of NIH-3T3 cells expressing NTRK1 fusions in soft agar (Fig. 16). KM12 cells were similarly sensitive to ARRY-470 and CEP-701, but less so to crizotinib (Fig. 22). A panel of lung cancer or colorectal cancer cell lines, which do not harbor an NTRK1 gene fusion, were not inhibited by ARRY-470 demonstrating the specificity of this drug only for cells harboring activating TRKA (Fig. 24). All three inhibitors induced cell-cycle arrest in G1 and apoptosis of KM12 cells (Fig. 25). Importantly, gefitinib, an epidermal growth factor receptor (EGFR) inhibitor, had no effect on the NTRK1 rearranged Ba/F3 or KM12 cells (Fig. 18b, Fig. 18d, Fig. 21, Fig. 22, Fig. 25a).

The index patient (MPRIP-NTRK1) had previously been treated with a number of standard lung cancer therapies prior to identification of the NTRK1 rearrangement. No clinical trials of potentially effective TRKA inhibitors were available, therefore the patient consented to treatment with crizotinib (250 mg twice daily) outside of a clinical trial. She experienced a minor radiographic response at first evaluation with a decrease in her serum levels of CA125 (Fig. 26). However, she had persistent malignant ascites and pleural effusion and developed clinical progression after ~3 months on treatment. This modest clinical activity of crizotinib is consistent with the in vitro results. Additional immunohistochemical analysis with thyroglobulin was performed to exclude the possibility of a thyroid carcinoma, which can also express TTF-1, (Fig. 27).

Discussion

We have identified novel, recurrent oncogenic NTRK1 fusions in a subset of patients (3/91; 3.3%) with lung adenocarcinoma that were negative for other common oncogenic
alterations. Our study further highlights the utility of targeted NGS as a method to discover novel drug sensitive genetic alterations in lung cancer. Based on our preclinical findings and the patient example, clinical studies of selective TRKA inhibitors in \( NTRK1 \) rearranged NSCLC are warranted.
Figure 6: Discovery and validation of oncogenic NTRK1 gene fusions in lung cancer samples. (a) Schematic of genomic rearrangement from tumor samples harboring MPRIP-NTRK1 and CD74-NTRK1 using the FoundationOne next-generation sequencing assay, including chromosomal breakpoints for each gene rearrangement. (b) Break-apart FISH analysis of MPRIP-NTRK1 and CD74-NTRK1 tumor samples showing clear separation of green (5') and red (3') signals corresponding to the NTRK1 gene. (c) TRKA (NTRK1) fusions are autophosphorylated and activate key downstream signaling pathways. Representative immunoblot analyses (n = 3) of cell lysates from 293T cells expressing RIP-TRKA and CD74-TRKA, but not their kinase-dead (KD) variants, display phosphorylation of known critical tyrosine residues (Y496, Y680 and Y681) in TRKA (pTRKA) and activation of ERK (phosphorylation of T202 and Y204, pERK). TPM3-TRKA was expressed in 293T cells as a positive control, as it has been previously demonstrated to be oncogenic. MW, molecular weight; HA, hemagglutinin. (d) NTRK1 fusions support cellular proliferation. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay of Ba/F3 demonstrated that cells expressing RIP-TRKA, CD74-TRKA, EML4-ALK or full-length TRKA supplemented with NGF proliferated in the absence of IL-3, whereas Ba/F3 cells expressing empty vector (EV) or the kinase-dead variant of RIP-TRKA did not proliferate (n = 3). Data are expressed as mean ± s.e.m. (e) MPRIP-NTRK1 or CD74-NTRK1 gene fusions induce tumorigenesis. NIH-3T3 cells expressing RIP-TRKA, RIP-TRKA-KD, CD74-TRKA, EML4-ALK or empty vector were injected into the flanks of nude mice and observed for tumor growth. Representative pictures taken at day 12 following injection are shown. The number of tumors induced in the injected animals are shown in parentheses (five mice injected per cell line, n = 1).
Figure 7: Next generation sequencing detects NTRK1 gene fusions. (a) Schematic highlighting the genomic DNA sequencing reads supporting the presence of the MPRIP-NTRK1 (top) or CD74-NTRK1 (bottom) gene rearrangements. (b) Examples of sequencing reads spanning the genomic breakpoint for MPRIP-NTRK1 (top) or CD74-NTRK1 (bottom).
Figure 8: DNA sequence of NTRK1 fusion cDNA. Complete cDNA sequence of (a) MPRIP-NTRK1 (M21;N14) with sequence derived from MPRIP in red and that of NTRK1 in blue and (b) CD74-NTRK1 (C8;N12) with sequence derived from CD74 in red and NTRK1 in blue.
Figure 9: Confirmation of mRNA expression of MPRIP- and CD74-NTRK1 samples. (a) and (b) RT-PCR demonstrates mRNA expression of the novel fusion transcripts. RNA extracted from frozen tumor sample harboring the MPRIP-NTRK1 (a) or CD74-NTRK1 (b) was subject to RT-PCR followed by agarose gel electrophoresis. (c) and (d) DNA sequencing of the RT-PCR product was performed and confirmed the identity and breakpoint of the fusion transcript. The following abbreviations are used: MPRIP (M), CD74 (C), NTRK1 (N), and exon (ex).
Figure 10: Design and testing of NTRK1 break-apart FISH probe. (a) Design of NTRK1 break-apart probe set aligned against the NTRK1 encoding region of chromosome 1q23.1. (b) Cell line GM09948 with a normal karyotype showing metaphase spread and interphase nuclei demonstrating close proximity of the 5' (green) and 3' (red) signals indicating an intact NTRK1 gene. (c) KM12 cells which harbor a TPM3-NTRK1 gene fusion showing clear separation of the 5' (green) and 3' (red) signals indicating a rearrangement of the NTRK1 gene. (d) NTRK1 FISH screening of 56 patients (Table 4) identified one additional positive patient. Representative images are shown for (b)-(d).
RNA sequencing on the KM12 cell line revealed 129 chimeric pairs supporting an intrachromosomal fusion on chromosome 1 between \textit{TPM3} and \textit{NTRK1}. A cartoon schematic of the inversion between \textit{TPM3} and \textit{NTRK1} on chromosome 1 in the KM12 colorectal cancer cell line is depicted.
Figure 12: Quantitative PCR for NTRK1 expression in lung cancer samples. Frozen tumor samples (n= 30) sent for the FoundationOne NGS assay were also assayed by quantitative PCR for mRNA expression of NTRK1 using primers specific to the 3' region NTRK1 relative to a housekeeping gene, GUSB. A negative control cell line, A549, and a positive control cell line, KM12 (TPM3-NTRK1), are shown. The tumor sample with CD74-NTRK1 is colored green and the MPRIP-NTRK1 is colored blue.
### Figure 13: Confirmation of protein expression induced by MPRIP-NTRK1 rearrangement.

Immunoblot analysis of 293T cells transiently transfected with empty vector (EV), full-length NTRK1 cDNA, or MPRIP-NTRK1 cDNA compared to tumor cells from a frozen pleural fluid sample or early passage cells in culture (CUTO-3) from the index patient with the MPRIP-NTRK1 fusion gene. MPRIP-NTRK1 is predicted to run at approximately 170 kDa, compared to full-length TRKA that runs at approximately 140 kDa, but can also run at a larger size when glycosylated following phosphorylation. The pleural fluid sample from the patient did not demonstrate autophosphorylation likely due to temperature conditions, and time until freeze from when the sample was obtained, as well as a freeze-thaw cycle for lysate preparation.
Figure 14: Predicted structures of fusion proteins resulting from MPRIP- and CD74-TRKA. Cartoon schematic demonstrating fusion break-points and critical domains of the predicted fusion protein products. The following abbreviations are used: transmembrane (TM) domain, coiled-coil domain (CCD), and exon (e). Portions of the genes involved in the rearrangements are marked as blue (NTRK1), grey (MPRIP), and brown (CD74).
Figure 15: Expression and drug inhibition of *NTRK1* fusions in NIH-3T3 cells. NIH-3T3 cells expressing (a) RIP-TRKA or (b) CD74-TRKA were treated with the indicated doses of drugs for 5h prior to cell lysis and immunoblot analysis of pTRKA, TRKA, pAKT, AKT, pERK1/2, ERK1/2, pSTAT3, and STAT3 as indicated. K544N and K544M represent kinase dead mutations for TRKA fusions. Representative blot images (n = 4) are shown.
Figure 16: NTRK1 fusions support anchorage independent growth and are inhibited by drugs with activity against TRKA. Representative images (n = 4) from anchorage independent growth assays of NIH-3T3 cells in soft agar. (a) NIH3T3 cells expressing empty vector, RIP-TRKA kinase dead, or RIP-TRKA were seeded in triplicate in soft agar and treated with DMSO (control) or 200nM of ARRY-470, crizotinib, or CEP-701 for 2 weeks (n = 4). (b) NIH3T3 cells expressing empty vector, CD74-TRKA kinase dead, or CD74-TRKA were seeded in triplicate in soft agar and treated with DMSO (control) or 200nM of ARRY-470, crizotinib, or CEP-701 for 2 weeks (n = 2). Values represent the mean ± SEM. Total colony area for each plate was quantified using MetaMorph software and plotted for each condition.
Figure 17: **CD74-NTRK1** induce refractory appearance in NIH-3T3 cells. (a)-(d) Phase-contrast microscopy images of NIH-3T3 cells expressing **CD74-NTRK1** with vehicle (a), CEP-701 (c) or gefitinib (c) treatment. (b) The kinase dead version, **CD74-NTRK1-K544M** did not induce a refractory appearance of the NIH-3T3 cells, and only treatment with the TRKA inhibitor CEP-701, not gefitinib disrupted refractory appearance. Representative images are shown.
Figure 18: Drug treatment inhibits activation of TRKA, downstream signaling, and proliferation in cells expressing NTRK1 fusions. (a) RNAi knockdown of NTRK1 inhibits cell proliferation in a cell line harboring TPM3-NTRK1. KM12 cells were analyzed by MTS proliferation assay 1–5 d after transfection with two different NTRK1-specific siRNAs (siRNA 1 and siRNA 2) or scrambled siRNA (control) (n = 3). Data are expressed as mean ± s.e.m. (b) Ba/F3 cells expressing MPRIP-NTRK1 (RIP-TRKA) or EV were lysed after 5 h of treatment with the indicated doses of drugs (G, gefitinib 1,000 nM) or DMSO control (c). Phosphorylation of TRKA (Y496, Y680 and Y681) or ERK (T202 and Y204) was assessed by antibodies specific to the indicated tyrosine or threonine residues. (c) CUTO-3 lung cancer cells harboring the MPRIP-NTRK1 gene fusion were treated with the indicated doses and drugs and subjected to immunoblot analysis. (d) Ba/F3 cells expressing the MPRIP-NTRK1 fusion demonstrate inhibition of proliferation as measured by MTS assay using the pan-TRK inhibitor, ARRAY-470, and the multikinase inhibitor, CEP-701, but not the EGFR inhibitor, gefitinib (n = 5). Data are expressed as mean ± s.e.m.
Figure 19: RNAi knockdown of TPM3-TRKA fusion in KM12 cells. RNAi knockdown of NTRK1 reduces TPM3-TRKA protein in the KM12 colorectal cancer cell line harboring the TPM3-NTRK1 rearrangement. Representative western blot showing decreased TPM3-TRKA protein and reduced pERK1/2 for siRNAs used in this experiment are shown.
Figure 20: Chemical structure of ARRY-470.

\[(S)-N-(5-((R)-2-(2,5\text{-}difuoro\text{-}phenyl)pyrrolidin-1\text{-}yl)pyrazolo[1,5-a]pyrimidin-3\text{-}yl)3\text{-}hydroxy\text{-}pyrrolidine-1\text{-}carboxamide\]
Figure 21: Drug treatment of Ba/F3 cells expressing CD74-NTRK1 inhibits activation of TRKA, downstream signaling, and proliferation. (a) Ba/F3 cells expressing CD74-NTRK1 (CD74-TRKA) or empty vector (EV) were lysed after 5h of treatment with the indicated doses of drugs (G = gefitinib 1000nM) or DMSO control. Representative image of n=3. (b) Treatment of Ba/F3 cells expressing CD74-NTRK1 with TRKA inhibitors inhibits cell proliferation as measured by MTS assay (n = 5). Values represent the mean ± SEM. Ba/F3 cells expressing CD74-NTRK1 demonstrate inhibition of proliferation by the pan-TRK inhibitor, ARRY-470, and the multi-kinase inhibitor, CEP-701, but not the EGFR inhibitor, gefitinib.
Figure 22: Drug treatment of KM12 cells expressing endogenous **TPM3-NTRK1** inhibits activation of TRKA, downstream signaling, and proliferation. (a) KM12 colorectal cancer cells harboring the **TPM3-NTRK1** fusion were lysed following 5h treatment with the indicated doses of inhibitors and subjected to immunoblot analysis. All western blot images are representative of at least \( n = 3 \). (b) Proliferation of KM12 cells is inhibited by ARRY-470, CEP-701, and crizotinib, but not gefitinib. Treatment of KM12 cells with TRKA inhibitors inhibits cell proliferation as measured by MTS assay \( (n = 5) \). Values represent the mean \( \pm \) SEM. The half maximal inhibitory concentration (IC50) values are listed (nM).
Figure 23: Treatment of Ba/F3 cells expressing oncogenic fusions with crizotinib. Crizotinib leads to inhibition of Ba/F3 expressing \textit{NTRK1} fusions, similar to Ba/F3 cells expressing ALK or ROS1 fusion constructs. Treatment of Ba/F3 cells expressing \textit{NTRK1}, \textit{ALK} or \textit{ROS1} fusions with crizotinib inhibits cell proliferation as measured by MTS assay ($n = 5$). Values represent the mean ± SEM. The half maximal inhibitory concentration (IC50) values are listed (nM).
Figure 24: Cells without NTRK1 fusions are resistant to ARRY-470. (a) Ba/F3 cells expressing mutant EGFR (deletion exon 19), EML4-ALK, or SDC4-ROS1 were treated with the indicated doses of ARRY-470 and cell proliferation was measured by MTS (n = 3). The indicated lung cancer (b) or colorectal cancer (c) cell lines were treated with the indicated doses of ARRY-470 and cell proliferation was measured by MTS (n = 4).
Figure 25: TRKA inhibition results in the accumulation of KM12 cells in G1 phase and induces apoptosis. (a) KM12 cells were treated with the indicated doses of drugs for 24hr. Cells were then stained with propidium iodide and analyzed by flow cytometry. ModFit analysis was used to quantify cell cycle profiles \((n = 3)\). Values represent the mean ± SEM. 

(b) KM12 cells were treated for 24h with the indicated drugs and doses, trypsinized, stained with YO-PRO® and propidium iodide (PI), and analyzed by flow-cytometry. Staurosporine was used here as a positive control for the induction of apoptosis. The percent of cells undergoing apoptosis (YOPRO® positive and PI negative) are plotted \((n = 4)\). Values represent the mean ± SEM. 

(c) TRKA inhibitors induce cleavage of PARP-1. KM12 cells were treated for 24h with the indicated drugs and doses. Cells were lysed, separated by SDS-PAGE and subject to immunoblot analysis with the indicated antibodies to detect cleaved PARP-1.
Figure 26: Treatment of the index patient harboring *MPRIP-TRKA* with crizotinib. (a) CT scan of the thorax demonstrating a left lower lobe mass before and after treatment with oral crizotinib 250 mg twice daily. (b) Serum CA125 levels at the time of diagnosis and while on treatment with crizotinib.
Figure 27: Histopathology from index patient harboring *MPRIP-NTRK1* demonstrating lung adenocarcinoma. (a) Needle core biopsy of primary lung left lower lung mass showing adenocarcinoma. (b) Cell block of fine needle aspirate from the same procedure showing tumor cells. (c) TTF-1 immunohistochemistry (IHC) demonstrating strong nuclear staining in tumor cells. (d) Thyroglobulin IHC demonstrating negative staining in tumor cells. Representative images are shown.
Table 2. Primers for RT-PCR and cloning.

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**Table 3.** Patient samples tested using FoundationOne next generation DNA sequencing assay.

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\(^*\)All patients tested for each of the molecular markers were negative for that oncogene, one patient was \( MET \) FISH positive

\(^6\)Age data was missing on 13 patients, median age not calculated for \( NTRK1 \) FISH positive patients
Table 4. Patient samples tested using *NTRK1* break-apart FISH assay

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Table 5. Kinase Selectivity Analysis of ARRY-470 at 1 μM (100 fold Trk enzyme IC50).

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Array TrkA enzyme (500 μM ATP) IC50 = 10 nM
Upstate panel run at 100 μM ATP or less
CHAPTER III

EGFR RESCUES DRUG-INHIBITED FUSION KINASES VIA FUSION KINASE REACTIVATION AND ADAPTOR SWITCHING.\(^3\)

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (2). The most tangible advances in treating this disease have occurred within the last decade with the advent of a precision medicine approach - matching biomarker-selected patients with oncogene-targeted therapies (252,257). Inhibition of these oncogenes, including gene fusion kinases such as ALK, ROS1, RET, or NTRK1, blocks cancer cell proliferation and survival, consistent with the model of oncogene addiction (3,21,37). ALK+ and ROS1+ patients treated with the inhibitor crizotinib demonstrate remarkable objective response rates and progression free survival times and in ALK+ patients this treatment is superior to standard chemotherapy (8,31). Early evidence also supports the efficacy of targeting NTRK and RET in lung cancer patients bearing oncogenic forms of these RTKs (39,292). However, complete radiologic responses are rare suggesting that a population of tumor cells display intrinsic drug resistance (8,293). Ultimately all patients will experience disease progression, most often from cellular resistance to the targeted therapies (195,211,212).

The primary focus of most drug resistance studies has been studying tumor samples from progressing tumor lesions or by use of established cancer cell lines that have undergone long-term selective pressure (211,212). These strategies have been valuable in predicting resistance mechanisms that arise from the outgrowth of resistant cancer cell clones, but do not necessarily yield insight into how to improve initial treatment with combinatorial therapy (209). Early adaptive signaling mechanisms could permit survival of a

\(^3\)Parts of this chapter were printed with permission from a manuscript currently under review at Cancer Research.
substantial number of cancer cells following the initial insult of a kinase inhibitor, thereby accelerating tumor progression (217-219).

In this study, we focused on oncogene fusions, which arise from genomic rearrangements resulting in expression of a chimeric protein with a constitutively activated kinase domain (169,294), herein referred to as a fusion kinase. Current estimates suggest that the oncogenic fusions ALK (~5-8%) (170,171), ROS1 (~1-2%) (102), RET (~1-2%) (168), and NTRK1 (~1-2%) (172) account for approximately 10% of lung adenocarcinoma, or ~100,000 patients per year globally (2). EGFR is one of the most well-studied receptor tyrosine kinases (RTKs) because it plays an essential role during embryonic development and adult homeostasis and is often aberrantly activated in cancer (249). EGFR is expressed at high levels or mutated in epithelial malignancies including lung, glioma, HCC, breast, colorectal, HNSCC, and ovarian cancers (249). Wild-type (WT) EGFR is a well-established therapeutic target of cetuximab in HNSCC and colorectal cancer (250,251). EGFR is overexpressed in ~80% of NSCLC and associated with a poor prognosis, but most recent trials in this disease focus on targeting only those tumors that harbor drug-sensitizing EGFR mutations, not the majority of patient tumors that express the WT receptor (261,295,296).

The studies presented here investigate the role of WT EGFR signaling in cancer cells that harbor oncogenic fusion kinases prior to the onset of acquired drug resistance, in an effort to improve our understanding of early adaptive survival signaling. We evaluated potential roles for EGFR signaling following treatment with a fusion kinase inhibitor (FKI), but also in the absence of any therapeutic stress in fusion oncogene positive NSCLC cell lines, mouse models, and patient samples.
Methods

Patients

Written informed consent was obtained from the patient prior to use of the patient’s tumor sample. The consent form and protocol was reviewed and approved by the Colorado Multiple Institutional Review Board. Excess tumor tissue from patients was collected for derivation of tumor xenografts (COMIRB #10-1386) or for additional research studies (COMIRB #11-1621).

Cell lines and reagents

H3122, H2228, HCC78, CUTO-3, CUTO-2, H1650, H520, DFCI-032, STE-1 lung adenocarcinoma cells, KM12 colorectal carcinoma cells, and HEK-293T cells were previously described (21,195,211,215,297,298). LC-2/ad lung adenocarcinoma cells were purchased from Sigma-Aldrich. H1993 lung adenocarcinoma cells were obtained from the University of Colorado Cancer Center tissue culture core. All cancer cell lines were cultured in RPMI1640 supplemented with 1-10% FBS. 293T cells were maintained in DMEM supplemented with 5% FBS. Gefitinib, crizotinib, and TAE-684 were purchased from Selleck chemicals. ARRY-470 was kindly provided by Array Biopharma, and foretinib (XL880) was kindly provided by GlaxoSmithKline. All EGF was purchased from R&D Systems. The following plasmids were previously described: pCDH-EML4-ALK, pCDH-SDC4-ROS1, pCDH-MPRIP-NTRK1, pCDH-MPRIP-NTRK1-KN (21,102,195,211). The FGFR1 cDNA was obtained from Origene, and EGFR was cloned from the EGFR-EGFP expression plasmid from the Sorkin Lab (Addgene, plasmid #32751) and both were cloned into the PLVX plasmid and kindly provided by Dr. Lynn Heasley.
**Lentivirus shRNA production and cell transduction, ectopic transfection, and siRNA transfection**

293T cell transient transfections were performed using Mirus TransIT LT1® (MIR 2304) or TransIT-X2® (MIR 6003) reagents according the manufacturer’s protocol. Briefly, 1-2 μg of cDNA was incubated with 7.5 μl of transfection reagent, and 250 μl of Opti-MEM® I serum reduced media (Thermo-Scientific) for 20 minutes at room temperature for every 9.6 cm² of cells seeded. Transfection complexes were added to 293-T cells drop wise at room temperature. Mission Non-Target shRNA Control #SHC002 and 2 EGFR shRNAs were ordered from the Functional Genomics Facility at the University of Colorado (Boulder, CO) TRCN0000010329 (sh#1) and TRCN0000039634 (sh#2). EGFR shRNAs were transfected into 293T cells and stably transduced via lentivirus into CUTO-3 and LC-2/ad lung adenocarcinoma cells as previously described in chapter 2.

**Co-immunoprecipitations**

Cell lysates were harvested using a modified RIPA lysis buffer (500 mM Tris-HCl, 1% NP-40, 0.25% Sodium deoxycholate, 150 mM NaCl, 1mM EDTA, and H₂O to volume) with Halt protease and phosphatase inhibitor cocktail (Thermo-Scientific) and incubated rotating overnight at 4°C with each designated antibody or antibody-bead conjugate (mouse EGFR sepharose beads, Cell Signaling, #8083). Lysate-antibody complexes were then incubated for 2 hours rotating at 4°C with PBS washed protein-G conjugated agarose beads (Merck Millipore catalog #539207.) Beads were washed through inversions and spun at 13,000 RPM for 30 seconds at 4°C 3x with ice cold PBS. Beads were re-suspended in PBS and 4x sample loading buffer, boiled, and stored at -80°C for immunoblot analysis. Antibodies were used for immunoblot analysis as described below.
Biotin conjugated crizotinib pull-downs

500 µg of H3122 cells or HCC78 cells were harvested in modified RIPA lysis buffer, and incubated with Millipore’s PureProteome™ streptavidin conjugated magnetic beads, following the specified biotinylated crizotinib or biotinylated cabozantinib, or unmodified drug or growth factor treatments, according to the manufacturer’s protocol. Biotinylated-crizotinib and biotinylated cabozantinib were provided by Uwe Rix at the H. Lee Moffitt Cancer Center. Beads were washed using an Invitrogen Lifetechnology magnet and inversions, and re-suspended with PBS and 4x sample loading buffer, boiled, and stored at -80°C for immunoblot analysis.

Immunoblotting

Immunoblot analysis was performed as described in detail in chapter 2 and previously (21). Precast 4-20% gradient gels (Bio-Rad) were used for EGFR and GRB2 immunoprecipitations. The following antibodies were used from Cell Signaling: pTRKA Y490 (9141 or 4619) and Y464/65 (C50F3), pEGFR Y1068 (D7A5) and Y1173 (53A5), total EGFR (2232L) and XP (D38B1), HA (C29F4 or 6E2), pALK Y1278/1282/1283 (3983), total ALK (D5F3, 3333, or 31F12), total ROS1 (D4D6), pRET Y905 (3221), total RET (C31B4), pERK XP T202/Y204, total ERK (3A7), pAKT S473 (D9E 4060 or 193H12), pMEK pS217 (4169) MEK (L38C12), GRB2 (3972), and pan-keratin (C11). Total RET was also purchased from Abcam (ab134100), GAPDH (MAB374) and pTYR (4G10) from Millipore. Total EGFR was purchased (610017; BD Biosciences). TRK (C-14) and tubulin antibodies were purchased from Santa Cruz Biotechnology. FGFR1 antibody (#TA301021, Origene) was purchased.

Proliferation assays

All proliferation assays were performed in media supplemented with 5% FBS as previously described using Cell Titer 96 MTS (Promega) (21). Cells were seeded at 750, 1000, or 2000 cells/well and treated for 72 hours at the drug concentrations described on
each graph. Percent control refers to percent of the DMSO only control. Each assay was performed in triplicate and graphed using GraphPad software. All statistical analysis was performed using a paired student’s t test.

**Proximity ligation assays (PLAs)**

Cells were seeded onto chamber slides at 25-50k cells/well. Cells were treated with the indicated doses and times then fixed for 15 minutes by shaking at room temperature in 4% paraformaldehyde. Cells were rinsed twice in PBS, and then the Duolink® In situ PLA® kit from SigmaAldrich in mouse/rabbit (with red detection) was used according to the manufacturer’s protocol (catalog # DUO92002, 92004, 92008). Antibody concentrations were optimized prior to PLA experiments. FFPE tissue PLAs from mice or patients were prepared as described in histology. Additionally, FFPE samples were treated with 300mM Glycine for 30 minutes at room temperature prior to the blocking step, then the assay was performed according to the manufacturer’s protocol. Cells were mounted using Duolink® In Situ Mounting Medium (with DAPI) and cured, and sealed with clear nail polish prior to imaging. Images were taken on a Nikon standard inverted fluorescent microscope at 40x. The following antibodies were used: Cell Signaling TRK (C17F1), ALK (D5F3), RET (C31B4), ROS1 (D4D6), EGFR (D38B1), SHC1 (3F4, H00006464) from Novus, GRB2 (26B04) from Invitrogen, GRB2 (610111) from BD, EGFR (29.1, E2760) from Sigma-Aldrich, and EGFR (SC-120; Santa Cruz) and GRB7 (A-12; Santa Cruz). The antibodies used in PLAs were tested for specificity using various cell lines (Fig. 33, and data not shown.) A stage micrometer from reticles.com (KR-85-11) was imaged identically to the PLAs and the scale bar inserted accordingly. Each scale bar shown represents approximately 50 microns, unless it is explicitly stated otherwise.
**CellProfiler quantification of PLAs**

CellProfiler software was used to quantify these assays (299). At least 3 independent experiments and at least 300 nuclei were assayed in every quantification analysis. The pipeline used in these studies to analyze images was: ColorToGray (to split fluorescent channels), EnhanceorSuppressFeatures (to enhance the red speckles), IdentifyPrimaryObjects (to specify the number of nuclei in the blue channel), and MeasureImageIntensity (of speckles in the red channel) for each image. Red fluorescence intensity was normalized to the number of nuclei in each image field. Each quantification graph is expressed as the mean ± the SEM. All statistical analysis was performed using a paired student’s t test.

**Fluorescence in-situ hybridization**

*NTRK1* FISH assays were performed as previously described in chapter 2 (21).

**Histology/immunohistochemistry**

Tumor specimens were fixed at room temperature (RT) in 10% neutral buffered formalin, processed for paraffin infiltration, paraffin-embedded at 65°C and cut at 4 microns. Slides were stained with hematoxylin-eosin, and examined microscopically. For IHC staining, the slides were de-paraffinized in xylene and rehydrated in graded concentrations of ethanol before antigen retrieval in EDTA pH 9 solution (Dako) in a pressure cooker at 121°F for 10 minutes. Next, the slides were cooled for 20 minutes before washing in 1X Wash Buffer (Dako). They were then treated with Dual Endogenous Enzyme Block (Dako) for 10 minutes, followed by Serum-Free Block (Dako) for 20 minutes. The samples were incubated with antibody for 60 minutes at RT followed by HRP-conjugated EnVision + Dual Link System (Dako) for 30 minutes at RT. Staining was developed using DAB+ chromogen solution (Dako). Slides were counterstained with automatic Hematoxylin (Dako), washed with wash buffer and distilled water, alcohol dehydrated and mounted with resinous media.
EGFR (D38B1) and pEGFR (D7A5) from Cell Signaling Technologies were used. H score analysis of EGFR IHC in patient samples was performed as previously described (300).

**Animals**

Animal care and procedures were approved by the Institutional Animal Care and Use Committee Office of the University of Colorado Anschutz Medical Campus. 40 nu:nu male mice were injected with 1 x 10^6 H3122 cells in each subcutaneous flank (left and right). Mice were kindly provided by the breeder colony run by Lynn Heasley and James DeGregori. Once tumors reached 250 mm³, mice were randomly distributed into 4 groups (n = ~20 tumors/group) and treated daily by oral gavage: diluent, crizotinib 50 mg/kg, gefitinib 25 mg/kg, or both for 17 days. Tumor size was evaluated twice weekly using calipers to measure and calculated using the formula: volume = (length × width^2)/2. Gefitinib was purchased from Selleck, and crizotinib was kindly gifted back to the Doebele lab from patients. Drugs were reconstituted in “diluent”, which was 1% polysorbate 80. Mice were euthanized when tumor volume or appearance exceeded 2000mm³, and tumor specimens were excised, and fixed in neutral buffered formalin, processed, paraffin-embedded and cut at 4 microns. Each tumor was blindly analyzed by PLA analyses for the pharmacodynamics part of the study. Final statistical comparisons of tumor volume change were calculated using an ANOVA on GraphPad Prism 5 software.

**Patient derived xenograft maintenance**

Animal care and procedures were approved by the Institutional Animal Care and Use Committee Office of the University of Colorado Anschutz Medical Campus. Propagation and maintenance of CULC001 and CULC002 xenografts was previously described by the Jimeno Lab (39,301). Briefly, fresh tumor tissue from lung cancer patients consented at the University of Colorado Hospital (Aurora, CO) in accordance with the protocol approved by the Colorado Multiple Institutional Review Board (COMIRB #08-0552) were collected.
Prepared 3 mm x 3 mm x 3 mm tumor pieces were dipped in Matrigel (BD Biosciences) and inserted into a “pocket” in both hind flanks of nude mice. Upon reaching 1,500 mm$^3$, tumors were passed to a new colony of animals.

**Results**

**EGFR transactivates fusion kinases in lung cancer cells**

Previous studies from our lab have shown that WT EGFR mediates intrinsic resistance to targeted inhibition of ROS1 in a ROS1+ cell line, and EGFR can facilitate the acquired resistance in that cell line when treated chronically with a ROS1 FKI (102,211). We also observed incomplete inhibition of critical downstream signaling pathways in gene fusion positive cell lines where phosphorylation of the fusion kinase was completely inhibited pharmacologically (Fig. 28a, 29). Consistent with this observation, other studies have shown a role for EGFR or other ERBB family members in mediating acquired resistance in ALK+ cell lines (223,224,226,227). A similar phenotype was also observed with RET fusions and EGFR in a thyroid cancer cell model (239), so we hypothesized that EGFR was playing a similar role for multiple different oncogenic fusions in lung cancer. In support of this hypothesis, the addition of the EGFR-specific tyrosine kinase inhibitor (TKI), gefitinib, increased inhibition of downstream signaling pathways while EGF stimulation had the opposite effect: EGF rescued phosphorylation of important downstream signaling pathways such as ERK1/2 and AKT in lung cancer cell lines bearing TRKA, RET, ROS1 and ALK fusions (Fig. 28a, Fig. 29). This indirect rescue of downstream signaling by the EGF/EGFR axis is consistent with prior studies observed with ALK, but our data using ROS1, RET and TRKA models demonstrate this is a more widespread trend in oncogene-driven lung cancer cells (302). Although EGF-induced bypass signaling was expected, we were surprised to see re-phosphorylation of the drug-inhibited fusion kinase (Fig. 28b-e, Fig. 29), suggesting an additional, currently unexplored role for EGFR signaling in cancer cells. To further
understand this fusion kinase re-phosphorylation, we tested whether EGF-induced activation of EGFR resulted in the dissociation of the FKI, or if the drug remained bound to the fusion kinase. To address this, we used a biotin-linked crizotinib or biotin-linked cabozantinib as a bait to immunoprecipitate the drug and its target kinase, ALK or ROS1, respectively, using streptavidin-conjugated beads (303). The biotin-labeled cabozantinib pulls down ROS1, and the biotin-labeled crizotinib pulls down ALK, and, as expected, the biotinylated drug-fusion kinase complex is lost upon addition of excess, unlabeled drug (Fig. 28f). Importantly, stimulation of cells with EGF also disrupted the biotin-cabozantinib pull down of ROS1 or biotin-crizotinib pull down of ALK, respectively (Fig. 28f). While ROS1 fusions are also a target of crizotinib, we were unable to observe pull-down of ROS1 using biotinylated-crizotinib in two different ROS1 cell lines, suggesting that the linker interferes with ROS1 binding. These data suggest that EGFR activation by EGF destabilized FKI binding to ROS1 and ALK.

To assess the influence of EGF stimulation on fusion kinases under conditions where the fusion kinase is not inhibited, we measured the kinetics of phosphorylation of EGFR, the fusion kinase, and ERK1/2 following EGF stimulation. We observed rapid, maximal activation of EGFR at 5 minutes and variable increased phosphorylation of the fusion kinases above levels that occur from constitutive activation, which also resulted in further amplification of downstream signaling (Fig. 29, Fig 30). These data provide evidence that the activity and signaling of oncogenic fusion kinases can be modified by non-oncogenic RTK signaling, such as EGFR, under normal constitutive signaling or FKI-treated conditions.

Proximity ligation assays reveal a novel role for EGFR-GRB2 signaling as a co-driver in fusion kinase positive lung cancer cell lines

We have shown that the fusion kinase is re-phosphorylated following EGFR activation, and that FKI binding was disrupted, but we wanted to ask whether the fusion
kinase was re-engaged in signaling. We first addressed this by using phosphotyrosine-specific antibodies, and were able to identify a common phosphorylated tyrosine residue(s) mapping to the conserved activation loop of the ALK, RET, and TRKA kinases (Fig. 31a-d). Next, based on the re-phosphorylation of tyrosines critical to kinase function, we tested whether this phosphorylation resulted in a rescue of tyrosine kinase function and signaling by the fusion kinase. Therefore, a proximity ligation assay (PLA) was employed to detect kinase-adaptor signaling complexes in cells. We designed assays to recognize each fusion kinase with a predicted, commonly used adaptor protein based on the phosphorylated tyrosine(s) detected following inhibition and rescue with EGF (Fig. 31a-c) (91,156,193). The PLA is designed to detect 2 proteins in close proximity, i.e. tens of nanometers (304,305). We have previously shown active signaling TRK-SHC1 complexes using this approach, and that these complexes can be disrupted by knockdown of one partner by RNAi or by pharmacologic inhibition of the kinase (39). TRKA-SHC1 (CUTO-3 cells), RET-GRB7 (LC-2/ad), ROS1-GRB2 (HCC78), and ALK-GRB2 (H3122 and STE-1) kinase-adaptor PLAs revealed that each fusion was signaling through the adaptor at baseline as expected, and that these complexes were disrupted by a cognate FKI (Fig. 31e, Fig. 31g, Fig. 32). Most importantly, the signaling complexes were restored after EGF stimulation (in the presence of the FKI), and were only fully disrupted by combination treatment of the FKI and gefitinib when EGF was also present (Fig. 31e, Fig. 31g, Fig. 32a). These data build on the re-phosphorylation results observed in the immunoblot experiments (Fig. 28, Fig. 29) and support a model where EGFR signaling is not only re-phosphorylating, but reactivating the signaling capacity of the inhibited fusion kinase.

We next queried the role of EGFR signaling in these gene fusion positive cell lines by implementing an EGFR-GRB2 PLA. This assay was validated using cell lines that encompass different levels of EGFR expression (Fig. 33) and also showed EGFR inhibitor
dose-dependent loss of EGFR signaling complexes (Fig. 33a, b). We observed 2 types of EGFR activation: in the first type, observed in ALK and TRKA fusion cell lines, EGFR signaling complexes were present in unperturbed cells (basal EGFR activation) (Fig. 31e, f, Fig. 32b). Alternatively, in ROS1 and RET fusion kinase positive cell lines, a striking increase in EGFR signaling complexes was observed following inhibition of the fusion kinase (induced EGFR activation) (Fig. 31e, f, Fig. 32b). Thus, these assays collectively provide additional evidence that EGFR can signal independently through GRB2 in these cells.

Switching of adaptor proteins from fusion kinases to EGFR following FKI treatment

In order to evaluate more closely the qualitative and quantitative increase in EGFR-GRB2 signaling with FKI treatment observed in Fig. 31, we performed a time course using the ROS1 cell line HCC78 to further understand the kinetics of the increase in EGFR-GRB2 signaling. We observed a significant change in EGFR signaling complexes within 5 minutes of addition of a ROS1 inhibitor, and this was readily reversible, reverting back within 15 minutes upon wash out of the ROS1 inhibitor (Fig. 34a, b). This switch in GRB2 adaptor binding stoichiometry from ROS1 to EGFR was also observed using co-immunoprecipitation following FKI treatment in the HCC78 cells (Fig. 34c). This dynamic rewiring of GRB2 from ROS1 to EGFR was specific, and did not occur with a different RTK, MET, that is both expressed and phosphorylated in these cells (Fig. 35a, b) (211). Use of H1993 cells, which display MET gene amplification and are sensitive to MET inhibitors such as crizotinib were used as a positive control to demonstrate that MET-GRB2 complexes can be detected using our assay and disrupted with MET inhibition (Fig. 35c, d) (306). Previous work in our lab showed ROS1 FKI-resistant cells had undergone addiction switching from ROS1 to EGFR following the loss of the ROS1 fusion gene, but the mechanism of EGFR activation in these cells remains unclear (211). To further evaluate the change in EGFR signaling in the
parental (HCC78) and drug-resistant cells (HCC78-TR), the EGFR-GRB2 PLA was performed. While there was not a substantial difference in EGFR expression or phosphorylation between the parental and resistant lines, the EGFR-GRB2 PLA was clearly and significantly activated only in the HCC78-TR cells (Fig. 35e-g). A similar result, albeit with slower kinetics, was seen in the RET+ LC-2/ad cells. Using both PLA and co-immunoprecipitation, both GRB2 and SHC1 adaptor proteins demonstrated a similar switch from RET to EGFR (Fig. 36). These experiments taken together suggest that while phosphorylation is necessary to initiate signaling, it may not be a sufficient indicator of adaptor binding and the initiation of a signaling cascade. These results demonstrate the plasticity of signaling in cancer cells as indicated by the ability of the GRB2 or SHC1 adaptors to switch from one kinase to another resulting from short-term kinase inhibition.

**EGFR and fusion kinases can form complexes**

Because EGFR signaling can transactivate fusion kinases in an inactive state (FKI-bound), we asked whether EGFR could also transactivate a different type of inactive state of the fusion kinase: a catalytically inactive mutant version of the fusion kinase. To address this, we co-expressed *EGFR* and kinase-dead, HA-tagged myosin phosphatase Rho-interacting protein (*MPRIP*)-*NTRK1* fusion genes in 293T cells (21). EGF stimulation resulted in phosphorylation of the catalytically inactive RIP-TRKA protein, and this effect was ablated with EGFR kinase inhibition (Fig. 37a). Similarly, EGFR-mediated transactivation was observed for a catalytically inactive CD74-TRKA (Fig. 38a). Taking the rapid phosphorylation of the fusion kinase by EGFR and the preference of the GRB2 adaptor switching onto EGFR (as opposed to other RTKs, such as MET (Fig. 35a, b) into consideration, we asked whether EGFR could exist in a complex with the fusion kinase, and perhaps that interaction or its proximity mediated both the phosphorylation and adaptor switching. RIP-TRKA co-immunoprecipitated with EGFR both in the CUTO-3 cells, where
both proteins are endogenously expressed, and also when ectopically co-expressed in 293T cells (Fig. 37b, c). This interaction was sustained under various kinase inhibitory and stimulatory conditions, including combined inhibition and EGF stimulation, suggesting the interaction may be kinase activity-independent (Fig. 37b, c). Similarly, complexes also formed between EGFR and ALK, ROS1, or RET fusion proteins in NSCLC cell lines (Fig. 38b-d) and by ectopic expression in 293T cells (Fig. 38e-g). Overexpression of a different RTK, FGFR1, did not generate complexes with ROS1, indicating a degree of specificity for the EGFR-oncogenic fusion kinase interaction (Fig. 38h, i). PLA complexes between each of the fusion kinases and EGFR were also observed in each respective cell line (Fig. 37d), but not with cytokeratins, another abundant class of proteins in these cells, nor was any antibody cross-reactivity detected (Fig. 33d, e). In conclusion, these data provide evidence of complexes between TRKA, ALK, RET, and ROS1 fusion kinases and EGFR both in NSCLC cell lines and with ectopic expression.

**EGFR is functionally important to fusion kinase addicted cell lines and tumors**

The ability of EGFR to phosphorylate and form complexes with fusion kinases suggests the potential for an important role for this RTK in tumor cells. We therefore asked whether EGFR was important for tumorigenic properties, such as cell proliferation in vitro or tumor maintenance in vivo, in cancer cells harboring oncogenic fusions. First, we expanded our initial studies to include 9 different fusion kinase positive cell lines (Table 6), and determine the influence of EGFR on cellular proliferation following inhibition with the addition of a fixed dose of gefitinib or inducing EGFR activation by EGF stimulation in the presence of the cognate FKI for each cell line. On average, the stimulation of EGFR with EGF significantly decreased the sensitivity of the cells by ~6-fold ($P = 0.004$) and the inhibition of EGFR with gefitinib significantly increased the sensitivity of the cell lines to their respective FKIs by an average of 12.7-fold ($P = 0.01$), demonstrating an important role for EGFR
signaling in cancer cells that express fusion oncogenes (Fig. 39a-i and Table 7). It is notable that EGFR inhibitors had an effect on proliferation even in the absence of exogenous EGF. Alternatively, we depleted EGFR by RNAi, further supporting the role of EGFR in proliferation of these cells in complement with the pharmacological inhibition already shown (Fig. 40). To determine the role of EGFR in vivo, we sought to establish whether the combination of the FKI crizotinib and gefitinib provided a more substantial reduction in tumor volume compared to monotherapy crizotinib in an ALK+ xenograft mouse model. The combination of gefitinib and crizotinib resulted in a significant decrease in tumor growth over crizotinib monotherapy (Fig. 39j). Additionally, pharmacodynamic analyses were conducted on tumors from each of the four treatment arms in this study to evaluate the target inhibition of ALK and EGFR at the termination of the study. There was a significant reduction of ALK-GRB2 and EGFR-GRB2 signaling complexes with combination therapy, compared to ALK monotherapy, consistent with the effects of combination therapy on tumor growth (Fig. 39k, l). Together these data provide evidence for a functional role for EGFR in fusion kinase-driven cell proliferation as well as tumor maintenance. We next asked whether EGFR activation could be detected in a patient-derived xenograft (PDX) mouse model derived from a NSCLC patient whose tumor harbored an NTRK1 fusion (Fig. 41a) (21,39). Tumors from the PDX model are positive for the NTRK1 rearrangement by FISH and demonstrate both high levels of EGFR expression as well as phosphorylation by immunohistochemistry (Fig. 41b, c). FFPE tumor samples from the NTRK1+ PDX model were positive for TRKA and EGFR signaling complexes by PLA, as well as TRK-EGFR PLA complexes whereas an NTRK1- PDX model derived from a patient with an unknown oncogene was negative for all 3 PLAs (Fig. 41d-i).

EGFR is highly expressed in unselected NSCLC (261). We retrospectively evaluated EGFR expression in a cohort of 26 ALK+ and ROS1+ patients who also had clinical EGFR
IHC testing performed. Similar to unselected NSCLC, the majority of ALK+ (and one ROS1+) tumors had high levels of EGFR expression with 21/26 samples exhibiting an IHC score ≥200 (out of 300) demonstrating that EGFR is available for signaling contribution in most ALK+ tumors (Fig. 42a).

Finally, we asked whether fusion kinase and EGFR signaling complexes could be detected in patient tumor samples. We analyzed ROS1, ALK, and RET positive patient tumor samples by PLA (Fig. 42, 43). Minimal EGFR-GRB2 signaling was detected by PLA prior to treatment with a FKI in a ROS1+ patient, but a significant increase in EGFR signaling was detected at disease progression post FKI treatment in that ROS1+ patient, as well abundant levels in resistant ALK+ or RET+ lung cancer patients (Fig. 42, 43). The fusion kinase-adaptor PLAs were also detected in each patient’s resistant sample following or while still on treatment with the FKI. None of the patient samples had evidence of a resistance mutation in the kinase domain to account for persistent fusion kinase activation suggesting the possibility of fusion kinase transactivation in these samples (Fig. 42b-e, 43a, c). The same patient samples were further tested for PLA specificity using mismatched oncogene PLAs (e.g., ALK-GRB2 PLA on RET fusion positive sample) and no PLA cross-reactivity was observed (Fig. 42f). An interesting observation was made in a tumor sample from an ALK+ patient’s brain metastasis. EGFR-GRB2 signaling was detected robustly throughout the sample, but at higher levels adjacent to stromal fibrovascular bundles, suggesting the possibility of a non-cell autonomous mechanism of EGFR activation in this particular patient’s metastatic tumor sample (Fig. 43c). The in situ patient tumor data reinforce the in vitro and in vivo findings, as well as provide evidence for a role for EGFR-GRB2 signaling in fusion kinase positive patients.
Discussion

Previous work has demonstrated that growth factor ligands can mediate resistance to targeted inhibition of dominant oncogenes in various cell types and activate by-pass signaling pathways (215,302,307) (Fig. 44). This growth factor mediated rewiring can require prolonged MEK inhibition in certain cancer models such as BCR-ABL+ CML, due to a MEK-dependent negative feedback loop (308). EGF and other EGFR ligands are produced in an autocrine or paracrine manner and expressed or upregulated in lung tumors, including upregulation in the setting of drug resistance to targeted agents (302,309-311). These prior studies, however, demonstrate a mechanism whereby ligand engagement leads to bypass of the dominant oncogene. The work presented here demonstrates two additional, novel roles beyond bypass signaling for EGFR in cancer cell lines harboring gene fusions, as well as a fusion kinase positive mouse model and patient samples: direct reactivation of the drug-inhibited oncogene and rapid, dynamic adaptor protein-mediated signaling rewiring. Thus, these findings provided evidence for a cooperative model of signaling (Fig. 44). These particular results are also supported by another recent, similar kinase transactivation report from gastrointestinal stromal tumors (GIST) (312).

The work presented here and elsewhere increasingly suggests that the original model of oncogene addiction should allow for the influence of supportive signaling input in cancer cells harboring constitutively activated oncogenes (3). The work presented here provides evidence against a strict top-down only signaling cascade driven entirely by the dominant oncogene, but rather suggests a more cooperative model, where the oncogene can receive input and be influenced by other proteins—in this particular example, EGFR (Fig. 28, 30, 31, 39, 44). Indeed, inspection of patient tumor responses in clinical trials of oncogene directed therapies in ALK and ROS1+ NSCLC suggest the need for a conceptual modification of the oncogene addiction model to account for the heterogeneous responses
observed between patients (cf figures 2a and 1a, respectively, in Kwak et al and Shaw et al) (31, 209, 313). While there may be many potential explanations for the interpatient heterogeneity, our results suggest that differential WT EGFR engagement could provide one such explanation. The critical role of EGFR described here in the context of a fusion oncogene is perhaps more consistent with the theory of non-oncogene addiction proposed in more recent years, in both tumor-intrinsic and tumor-extrinsic manners (314).

Previous studies have described a critical role for WT EGFR in KRAS mutant and EGFR mutant lung cancer, suggesting a requirement for the WT receptor even when a constitutively active oncogene is present in a cancer cell (262, 263). Prior work has also identified EGFR signaling dependence in cancers without a clear genetically-mediated activation mechanism or phosphorylation of EGFR detected by immunoblot analysis, one of the standard detection methods for RTKs in cancer (211, 250, 251, 315). Importantly, detectable autophosphorylation of EGFR is not necessarily associated with its kinase activity or signaling engagement (229). Furthermore, WT EGFR can be activated in the absence of ligand via the formation of ionic bonds between the juxtamembrane region of EGFR with the lipid PIP$_2$ in the membrane (255) and other ligand-independent mechanisms (256). More recent work has also revealed EGF-containing secretomes induced by oncogene-targeted therapies for different oncogenes, including ALK, can support the survival of otherwise drug sensitive clones (311).

The RTK-adaptor PLAs used in this study enabled the discovery of activated EGFR signaling complexes in cell lines, as well as in FFPE sections of PDX or patient tumor samples in the absence of any detectable genetic abnormalities. The concept of GRB2 adaptor switching demonstrated here may be relevant to other previously established models when by-pass signaling mediated therapeutic resistance, and where the role of the adaptor was not previously explored. The work from this study indicates that an adaptor
protein may demonstrate binding plasticity and simply “switch” from ROS1 to EGFR under therapeutic stress (Fig. 31c, 34a). However, washing out the drug allows GRB2 to revert to ROS1 binding, suggesting a preference for the dominant fusion oncogene (Fig. 34d). The adaptor binding is likely subject to many other factors, including concentration and competition in its environment, and thus may not automatically occur following phosphorylation. Using the ROS1+ HCC78 cells as an example, both the acute loss of the ROS1 signaling (through drug treatment) and the chronic loss of ROS1 signaling (via the loss of a copy of ROS1 fusion in the HCC78-TR cells) was required for EGFR’s signaling cascade to take over (Fig. 31, 33, 35) (211).

The ability of EGFR to transactivate the fusion kinase in the presence of a cognate FKI may be relevant in other drug resistance models and explain previous paradoxical results that have been enigmatic. Notably, acquired drug resistance in the ALK+ H3122 cell line using two next generation ALK inhibitors, alectinib and ceritinib, induced increased EGFR phosphorylation and EGFR dependence (226). However, more interesting was the unexplained persistence of ALK phosphorylation in these cells despite the presence of a potent ALK inhibitor and the lack of a kinase domain mutation that would decrease ALK inhibitor binding (cf Figure 2a, b in Dong et al.). These data suggest the possibility of a chronic state of ALK transactivation by EGFR (and ERBB3) and further implicate the importance on EGFR in gene fusion positive lung cancer.

The complex signaling networks in cancer cells create the potential for growth factor-mediated co-activation of RTKs to mediate resistance to oncogene-targeted therapy (215,302,316,317). RTK-mediated bypass signaling or cross-talk are potential mechanisms of acquired resistance through re-activation of key downstream pathways, as well as addiction-switching of oncogenes (295,318). Signaling cross-talk can occur through a direct mechanism, such as heterodimerization, or through various indirect methods including
scaffolding, feedback loops, transcriptional regulation, or the reactivation of shared
downstream signaling components (295). EGFR has been implicated in many of these
cross-talk mechanisms and can heterodimerize with other RTKS, including PDGFR and
IGF-1R, as well as the other members of the ERBB family of RTKs (230,236,319,320).
EGFR has been shown to transactivate many other receptors in other models, such as
TRKB and TRKC in certain neuronal cell types, TRKB in lung cancer, RON in HNSCC and
RET fusion kinases in thyroid cancer (238-240,321). Additionally, full-length TRKA and
EGFR signaling crosstalk have been demonstrated in non-disease models such as
monocytes and renal epithelial cells (322,323). Recognizing and understanding the ability of
cancer cells to signal by novel or complex mechanisms, especially in response to targeted
therapy, is necessary to maximize initial tumor cell killing and to delay clinically significant
drug resistance.

These studies are the first to demonstrate that EGFR can influence the activity of an
oncogenic fusion kinase in cell lines derived from human tumors. Our results support
previous work demonstrating that EGFR could interact with and induce phosphorylation of
both a catalytically active and inactive RET fusion when expressed exogenously in a model
cell line and expand these findings to additional fusions including ALK, ROS1, and TRKA,
suggesting a common functional role for EGFR in cancer cells harboring oncogenic fusions
(239). This manuscript is also consistent with studies indicating a potential for reactivation of
kinase dead mutations (324). These data suggest that WT EGFR, which is highly expressed
on the majority of ALK+ tumor samples (Fig. 42a), plays an onco-requisite role in cancer
cells harboring gene fusions. This work demonstrates a potential role for inhibiting non-
mutated EGFR using EGFR inhibitors in ALK+ or ROS1+ patients being treated with
crizotinib, and potentially for RET+ and TRKA+ patients once targeted drugs are approved
for these indications. Our work in a colorectal cancer cell line KM12 harboring the TPM3-
*NTRK1* fusion suggests that this role for EGFR in the context of fusion kinases might also apply to tumor types other than lung cancer harboring fusion genes (36,165,294). While it is clear from clinical trials crizotinib in ALK+ and ROS1+ patients that EGFR cannot sustain sufficient signaling in all patient tumors or in all cancer cells, EGFR rescue may explain the incomplete or heterogeneous response seen in the majority of patients (209).

In summary, these studies demonstrate a unique, direct contribution of EGFR’s signaling to the function of four different fusion kinases and demonstrate that EGFR can enable an early, adaptive rescue of signaling in cancer cells whose dominant oncogenes are inhibited. Importantly, exogenous EGF is not necessary for all of the EGFR roles described here in maintaining critical signaling in cancer cells. Notably, all of the EGFR contributions could be abrogated with the EGFR TKI gefitinib. On-going work will determine other potential pharmacologic strategies to inhibit WT EGFR in the clinic. Further investigation of inhibition of EGFR in combination with oncogene-specific inhibitors is warranted to determine if these combinations will deepen and/or prolong initial patient responses, and perhaps ultimately delay the onset of disease progression by acquired resistance.

**Acknowledgements**

I would like to sincerely thank Adriana Estrada-Bernal, Chad G. Pearson, Ricardo Pineda, and Barbara A. Helfrich for their assistance with the studies presented in this chapter. I also thank Pasi A. Janne from the Dana Farber Cancer Institute and Christine M. Lovly from Vanderbilt-Ingram Cancer Center for providing reagents used in this study. I also would like to thank also thank GlaxoSmithKline and Array Biopharma for providing foretinib (XL-880) and ARRY-470, respectively. I would also like to thank Patrick Chestnut and the Molecular Pathology Shared Resource for their assistance with collection of patient specimens for these studies.
Figure 28
Figure 28: EGFR blockade improves inhibition of downstream signaling, and stimulation re-activates targeted inhibition of TRKA, RET, ROS1, and ALK fusion kinase domains. (a) NSCLC fusion kinase positive cell lines HCC78 (ROS1), H3122 (ALK), LC-2/ad (RET), CUTO-3 (TRKA), and CUTO-2 (ROS1) were treated with either a vehicle control, cognate fusion kinase inhibitor: 250 nM TAE-684 (HCC78 ROS1), 250 nM crizotinib (ALK or ROS1), 250 nM foretinib (RET), or 100nM ARRY-470 (TRKA), or FKI with 1 µM gefitinib. Downstream signaling changes were evaluated with pMEK1/2 (S217/221), S473 for pAKT, T202 and Y202 for pERK. n = 2. (b) NSCLC fusion kinase positive cell lines CUTO-3 (c) LC-2/ad, (d) HCC78 cells, and (e) H3122 cells were treated for 3 hours with vehicle control (DMSO), a cognate fusion kinase inhibitor: 100nM ARRY-470 (TRKA), 250 nM foretinib (RET), 500 nM TAE-684 (ROS1), or 250 nM crizotinib (ALK), 1 µM gefitinib, or the combination of FKI and gefitinib, in the absence or presence of 30 minutes of 100ng/mL EGF stimulation. Red boxes highlight rescued fusion phosphorylation with the addition of EGF in the presence of the FKI. Immunoblot analysis was performed using the antibodies indicated. Phospho-specific antibodies to Y496 Y680/681 were used for pTRKA, and 4G10 was used for pROS1, pRET, and pALK as shown. A more comprehensive evaluation of the signaling changes under each treatment condition is shown in figure 29. n = 3. (f) Immunoblot analysis of HCC78 or H3122 cell lysates treated with vehicle control (DMSO), 250 nM cabozantinib or crizotinib 250 nM of biotin conjugated cabozantinib or biotin conjugated crizotinib in the presence of 100x unconjugated drug, or 100 ng/mL EGF and immunoprecipitated with streptavidin conjugated magnetic beads. Blots were then probed for total ROS1 or ALK to assess the ability of cabozantinib or crizotinib to bind specifically to ROS1 or ALK, under the inhibitor only and inhibitor plus EGF stimulated conditions. Lysates were also probed for downstream pERK to indicate potency and no interference of the linker of biotinylated crizotinib compared to unlabeled crizotinib.
Figure 29: EGFR inhibition or stimulation affects fusion oncogene signaling. (a) CUTO-3 (b) LC-2/ad, (c) HCC78 cells, and (d) H3122 cells were treated for 3 hours with vehicle control (DMSO), a cognate fusion kinase inhibitor: 100nM ARRY-470 (TRKA), 250 nM foretinib (RET), 250 nM TAE-684 (ROS1), or 250 nM crizotinib (ALK), 1 µM gefitinib, or the combination of FKI and gefitinib, in the absence or presence of 30 minutes of 100ng/mL EGF stimulation. Immunoblot analysis was performed using the antibodies indicated. Phospho-antibodies to Y1068 and Y1173 or 4G10 were used for pEGFR, S473 for pAKT, T202 and Y202 for pERK, Y496 Y680/681 for pTRKA, and 4G10 for pROS1, pRET, and pALK.
Figure 30: EGF stimulation increases unperturbed fusion kinase phosphorylation. (a) Immunoblot analysis of CUTO-3 cells phosphorylation kinetics for EGFR, RIP-TRKA, and ERK following vehicle control (PBS) stimulation, 5, 15, and 30 minutes of 100 ng/mL EGF stimulation. Blots were probed with Y496 and Y680/681 for pTRKA. Representative images of blots are shown. Identical immunoblot and quantification analysis of (b) HCC78 cells (ROS1) (c) STE-1 cells (ALK) and (d) LC-2/ad (RET) cells, pEGFR, the corresponding fusion kinase phosphorylation, and pERK following the indicated time course of 100 ng/mL EGF stimulation (5, 15, and 30 minutes.) n = 3.
Figure 31
Figure 31: EGFR restores pharmacological disruption of conserved fusion kinase tyrosines, signaling complexes and signals independently through GRB2 in fusion kinase NSCLC cell lines. (a) Immunoblot analysis of CUTO-3 cells using an antibody against Y480/481 after inhibition with the TRK inhibitor ARRY-470 at 100 nM, the combination of the FKI with 1 µm gefitinib, with and without 100 ng/mL EGF stimulation. n = 3. (b) Immunoblot analysis of LC-2/ad cells treated with 250 nM foretinib, the combination of the FKI with 1 µm gefitinib, with and without 100 ng/mL EGF stimulation. The blot was probed using an anti-pRET Y905 antibody. n = 3. (c) Immunoblot analysis of H3122 cells using the anti-pALK Y1278/82/83 antibody. Cells were treated with crizotinib, the combination of the FKI with 1 µm gefitinib, in the presence or absence of 100 ng/mL EGF to demonstrate which ALK tyrosine residues were being phosphorylated. Representative blot images are shown. n = 3. (d) Protein amino acid alignment performed using PROMALS software (http://prodata.swmed.edu/promals/promals.php) for TRKA, RET, ALK, and ROS1 kinase domains. Y905 of RET, Y480/481 of TRKA, and Y1282/83 of ALK are the conserved activation loop tyrosines for each kinase, shown in red for each. No phosphotyrosine-specific antibody was available for ROS1 at Y2115 and/or Y2116, but ROS1 is shown for comparison. (e) Quantification of fusion kinase-adaptor PLAs in the indicated cell lines: TRKA-SHC1 (CUTO-3; FKI = 250 nM foretinib), RET-GRB7 (LC-2/ad; FKI = 250 nM foretinib), ROS1-GRB2 (HCC78; FKI=200 nM TAE-684), and ALK-GRB2 (H3122; FKI=250 nM crizotinib; STE-1; FKI=250 nM crizotinib) were treated with DMSO, FKI, FKI + 20-minute stimulation with 10 ng/ML EGF, or FKI + 1 µm gefitinib + EGF. n = 3. Statistical analysis was performed across average fold changes in red fluorescence intensity across all 5 cell lines. P values were calculated using a paired student’s t test. * indicates p < 0.05, ** p < 0.01, *** p < 0.005. (f) Quantification of the EGFR-GRB2 PLAs in the indicated cell lines, with the same drug treatment conditions as described in (e). n = 3. Statistical analysis was performed across average changes in red fluorescence intensity across all 5 cell lines. P values were calculated using a paired student’s t test. * indicates p < 0.05, ** p < 0.01. Data in both (e) and (f) is expressed as the mean ± the SEM. (g) Representative images from experiments in (a) and (b) are shown for the HCC78 (ROS1) cell line under the 4 treatment conditions. Scale bars shown represent approximately 50 microns.
Figure 32: Representative image analyses of fusion kinase and EGFR signaling PLAs. (a) Representative images from fusion kinase-adaptor PLAs in the indicated cell lines: TRKA-SHC1 (CUTO-3; FKI = 100 nM AR470), RET-GRB7 (LC-2/ad; FKI = 250 nM foretinib), and ALK-GRB2 (H3122 and STE-1; FKI=250 nM crizotinib) were treated with DMSO, FKI, FKI + 20-minute stimulation with 10 ng/ML EGF, or FKI + 1 µm gefitinib + EGF. (b) Representative images from EGFR-GRB2 PLAS in the same cell lines and with the same treatment conditions as described in (a). n = 3. Scale bars shown represent approximately 50 microns.
Figure 33: Optimization of the EGFR-GRB2 PLA and fusion kinase PLA antibody controls. Demonstration of the specificity of the EGFR-GRB2 PLA (a) Representative images of H1650 NSCLC cell line that express the EGFR activating mutation deletion exon 19 (delE746A-750) treated with DMSO or treated with 1 µM gefitinib. (b) H1650 cells were treated with a dose range of gefitinib to demonstrate a corresponding linear reduction in EGFR-GRB2 signaling complexes, and a significant reduction with the maximal dose, and P values were calculated using a paired student’s t test. * p < 0.05. Data is expressed as the mean ± the SEM. n = 3. (c) H520 lung squamous cell line that does not express detectable levels of EGFR had no PLA signal. n = 3. (d) PLA assays were used to demonstrate antibody and corresponding PLA specificity. The TRKA and ALK fusion proteins did not form complexes with another abundant class of proteins, cytokeratins (a pan-cytokeratin antibody was utilized), using a TRK- or ALK-cytokeratin PLA in CUTO-3 and H3122 cells. The green channel was included in merged pictures to demonstrate functionality of the cytokeratin antibody. (e) EGFR-fusion kinase PLA complexes for RET, ROS1, TRKA and ALK were not detected in cell lines that do not express the appropriate fusion kinase, indicating the antibodies do not cross-react with other kinases. n = 3. Scale bars shown represent approximately 50 microns.
Figure 34: Time course of GRB2 signaling rewiring in a ROS1+ cell line treated with a fusion kinase inhibitor (FKI) reveals the kinetics are rapid and reversible. (a) Time course of FKI treatment in HCC78 ROS1+ cells fixed at the indicated time points, and assayed by ROS1-GRB2 or EGFR-GRB2 PLAs. FKI= 250 nM TAE-684. Representative images are shown. n = 3. (b) Quantification of experiments described in (A). Data is expressed as the mean ± the SEM. * indicates p < 0.05, ** p < 0.01, *** p < 0.005. (c) ROS1 or EGFR was immunoprecipitated from HCC78 parental cells followed by immunoblot analysis using anti- ROS1, EGFR, or GRB2 antibodies after treatment with vehicle (DMSO) or FKI; 250 nM TAE-684. (d) After two hours of FKI treatment, cells were washed twice in PBS and maintained in fresh media at the designated “washout” time until fixation. Cells were assayed by ROS1-GRB2 or EGFR-GRB2 PLA accordingly. Representative images are shown. n = 3. (e) Quantification of experiments described in (d). Data is expressed as the mean ± the SEM. * indicates p < 0.05, ** p < 0.01. Scale bars shown represent approximately 50 microns.
Figure 35: The GRB2 adaptor switches from ROS1 to EGFR with acute and chronic (resistant) ROS1 FKI treatment. (a) A MET-GRB2 PLA was performed following treatment of the HCC78 ROS1+ cells with the FKI to demonstrate that the reported signaling increase of EGFR-GRB2 was unique, and not observed for all RTKs. Representative images are shown. n = 3. (b) Quantification of PLA analysis described in (a). Changes in MET-GRB2 signaling complexes were not significant by a student’s paired t-test. (c) Representative images demonstrating MET-GRB2 signaling PLA in a MET gene amplified NSCLC cell line, H1993, with and without 250 nM treatment of the MET inhibitor crizotinib. n = 3. (d) Quantification of PLA analysis described in (c). * indicates p < 0.05, ** p < 0.01. (e) ROS1-GRB2 and EGFR-GRB2 PLAs were performed on untreated parental HCC78 cells and on TAE-684 resistant HCC78 cells (HCC78-TR). Representative images are shown. n = 3. (f) Quantification of PLA analysis described in (e). * indicates p < 0.05, ** p < 0.01. (g) EGFR immunoprecipitation from HCC78 (parental) and HCC78-TR (resistant) cells followed by immunoblot analysis with anti-pEGFR, or EGFR antibodies. pEGFR was detected using Y1068 and Y1173 antibodies. Representative images of immunoblot analysis are shown. All data is expressed as the mean ± the SEM. All P values were calculated using a paired student’s t test. n = 3. Scale bars shown represent approximately 50 microns.
Figure 36: GRB2 and SHC1 signaling rewire in a RET+ cell line treated with an FKI.  (a) and (b) Time course of FKI treatment in LC-2/ad RET+ cells fixed at the indicated time points, and assayed by RET-GRB2, EGFR-GRB2, RET-SHC1, or EGFR-SHC1 PLAs. FKI= 250 nM foretinib. Representative images are shown. n = 3. (c) and (d) Quantification of experiments described in (a) and (b). Data is expressed as the mean ± the SEM. P values were calculated using a paired student’s t test  * indicates p < 0.05, ** p < 0.01, *** p < 0.005. (e) RET or EGFR was immunoprecipitated from LC-2/ad cells followed by immunoblot analysis using anti-RET, EGFR, GRB2, or SHC1 antibodies after treatment with vehicle (DMSO) or FKI; 250 nM foretinib. Scale bars shown represent approximately 50 microns.
Figure 37: EGFR transactivates a kinase dead fusion and forms a complex with kinase fusion proteins. (a) EGFR and HA-tagged catalytically inactive RIP-TRKA (K544N) or an empty-PCDH vector cDNA were transiently expressed in 293T cells for 24 hours and treated with DMSO vehicle, stimulated with 10 ng/mL EGF for 60 minutes, or with 1 µM gefitinib for 2 hours, and immunoprecipitated with the HA antibody, and immunoblotted for phospho-TRKA at Y496, and Y680/481 or HA. Immunoblotted lysates from the same experiment are shown. n = 3. (b) Immunoprecipitation of TRKA was performed in CUTO-3 cells following treatment for 3 hours of vehicle control (DMSO), 100nM ARRY-470, 1 µM of gefitinib, the combination, and in the absence or presence of 20 minutes of stimulation with 10ng/mL EGF. Corresponding immunoblot analysis of TRKA and EGFR is shown, including analysis of lysates from the same immunoprecipitation experiment, n = 3. (c) EGFR, RIP-TRKA-HA, or empty-vector (EV) cDNA were transiently expressed in 293T cells for 24 hours and treated for 3 hours of vehicle control (DMSO), 100nM of ARRY-470, 1 µM of gefitinib, the combination, and in the absence or presence of 30-minute stimulation with 10ng/mL EGF. Lysates were immunoprecipitated using an anti-HA antibody. Immunoblotted lysates from the same experiment are shown, n = 3. (d) Proximity ligation assays were used to further assess potential protein-protein interactions between TRKA, RET, ALK, and ROS1 (rabbit) fusion kinases and EGFR (mouse) endogenously in CUTO-3, LC-2/ad, H3122, and HCC78 cells lines, respectively. Representative images are shown. n = 3. Scale bars shown represent approximately 50 microns.
Figure 38
Figure 38: RET, ROS1 and ALK fusion kinases co-immunoprecipitate specifically with EGFR. (a) EGFR and FLAG-tagged catalytically inactive CD74-TRKA (K544M) or an empty-PCDH vector cDNA were transiently expressed in 293T cells for 24 hours and treated with DMSO vehicle, stimulated with 10 ng/mL EGF for 60 minutes, or with 1 µM gefitinib for 2 hours. Immunoblotted lysates from the experiment were probed for phospho-TRKA at Y496, and Y680/481 or FLAG and EGFR antibodies. n = 3. (b) Immunoprecipitation of EML4-ALK (E13:A20) with EGFR using an anti-EGFR antibody under basal, FKI, FKI + 20 minutes of 10 ng/mL EGF, and FKI + 1 µM gefitinib + EGF in H3122 cells (FKI = 250 nM crizotinib). (c) SLC34A2-ROS1 and EGFR in HCC78 cells, (FKI = 250 nM TAE-684) and (d) CCDC6-RET and EGFR in LC-2/ad cells (FKI = 250 nM foretinib). Representative immunoblot and corresponding cell lysate images are shown. n = 3. (e) Immunoprecipitation of ALK in 293T cells expressing an empty PCDH vector, EML4-ALK (E13:A20) and EGFR followed by immunoblot analysis with ALK and EGFR antibodies under unstimulated conditions (DMSO) or treatment with both 250nM crizotinib and 10ng/mL EGF. (f) Immunoprecipitation of ROS1 in 293T cells expressing empty vector, SDC4-ROS1 (S2:R32) and EGFR followed by immunoblot analysis with ROS1 and EGFR antibodies under unstimulated conditions or treatment with both 250nM TAE-684 and 10ng/mL EGF. (g) Reciprocal immunoprecipitation of EGFR in 293T cells expressing empty vector, EGFR and ALK or ROS1 followed by immunoblot analysis with anti-EGFR and ALK or ROS1 antibodies under unstimulated conditions or treatment with both 250nM crizotinib and 10ng/mL EGF. Pre-immunoprecipitation lysates are shown below each panel. Empty vector (EV) or EGFR expression alone in 293T cells are shown as negative controls. Representative images are shown, n = 2. (h) Immunoprecipitation of FGFR1 in 293T cells co-transfected with FGFR1 and SDC4-ROS1 cDNAs followed by immunoblot with anti-FGFR1 or ROS1 antibodies under unstimulated (DMSO) or 200 nM TAE-684 plus 10 ng/mL FGF2 conditions. (i) Reciprocal immunoprecipitation against ROS1 was also negative under the same conditions as shown in (g). Pre-immunoprecipitation lysates are shown below each panel. Empty vector (EV) transfection is shown as a negative control. Representative images are shown, n = 2. Scale bars shown represent approximately 50 microns.
Figure 39
Figure 39: EGFR contributes to the oncogenic program in fusion kinase driven lung cancer in vitro and in vivo. (a)-(i) Cell lines that harbor gene fusions were treated with the indicated FKI alone (dark blue dose curve, x-axis), FKI + 1 µM gefitinib (green), or FKI + 100 ng/mL EGF (light blue) for 72 hours and assessed using MTS. Each condition was performed in triplicate, data are expressed as the mean ± the SEM, n = 3. IC50 values and statistical analysis for each condition are shown in Table 7. (j) Graph depicting changes in tumor volume in an H3122 xenograft mouse model over the course of 17 days of treatment with vehicle (black), gefitinib (blue), crizotinib (red), or the combination (green) of crizotinib and gefitinib. Each treatment group started with 20 tumors at approximately 250 mm3 at study baseline. Statistical analysis was performed using Bonferroni’s multiple comparison ANOVA test: crizotinib vs. control, p < 0.05; crizotinib vs. gefitinib, p < 0.001; gefitinib vs. control, not significant; crizotinib+gefitinib vs. control, p < 0.001; crizotinib + gefitinib vs. gefitinib, p < 0.001; and crizotinib vs. crizotinib + gefitinib, p <0.05 (n = 20 tumors per group, except control n = 19). (k) 5 FFPE tumors from each of the 4 treatment groups were blindly analyzed by ALK-GRB2 or EGFR-GRB2 PLA signaling analysis for a pharmacodynamics assessment of the effects of the drugs on the indicated signaling pathways in each mouse tumor. Representative images from each group are shown. (l) Quantification of all ALK-GRB2 and EGFR-GRB2 PLAs across tumors from each treatment group. Data is expressed as the mean ± the SEM. All P values were calculated using a paired student’s t test. * indicates p < 0.05, ** p < 0.01. Scale bars shown represent approximately 50 microns.
Figure 40: EGFR knockdown reduces fusion kinase cancer cell proliferation. (a) Knockdown of EGFR was performed by stable expression of two different shRNA constructs against \textit{EGFR} or non-targeting control (NTC) shRNA vector (PLKO) in CUTO-3 cells followed by immunoblot analysis with pEGFR, EGFR, and Tubulin antibodies. (b) Measurement of cell proliferation by MTS of CUTO-3 cells expressing NTC or two different shRNA constructs against EGFR. (c) Knockdown of EGFR was performed by stable expression of two different shRNA constructs against \textit{EGFR} or non-targeting control (NTC) shRNA vector (PLKO) in LC-2/ad cells followed by immunoblot analysis using pEGFR, EGFR, and Tubulin antibodies. (d) Measurement of cell proliferation by MTS of LC-2/ad expressing NTC or two different shRNA constructs against EGFR as in (b).
Figure 41: EGFR is highly expressed, phosphorylated, and both EGFR and TRKA signaling complexes are present in vivo in an NTRK1+ patient-derived xenograft (PDX) model. (a) Images of F1 generation flank tumors from NTRK1+ PDX in a nude mouse. (b) 100x FISH images for specimen CULC 001 (NTRK1+) F1 Spleen metastases showing nuclei with single 3’NTRK1 (red arrows), single 5’NTRK1 (green arrows) and split 3’NTRK1 and 5’NTRK1 signals (yellow arrows.) (c) 100x immunohistochemistry analysis of NTRK1 PDX spleen metastatic tumors showing H&E, human total EGFR, or Y1068 for pEGFR staining. (d) and (g) TRKA-SHC1, (e) and (h) EGFR-GRB2, and (f) and (i) TRKA-EGFR PLAs were performed on F3 generation FFPE tumor tissue from either the NTRK1(+) PDX CULC-001 mouse model (a-c) or an NTRK1(-) PDX CULC-002 model (d-f). Scale bars shown represent approximately 50 microns.
Figure 41
Figure 41 (cont.)
Figure 42: Fusion kinase, and EGFR signaling complexes are present in RET, and ALK, fusion resistant patient samples. (a) Vertical scatter plot analysis of H-score quantification of EGFR immunohistochemical analysis of 26 ALK+ and ROS1+ NSCLC patients. The line represents the median of the scores. EGFR IHC grading by H score is standardized, and occurs on a scale of 0 to 300 based on the number of tumor cell membranes that are positive. (b) FFPE tumor samples from 1 RET+ patient taken 3 days after treatment with the FKI ponatinib (c) – (e) FFPE tumor samples from 3 different ALK+ patients post treatment with the FKI crizotinib were also assessed by ALK and EGFR PLAs. (f) FFPE tumor samples from RET+ and ALK+ patients were used as negative controls to show specificity of each fusion kinase-adaptor assay in different patient samples positive for a different fusion kinase. No PLA cross-reactivity was detected in any of the indicated samples. Scale bars shown represent approximately 50 microns.
Figure 43: Fusion Kinase and EGFR signaling complexes are present in ROS1+ and ALK+ patient samples resistant to crizotinib. (a) FFPE tumor samples from a ROS1+ patient pre- and post-treatment (at disease progression) with the FKI crizotinib were analyzed using ROS1-GRB2 and EGFR-GRB2 PLA. Representative images are shown. (b) Quantification of PLA signal for both ROS1-GRB2 and EGFR-GRB2 signaling complexes is shown. Data are expressed as the mean ± the SEM. P values were calculated using a paired student’s t-test. ** p < 0.01 (c) ALK-GRB2 and EGFR-GRB2 analysis in primary lung and metastatic brain lesions in a crizotinib resistant ALK+ patient. Scale bars shown represent approximately 50 microns.
Figure 44: EGFR and fusion kinase cooperative signaling model. Cartoon schematic summarizing the previous model for (a) canonical by-pass signaling or (b) a modified model demonstrating the cooperative signaling that is going on between EGFR and an oncogenic fusion kinase. FKI = fusion kinase inhibitor.
Table 6: Cell lines and inhibitors used in this study.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Gene Fusion</th>
<th>Fusion Kinase Inhibitor (FKI)</th>
<th>Other significant drug targets</th>
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</thead>
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<tr>
<td>H3122</td>
<td><em>EML4-ALK</em>¹</td>
<td>crizotinib</td>
<td>MET, ROS1</td>
</tr>
<tr>
<td>H2228</td>
<td><em>EML4-ALK</em>²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STE-1</td>
<td><em>EML4-ALK</em>³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFCI032</td>
<td><em>EML4-ALK</em>⁴</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCC78</td>
<td><em>SLC34A2-ROS1</em></td>
<td>TAE-684</td>
<td>ALK</td>
</tr>
<tr>
<td>CUTO-2</td>
<td><em>SDC4-ROS1</em></td>
<td>crizotinib</td>
<td>MET, ALK</td>
</tr>
<tr>
<td>CUTO-3</td>
<td><em>MPRIP-NTRK1</em></td>
<td>ARRY-470</td>
<td>None (at 1µM)</td>
</tr>
<tr>
<td>KM12</td>
<td><em>TPM3-NTRK1</em></td>
<td>foretinib</td>
<td>MET, ROS1, TRKA, AXL</td>
</tr>
<tr>
<td>LC-2/ad</td>
<td><em>CCDC6-RET</em></td>
<td>foretinib</td>
<td>MET ROS1, TRKA, AXL</td>
</tr>
</tbody>
</table>

¹*EML4* exon 13–*ALK* exon 20. ²*EML4* exon 6–*ALK* exon 20
**Table 7:** EGFR significantly contributes to cellular proliferation in fusion kinase positive cell lines. IC\(_{50}\) values and statistical analysis for proliferation assays in Figure 39 are shown. *P* values were calculated using a paired student’s t test.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>FKI</th>
<th>FKI</th>
<th>FKI + EGF</th>
<th>FKI + gefitinib</th>
<th>FKI + EGF + gefitinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3122 (ALK)</td>
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<td>100.2</td>
<td>923.7</td>
<td>63.0</td>
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<td>H2228 (ALK)</td>
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<td>400.8</td>
<td>953.1</td>
<td>157.3</td>
<td>89.7</td>
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<tr>
<td>STE-1 (ALK)</td>
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<td>48.9</td>
<td>267.8</td>
<td>23.8</td>
<td>24.3</td>
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<tr>
<td>DFCI032 (ALK)</td>
<td>crizotinib</td>
<td>280.5</td>
<td>1114.0</td>
<td>21.4</td>
<td>25.6</td>
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<tr>
<td>HCC78 (ROS1)</td>
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<td>124.5</td>
<td>368.6</td>
<td>15.9</td>
<td>23.0</td>
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<td>7.6</td>
<td>15.7</td>
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<td>44.1</td>
<td>57.6</td>
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<td>2.8</td>
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<td>LC2/ad (RET)</td>
<td>foretinib</td>
<td>26.8</td>
<td>121.7</td>
<td>3.1</td>
<td>6.9</td>
</tr>
</tbody>
</table>

**AVERAGE FOLD CHANGE:**  
\[+5.9 \quad **P=0.004 \quad -12.7 \quad *P=0.01 \quad -8.5 \quad *P=0.01 \quad -55.1 \quad **P=0.004\]
CHAPTER IV
MECHANISMS DRIVING COOPERATIVE SIGNALING IN FUSION KINASE POSITIVE CANCER CELLS

Introduction

Work in chapter three described a more cooperative signaling model than was previously described by oncogene addiction. It was demonstrated that a wild-type kinase, EGFR, is able to modify the role of a constitutively active fusion kinase oncogene. EGFR’s signaling axis can phosphorylate each of the 4 fusion kinases (both in unperturbed cells as well as under conditions of therapeutic stress), as well as form complexes that can be detected using various biochemical methods. It was demonstrated that EGFR’s signaling cascade functionally contributes to the oncogenic program in these four molecular contexts in vitro, in vivo, and in situ. However, many important questions regarding these observations remain unanswered.

We have demonstrated using co-immunoprecipitations and proximity ligations assays that complexes can be formed between these proteins, but how are these fusions and EGFR interacting at the molecular level, and could this interaction represent a potential therapeutic opportunity? Importantly, we showed that the rescue of signaling and interactions between the fusion oncogenes and EGFR did not occur with other RTKS like MET or FGFR, but what is it that is unique about EGFR that distinguishes it from other RTKs? We described a novel early adaptive signaling mechanism we referred to as “adaptor switching” with the ROS1 and RET fusion cells. If GRB2 or SHC1 can really “switch” from the fusion kinase to EGFR with FKI treatment, as we have described, then we should be able to block this adaptive phenotype using point mutations. Why is unmutated EGFR being used by these cancer cells in the presence of a potent oncogene, but its role is not causing oncogene overdose (325,326)? The theory of genetic streamlining, an underlying principle of oncogene addiction, would suggest that the cancer, driven by the natural selection of
advantageous, usually pro-tumorigenic events, will ultimately shed any unnecessary pathways because there is no evolutionary advantage to retaining them and they may present an energetic cost to the cancer cell (327,328). What advantage is EGFR providing these fusion kinase-driven cancers? We know that EGFR is making a contribution to the downstream signaling in these fusion kinase positive cells (Fig. 28, Fig. 29), and that it is both signaling through the fusion oncogene itself (to amplify the downstream signaling), but our PLA data also suggests it is signaling independently through GRB2, and potentially diversifying the downstream signaling (Fig. 31, Fig. 32). Could the survival advantage EGFR be providing to these cells be related to downstream signaling changes? Preliminary answers to each of these questions will be addressed in this chapter.

**Potential interactions between fusion kinases and EGFR**

The phosphorylation of ALK, ROS1, RET or TRKA fusion kinases by EGFR and the presence of complexes between the two proteins led to the hypotheses of three potential interaction mechanisms (Fig. 45): (a) a kinase-substrate model, (b) an intermediary protein model, and (c) a heterodimerization model. (a) The simplest explanation for the data presented in chapter 3 is that EGFR can phosphorylate the 4 fusion oncogenes, and the fusion oncogene is serving as a substrate. Preliminary evidence has demonstrated that EGFR’s signaling cascade can phosphorylate a fusion kinase under unperturbed conditions or in the presence of an inhibitor, and that it can also phosphorylate a catalytically inactive version of the fusion expressed in a different cell microenvironment (Fig. 31, 37, 38). However, the co-immunoprecipitations and PLA complexes observed in chapter 3 or by other groups were detected even when a potent kinase inhibitor against either protein was utilized, providing evidence against this proposed model (Fig. 37, Fig. 38) (239).

(b) This intermediary protein model is driven by the fact that we have not shown data demonstrating a direct interaction between EGFR and the fusion oncogenes. Moreover, it has not been ruled out that another protein could be mediating the phosphorylation and also
present in the protein complexes we have detected. Previous work has shown interactions between the ETV6-NTRK3 fusion and IGFR1 are mediated by IRS-1, a common signaling adaptor (101). Building off this idea of an adaptor bridging the interactions, candidate signaling proteins that could be mediating interaction between EGFR and a fusion kinase in our model include SRC, GRB2, or SHP2, all of which can bind to EGFR (329-332). One could even speculate that different signaling adaptors could be mediating interactions between EGFR and each of the 4 fusion kinases, and those may change in different tumor microenvironments based on expression or other factors (106,333,334). The proximity ligation assays used extensively in this thesis are a powerful tool, but their “range” for detecting protein-protein interactions in the version utilized is approximately 20-40 nm (the exact distance is proprietary). Based on the average size of a protein and the antibodies we are using to detect them it is feasible that another protein is present in these complexes (304). Indeed, multiple phosphorylation events investigated in chapter 3 were rapid (as fast as 5 minutes), but the kinetics of most signaling cascades, particularly with upstream kinases of interest such as RTKs, can occur more rapidly than 5 minutes (335). The kinetics observed in our studies are consistent with growth factor mediated kinase signaling, and no obvious differences are expected with a fusion kinase (336). An intermediary protein would need to be expressed relatively ubiquitously; not just in the human tumor derived cancer cell lines used in these studies, but also in the 293T cells used for the ectopic expression studies. Expression of all 3 candidate intermediate proteins: SRC, GRB2, and SHP2 has been validated in the literature in various previous studies (337-339).

(c) The last model proposed is a heterodimerization model between EGFR and fusion kinases. Tyrosine kinases are known to require homo- or heterodimerization in order to become enzymatically activated (340). Interestingly, the intracellular portion of the ERBB family of RTKs has been characterized to dimerize uniquely from other RTK families and this could be relevant to the studies presented in this thesis. This evidence provides particular
appeal to this model, because we have shown a fusion kinase preference towards a role for EGFR over other RTKS like MET and FGFR. EGFR’s kinase domain homodimerizes or heterodimerizes asymmetrically, where the low basal level of activity from the c-lobe of an “activator” kinase allosterically activates the n-lobe of a “receiver” (229). That contact induces a conformational change in the receiver kinase that relieves cis-autoinhibitory interactions from its own activation loop (229). This is critical, because as long as those cis-autoinhibitory interactions are broken, EGFR can take on a fully activated configuration, without phosphorylation of its activation loop or ligand binding (341,342). This is the structural basis for activating mutations for EGFR identified in NSCLC (262,343). An additional unique aspect of EGFR’s dimerization biology worth highlighting is the dual necessary and sufficient roles of the juxtamembrane domain for the regulation and successful execution of asymmetric dimerization (341,344). Collectively, the knowledge of these studies led to the hypothesis that interactions between the fusion kinases and EGFR could be sufficient to activate EGFR beyond low basal levels.

**To dimerize or not to dimerize: that is the question**

Limited structural information has been collected on full-length oncogenic fusion kinase proteins, but many relevant 5’ fusion partner domains and kinase domains have crystal structures available (345-351). The final chimeric protein product will likely vary substantially based on the exact genomic breakpoints and the protein domains that were retained. One conserved biological aspect amongst the variety of oncogenic fusion kinases studied here, and relevant for this comparison, is that they all contain a constitutively activated tyrosine kinase domain. Most activated kinase domains harbor high levels of architectural homology (228). This plasticity of kinase structure, especially within RTK sub-families, is one rationale for why certain types of TKIs can be so promiscuous. Type 1 ATP competitive inhibitors target an “active” state of the kinase, and obtain less specificity than with type 2 inhibitors that bind an inactive state (352). Many structural analyses on
oncogenic kinases are pharmacologically driven towards kinase inhibition, i.e. TKIs bound to pure kinase domains or amino acid contacts relevant to drug-binding or resistance driving mutations (345-348). Current literature searches have revealed no studies solving the full structure (or at least a partial one crossing the breakpoint) of an oncogenic fusion kinase protein.

Interestingly, fusion kinase transformation studies that frequently assess the importance of domains speculated to mediate dimerization, are generally conducted using artificial models such as 293T, NIH-3T3, or Ba/F3 cells (37,54,69). Using these artificial model systems, a targeted deletion approach is often conducted with specific deletion constructs to identify key regions of the gene that are critical for oncogenic transformation, and are suspected to mediate dimerization (22,54,353,354). Accordingly, in the context of those immortalized cell microenvironments, it has been shown that predicted dimerization domains are important, i.e., the coiled-coil basic domain in EML4 is necessary for ALK fusion cell transformation when expressed in NIH-3T3 cells (22). Indeed, it has not been shown, in a true lung cancer cell line derived from human tumors that these predicted dimerization domains are necessary. The ability of the fusion kinase to homodimerize in artificial systems does not indicate that they will if there is a partner with a higher binding affinity available. This is of interest, because unselected lung cancers, as well as those that are ALK+ and ROS1+ express high levels of EGFR (Fig. 42a) (261). Furthermore, not all oncogenic fusions have a known or even predicted dimerization domain in the 5’ fusion partner; ROS1 is a frequent example already highlighted in this thesis (37,102,165,211). CD74, SDC4, SLC34A2, and LRIG3 are examples of ROS1 5’ fusion partners from these studies that do not harbor a known dimerization domain. Accordingly, previous work suggests the loss of certain 5’ regulatory domains may be sufficient to activate certain kinases (1). It has also been reported that not all mutated kinases require dimerization for their constitutive kinase activity, including EGFR deletion 19 and exon 20 insertion
mutations in lung cancer (355). We hypothesized that EGFR and the fusion kinases could be interacting by forming heterodimers, and that a constitutively active fusion kinase could act as an “activator” in the asymmetrical dimer. We sought to explore this model initially through structural modeling of the EGFR and ALK kinase domains.

**Methods**

**Patients**

Written informed consent was obtained from the patient prior to use of the patient’s tumor sample. The consent form and protocol was reviewed and approved by the Colorado Multiple Institutional Review Board. Excess tumor tissue from patients was collected for derivation of tumor xenografts (COMIRB #10-1386) or for additional research studies (COMIRB #11-1621).

**PyMOL modeling**

Help with PYMOL modeling was provided by Dr. Jeffrey Kieft from the Department of Biochemistry and Molecular Genetics at the University of Colorado Denver.

**Cell lines and reagents**

NIH-3T3 (mouse fibroblasts), PCDH-3T3 (3T3 fibroblasts stably expressing an empty vector), ALK-3T3 (3T3 fibroblasts stably expressing EML4-ALK), ROS1-3T3 (3T3 fibroblasts stably expressing SDC4-ROS1), H3122 lung adenocarcinoma cells, STE-1 lung adenocarcinoma cells, and KM12 colorectal cancer cell lines were previously described (21,195,211,215,297,298). All cancer cell lines were cultured in RPMI1640 supplemented with 1-10% FBS. HEK-293T cells were maintained in DMEM supplemented with 5% FBS. Gefitinib, crizotinib, and TAE-684 were purchased from Selleck chemicals. All EGF was purchased from R&D Systems. Expression vectors WT EGFR-pBABE-HA, L704N EGFR-pBABE-HA, I941R EGFR-pBABE-HA, and the catalytically inactive D837A EGFR-pBABE-HA were kindly provided by Dr. Jeonghee Cho, at Sungkyunkwan University. EGFR-GRB2 binding mutations were created using Agilent’s QuikChange II XL site-directed mutagenesis
kit according to the manufacturer’s protocol. Y1068F, Y1086F, Y1148F, Y1173F, and Y1068F + Y1086F were created against WT EGFR-pBABE-HA as a template. The following primers were used for each mutation: Y1068F FOR: 5'-cctcccagtgcctgaattcataaaccagtccgttc-3'. REV:5'-gaacggactgttatgaattcaggctgaggagggg-3'. Y1086F FOR: 5'-ctctgtgcagaatctctttcacaactcagcctc-3'. REV: 5'-gaggctgatttgtaaaagacaggctttgtgacag-3'. Y1148F FOR: 5'-agcctggacaaccctgacctccagccagag-3'. REV: 5'-gtctgtgctcagctgacggtttcctccagctc-3'. Y1173F FOR: 5'-ctccacagctgaaatgcaatcacttggagttgc-3'. REV: 5'-gcgaccccagagttgtagctttctgcagagttgag-3'. The double mutant was created using Y1068F FOR with Y1086F REV primers.

Transfections were performed using Mirus TransIT X2 reagent according to the manufacturer’s protocol in the cell lines as described into a 6-well plate. Transfected cells used in PLAs were transfected for 24 hours first in a 6-well plate, trypsinized and re-seeded into a chamber slide.

Immunoblotting and antibodies

Immunoblot analysis was performed as previously described in detail in chapter 2 of this thesis. The following antibodies were used from Cell Signaling: pEGFR Y1068 (D7A5) and Y1173 (53A5), total EGFR XP D38B1, HA (C29F4 or 6E2), pALK Y1278/1282/1283, total ALK (D5F3 or 31F12), total ROS1 (D4D6), pMEK pS217 (4169), MEK (L38C12), pS338 CRAF (56A6), and GRB2 (3972). GAPDH (MAB374) and pTYR (4G10) were purchased from Millipore. Total EGFR was also purchased (610017; BD Biosciences).

Proximity ligation assays (PLAs)

Cells were seeded onto chamber slides at 25-50k cells/well. Cells were treated with the indicated doses and times then fixed for 15 minutes by shaking at room temperature in 4% paraformaldehyde. Cells were rinsed twice in PBS, and then the Duolink® In situ PLA kit from SigmaAldrich in mouse/rabbit (with red detection) was used according to the manufacturer’s protocol (catalog # DUO92002, 92004, 92008). Antibody concentrations
were optimized prior to PLA experiments. FFPE tissue PLAs from mice or patients were prepared as described in histology. Additionally, FFPE samples were treated with 300 mM Glycine for 30 minutes prior to the blocking step, then the assay was performed according to the manufacturer’s protocol. Cells were mounted using Duolink® In Situ Mounting Medium (with DAPI) and cured, and sealed with clear nail polish prior to imaging. Images were taken on a Nikon standard inverted fluorescent microscope at 40x. The following antibodies were used: Cell Signaling ALK (D5F3), ROS1 (D4D6), EGFR (D38B1). GRB2 was purchased (610111; BD Biosciences), and EGFR (SC-120), CRAF (SC-227 for immunoblot and C-12 for PLA), and BRAF (F-7) were purchased from Santa Cruz Biotechnology. RAS (1-; MA1-012X) was purchased from Pierce, and RAS (ab108602) was purchased from Abcam. A stage micrometer from reticles.com (KR-85-11) was imaged identically to the PLAs and the scale bar inserted accordingly. Each scale bar shown represents approximately 50 microns, unless it is explicitly stated otherwise.

**CellProfiler quantification of PLAs**

CellProfiler software was used to quantify these assays (299). The pipeline used in these studies to analyze images was: ColorToGray (to split fluorescent channels), EnhanceorSuppressFeatures (to enhance the red speckles), IdentifyPrimaryObjects (to specify the number of nuclei in the blue channel), and MeasureImageIntensity (of speckles in the red channel) for each image. Red fluorescence intensity was normalized to the number of nuclei in each image field. Each quantification graph is expressed as the mean ± the SEM. All statistical analysis was performed using a paired student’s t test.

**Flow cytometry**

Apoptosis was measured in KM12 cells using the Vybrant apoptosis YO-PRO/PI kit (Invitrogen). Briefly, KM12 cells were seeded 24 hours prior to treatment prior to trypsinization and staining.
Results

Upstream signaling mechanisms of cooperative signaling: modeling EGFR and ALK heterodimers

For a preliminary evaluation of fusion kinase-EGFR heterodimers, we modeled known fusion kinase domain structures into the EGFR dimer interface using PyMOL molecular visualization software (Fig. 46). This approach does not take into account the effect of the protein structure contributed by the 5' partner on the full-fusion protein structure, but it enabled us to evaluate the geometric feasibility of our hypothesis in the dimer interface. Additionally, most fusion partners do not have crystal structures available for such modeling, so we chose to focus on the kinase domains for this initial step. A similar approach that utilizes modeling of known structures for pieces of fusions has been conducted with known structures of RAD51 and the EGFR kinase domain (26). Our modeling demonstrated that the ALK kinase domain overlaid with HER3 in the EGFR-HER3 heterodimer interface (356).

Previous work has shown that mutations at the EGFR asymmetrical dimer interface at L704N impair its “receiver” activity, and I941R impair its “activator” activity (355). Based on this previous work, I hypothesized that a constitutively active fusion kinase could serve as the activator of the asymmetrical dimer (Fig. 47a). If this hypothesis were correct, only the L704N EGFR “receiver” mutant should disrupt the interaction between EGFR and ALK. Constructs expressing each of these EGFR mutants were expressed transiently, and assayed by PLA for interactions between ALK and EGFR (Fig. 47c). NIH-3T3 cells were chosen because they express very low to undetectable endogenous levels of HER family members, and these cell lines stably expressing EML4-ALK were previously established, named ALK-3T3 (195,357). Robust PLA signal was detected between wild-type EGFR and ALK as well as with the activator-impaired mutant, I941R. The receiver-impaired mutant led to a reduced quantitative signal between EGFR and ALK (Fig. 47c, d). Importantly, a
catalytically inactive mutant of EGFR, D837A, did not disrupt interactions between EGFR and ALK, a result that is consistent with our previous studies using kinase inhibitors in chapter 3 (Fig. 47c, d). This preliminary experiment showed that of the 3 EGFR mutants expressed in ALK-3T3 cells, only the L704N mutant disrupted interactions between EGFR and ALK.

**EGFR-GRB2 binding mutations do not block interactions between ROS1 and EGFR**

An observation in chapter 3 was previously described in this thesis as “adaptor switching (Fig. 35, 36). In order to improve our understanding of the molecular mechanisms associated with this phenotype we initially set out to block the observed switch of GRB2 binding from ROS1 to EGFR with FKI treatment. GRB2 has two direct binding tyrosine sites on EGFR (Y1068 and Y1086), and 2 indirect tyrosine sites (Y1148 and Y1173) where it can bind onto the SHC1 adaptor when it is already docked (358). Individual point mutations converting each of these critical tyrosines to phenylalanines, and one 1068+1086 double mutant were designed to prevent GRB2 from switching or binding onto EGFR (Fig. 48a, b). The ROS1+ HCC78 cells and RET+ LC-2/ad cells were originally used to characterize adaptor switching, but both express high endogenous levels of EGFR. A fusion kinase+ model with essentially no endogenous expression of EGFR to test these EGFR mutants was needed. NIH-3T3 cells stably expressing SDC4-ROS1, named ROS1-3T3s were selected for these experiments (102). Interestingly, transient overexpression of WT EGFR in these cells resulted in an increase of ROS1 fusion phosphorylation, indicating that the overexpression of EGFR in these cells, without ectopic EGF stimulation, can induce similar phosphorylation events observed in the ROS1+ lung cancer cells in chapter 3 (Fig. 30, 48c). pEGFR was detected clearly in these cells by western blot, and lead to an increase in pROS1 (Fig. 48c). However, the transient overexpression of WT EGFR in these cells was unable to induce a positive EGFR-GRB2 PLA signal in the ROS1-3T3 under control or ROS1 FKI treated conditions (Fig. 48d). It was not possible to test the mutants’ ability to
prevent GRB2 adaptor switching in this model since a positive PLA signal for adaptor switching to WT EGFR was not detectable. ROS1-GRB2 PLA was positive in the cells, indicating the assay was working (Fig. 48d). Consistent with this data, GRB2 was co-immunoprecipitated with ROS1, but not EGFR in these cells (Fig. 48e). The ROS1-GRB2 pulldown was disrupted with FKI treatment, suggesting the importance of GRB2 binding to ROS1. Therefore, we were unable to test the ability of these GRB2 binding mutants to disrupt the switching of GRB2 from ROS1 to EGFR with FKI treatment in this model.

Previous work has indicated a signaling adaptor, IRS-1, to facilitate interactions between a different fusion kinase, TEL-TRKC, and the RTK IGFR1 (101). Based on these studies, it was hypothesized that GRB2, a critical signaling mediator in lung cancer cell lines, could facilitate interactions between the fusion kinases and EGFR in these models. To test this, the EGFR binding double mutant for GRB2 (Y1068F + 1086F) was co-expressed alongside WT EGFR in ROS1-3T3 and assayed by co-immunoprecipitation. The GRB2 binding mutant did not appear to impair or reduce the amount of HA or EGFR coming down with ROS1 in these cells qualitatively (Fig. 48f). This result indicates that GRB2 binding to EGFR is not important for interactions between ROS1 fusions and EGFR.

**Downstream signaling mechanisms of cooperative signaling: RAS-CRAF and RAS-BRAF PLAs**

Previous work in chapter 3 has shown a clear signaling contribution from EGFR in fusion kinase positive lung cancer cells. We demonstrated that the FKI alone decreased downstream signaling at critical points in both the MAPK (pERK) and AKT (pAKT) signaling pathways (Fig. 28, Fig. 29). We saw an additional contribution from EGFR, measured by the increased reduction of pMEK with gefitinib (Fig. 49a), suggesting an important role for both the fusion and EGFR’s signaling cascades through the MAPK pathway. However, an unanswered question is whether EGFR was just amplifying signaling through the fusion kinase, or further diversifying the signaling independently. PLA data from chapter 3 would
suggest that EGFR is signaling independently through GRB2 and SHC1, in addition to amplifying signaling through the fusion kinase (Fig. 30, Fig. 31). Both GRB2 and SHC1 are important adaptors for MAPK signaling. Important nodes in MAPK signaling upstream of MEK include RAS and RAF, but both nodes harbor different isoforms. We questioned whether EGFR was signaling through the same or different MAPK effectors to signal in these cells. To test which effectors may be important in our models, signaling PLAs were designed to assess interactions between RAS and BRAF as well as RAS and CRAF.

Interactions between different RAS isoforms and RAF isoforms are crucial for the recruitment of RAF to the plasma membrane, its subsequent dimerization and activation, and the effective activation of the MAPK signaling pathway (359-361). Notably, BRAF has higher basal kinase activity than CRAF, likely because CRAF lacks a constant negative charge in the N-terminal region of the kinase domain (362). This negatively charged region is essential for the allosteric dimerization observed amongst RAF family members, similar to ERBB family members (229,363). This PLA detects interactions between “pan” RAS family members (H, K, and NRAS), but specifically with either BRAF or CRAF isoforms. This approach allows for the dissection of differential contributions of BRAF or CRAF using FKI or EGFR specific inhibitors. The BRAF and CRAF antibodies were previously tested for specificity in assaying BRAF-CRAF heterodimers by PLA (364,365). These 2 RAS-RAF and BRAF-CRAF heterodimer PLAs were employed in ALK+ and ROS1+ cell lines to determine differential contributions from BRAF and CRAF. Robust PLA signal was detected for all 3 PLAs under control treated conditions in these cells (Fig. 49b, c). Inhibition of the fusion only, using an FKI, strongly decreased signaling interactions from RAS to CRAF (Fig. 49b, c). Targeted inhibition of EGFR with gefitinib resulted specifically in reduced RAS and BRAF signaling PLA complexes (Fig. 49b, c). BRAF-CRAF heterodimers were reduced with inhibition of the fusion kinase alone in the ALK+ cells, but further reduced with the addition of the EGFR inhibitor gefitinib (Fig.49b). These results are consistent with the downstream

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signaling inhibition effects observed in chapter 3 (Fig. 28, 29), and shown in (Fig. 49a). Collectively, these results suggest that EGFR might be controlling BRAF recruitment to RAS isoforms, and the fusion kinase may be regulating the CRAF recruitment by RAS.

RAS-RAF PLAs were helpful in our cell line models to understand the regulation of MAPK signaling through EGFR and the fusion kinase cascades. We wanted to know if these PLAs could be useful in measuring MAPK signaling intensities in other contexts. The RAS-CRAF PLA was tested in ALK+ H3122 cells made chronically resistant to the FKI crizotinib by a collaborating lab (182). A RAS-CRAF PLA was used here, because the quantification of the RAS-BRAF PLA showed no reduction in response to FKI treatment in ALK+ cells, but RAS-CRAF was sensitive to ALK FKI treatment (Fig. 49b). The resistant cells, named crizotinib acquired resistant (CARs), cells developed a KRAS amplification that empowered them to survive the upstream signaling blockade of the ALK fusion. RAS-CRAF signaling was abrogated in the parental H3122 cells, but the signal remained strong in the presence of crizotinib in the CAR resistant cells (Fig. 50a). RAS gene amplification can be identified by next-generation sequencing or through FISH, but the RAS-CRAF PLA enabled detection of the functional output of that RAS amplification, and the resultant signaling complexes with CRAF. In order to understand if this assay could help us evaluate RAS signaling in situ, patient samples from a cohort at the University of California San Francisco and the University of Colorado were also measured by RAS-CRAF PLA (Fig. 50b). This analysis was able to qualitatively detect different amounts of RAS signaling through CRAF in situ within the cohort of ALK+ resistant patients. A proposed gradient for the differences is shown (Fig. 50c). Single cell heterogeneity of RAS signaling was evident using the PLA approach, with clear areas of enhanced RAS signaling (dotted circle, Fig. 50b). These results suggest the potential of this assay for detecting different intensities of RAS signaling in fusion kinase positive patient cells.
**EGFR and a TRKA fusion kinase control different cell fates**

Subtle changes in the kinetics of MAPK pathway signaling can result in different cell fates within the same model system (197-199). The magnitude of MAPK pathway activation has also been shown to regulate different hallmarks of oncogene-induced transformation such as proliferation, or alternatively cell-cycle arrest and senescence (reflecting incomplete neoplastic transformation) (366). ERK signaling has been shown to regulate diverse downstream transcription factor targets, allowing it to modulate an extensive variety of cellular functions, such as proliferation, survival, differentiation, and migration (367-371). Collectively, these previous studies substantiate the importance of the spatial and temporal control of the MAPK pathway in cancer. We hypothesized that the differential engagement of BRAF and CRAF by the fusion kinases and EGFR may result in different cell fates. These different cell fates may contribute to the overall oncogenic program by promoting different hallmarks of cancer, and ultimately help support a more complete disease program (372,373). In order to evaluate the functional consequences of the differential signaling programs observed in (Fig. 49) proliferation and apoptosis assays were conducted using the KM12 cell line (TRKA+). These cells were treated with a specific TRKA FKI, ARRY-470, or treated with the EGFR inhibitor gefitinib to dissect out the EGFR signaling contribution. Inhibition of TRKA led to a significant reduction in cellular proliferation, whereas EGFR inhibition had no effect on proliferation even at the highest doses (Fig. 51a). In contrast to this, inhibition of the fusion resulted in only a modest increase of apoptosis, but there was a larger single agent effect with the EGFR inhibitor (Fig. 51b). These data suggest that the fusion kinase, TRKA, may principally signal to regulate cellular proliferation and growth, and EGFR may control survival through anti-apoptotic signaling in these cells (Fig. 51c).

**Discussion**

Upstream and downstream mechanisms enabling the cooperative signaling observed between ALK, ROS1, RET, and TRKA fusion kinases and EGFR were
investigated in this chapter. The potential of EGFR and fusion kinases to form heterodimers was tested by using mutations that disrupt the molecular contacts at the dimer interface. This initial PLA analyses suggested ALK and EGFR interactions were disrupted by only one of the EGFR dimerization mutants, the receiver impaired mutant (Fig. 47c, d). This is consistent with the heterodimer hypothesis, of a constitutively active fusion kinase serving as an “activator” at the asymmetrical interface. However, since limited cells were imaged and quantified for this experiment, PLAs need to be repeated with more nuclei included for each condition, and followed-up with co-immunoprecipitations of the two different EGFR dimerization mutants and ALK or other fusion kinases. Another potential explanation for this result is that the receiver impaired mutant disrupts the epitope for the EGFR antibody that is exposed during PLA fixation. Immunofluorescence analysis should be performed to follow-up and confirm the EGFR antibody used in the PLA can detect this mutant form of EGFR following these fixation conditions. An alternative approach would be to design a PLA using the anti-HA antibody and ALK, as each of these EGFR constructs contains a c-terminal HA tag.

It was surprising that despite high levels of pEGFR, EGFR-GRB2 signaling complexes were not detectable (Fig. 48c). The levels of pEGFR observed were likely functionally relevant, because they resulted in an increase of pROS1, similar to those seen in chapter 3 when EGFR was ligand activated. This confounding result suggested there may be a “tissue tropism” effect preventing EGFR to signal through GRB2 in this particular cell context. These data reinforce the importance of measuring adaptor binding in functionally relevant signaling cascades demonstrated in chapter 3 (Fig. 35), not just detecting kinase phosphorylation. While phosphorylation may be necessary for signaling complexes, it is not sufficient to determine whether a signaling cascade was initiated. These data drive the importance of our use of signaling PLAs, and not just kinase phosphorylation as a measure of kinase activity. The importance of adaptor binding to a kinase is supported by biochemical
data that GRB2 was immunoprecipitated with ROS1 in this model cell line, but not EGFR (Fig. 48d). EGFR may be prone to signal through a different adaptor molecule in these cells, such as SHC1 or the P85 subunit of PI3K. Different EGFR-adaptor PLAs may need to be utilized to determine their relevance in different contexts. The transient overexpression of the WT EGFR expression plasmid may perplex the adaptor switching observation if signal levels detected with transient overexpression become too high, prior to FKI treatment. A different model cell line would be required to test the ability of these mutations to block GRB2 adaptor switching. Ideally, a lung cancer cell line like the HCC78 (ROS1+) or LC-2/ad (RET+) with endogenous EGFR removed via a genome editing mechanism like CRISPR would be used to test this (354).

GRB2 may not be a mediator of ROS1 and EGFR molecular interactions in ROS1-3T3 cells, based on mutating GRB2 binding sites on EGFR. The cis-mutations of both GRB2 binding sites on EGFR from tyrosines to phenylalanines did not prevent EGFR and ROS1 from co-immunoprecipitating with each other in ROS1-3T3 cells. These data showed ROS1 was still able to interact with GRB2 in the presence of WT EGFR (Fig. 48e). The GRB2 mutant EGFR and ROS1 were still able to form a complex independent of GRB2 binding to EGFR (Fig. 48e). This experiment suggests that GRB2 is not serving a mediator of these interactions. Notably, GRB2 also did not immunoprecipitate with ROS1 when the mutant EGFR construct was expressed, suggesting expression of mutant EGFR may disrupt ROS1-GRB2 interactions. These findings indicate GRB2 binding to EGFR may be important for ROS1’s own interaction with GRB2, but not with EGFR. SRC and SHP2 are two proteins that were not evaluated in these studies that may potentially be facilitating the complex formed between ROS1 and EGFR.

These studies reveal potential differences in the use of downstream RAF effector isoforms by EGFR and the fusion kinases. Embryonic studies of BRAF and CRAF in mice have shown both isoform specific and redundant functions of the two isoforms, such as
CRAF’s specific role in embryonic progression through the 2-cell stage and the induction of the posterior mesoderm, and redundant functions for both in promoting development until mid-gestation (374,375). The loss of either isozyme is homozygous lethal at different stages in embryogenesis and mutants are phenotypically different, and suggesting a specific requirement for both (376,377). CRAF can homodimerize, even if it does have weaker basal kinase activity (362,365,378). The higher basal kinase activity of BRAF may additionally highlight the importance of the signaling contribution of EGFR in these cells (362). ARAF was not included in these initial PLA analyses, but previous work has shown that ARAF can behave as a stabilizing scaffold for BRAF-CRAF heterodimers, and may prove to play an important role here when investigated (379). RAS-ARAF, or additional RAF heterodimer PLAs may be tested here to further improve the understanding of each isoforms’ contribution.

The distinct roles of RAF isoforms shown in this chapter may drive unique cellular fates important to the cancer’s survival (Fig. 51d). Studies have shown that RTKs can be grouped into different classes based on their signaling abilities and diversity (380). Certain kinases such as EGFR, have the capacity to signal through more adaptors than other receptor tyrosine kinases and that may enable them to diversify the downstream signaling in certain cell contexts (380,381). A systematic RNAi perturbation approach was used to identify which kinases could control more downstream signaling pathways. In this analysis, the most potent kinases (EGFR and MET) could signal through the shared, common pathways, but also additional, diverse pathways. A weaker one, like TRKB, was more limited to a common, minimal backbone of signaling nodes (380). This is of interest, because the example of a weaker kinase signaling network, TRKB, is a member of a family of kinases commonly found in oncogenic fusions. A contributing factor to this type of analysis may be the number of autophosphorylation and adaptor binding tyrosines on each kinase, which may contribute to an increase in signaling network potential. EGFR has 12
autophosphorylation sites, and has been known to engage many different adaptor or scaffolding proteins illustrated in (Fig. 5). A cooperative signaling network that pairs a kinase with a limited signaling network, such as a TRK family member, with a larger one such as EGFR, may result in a survival advantage and an explanation for why the cancer maintained high levels of a WT protein, EGFR. Kinases from the different classes reported in this analysis are often co-expressed in human tumors, supporting this hypothesis (380). Even a catalytically inactive form of EGFR has been shown to activate many other kinases through protein-protein interactions, acting to further diversify signaling in certain cellular contexts (237). Our results in the KM12 cell line showed that EGFR could do this by driving anti-apoptotic signaling in these cells (Fig. 51). Collectively, our data suggests that one of the functional roles of EGFR in the context of fusion kinase driven cancers is to diversify the downstream signaling, and drive survival signaling independent of the fusion kinase oncogene. Substantial additional work will be required to follow up and build on the early observations made in this chapter.
Figure 45: Modeling potential molecular interactions between EGFR and fusion kinases. Cartoon schematics of (a) Kinase-substrate model: fusion kinases may serve as a direct phosphorylation substrate of the EGFR signaling axis. (b) Intermediary protein model: an intermediate protein, such as a signaling adaptor, may be responsible for “sandwiching” interactions between EGFR and fusion kinases. (c) Heterodimerization model: a constitutively active fusion kinase may serve as an ‘activator’ in the EGFR asymmetrical dimer interface.
Figure 46: Structural modeling of EGFR dimer interface with different kinase domains with PyMOL. Structure of the heterodimer between the EGFR (cyan) and HER3 (green) kinase domains (PDB: 4RIW). Model of the predicted EGFR intermolecular contacts with the kinase domains of c-MET (red) (PDB: 1R1W) and ALK (dark blue) (PDB: 3L9P) overlayed with HER3. These models were built using the homology between the kinase domains of HER3, c-MET, and ALK. All kinase domains should be able to form nearly all of the same interactions with EGFR. PDB represents the protein data base, and the 4-letter code for each protein's structural coordinates is indicated. Modeling help provided courtesy of Dr. Jeffrey Kieft.
Figure 47: EGFR receiver impaired dimerization mutant may disrupt EGFR and ALK interactions by PLA. (a) Cartoon schematic of dimerization hypothesis, modified from Cho et. al. 2013. (b) Immunoblot analysis of transient expression of EV, WT EGFR, L704N EGFR (receiver impaired), I941R EGFR (activator impaired), and D837A (catalytically inactive). Blots were probed with the indicated antibodies. (c) Representative images of EGFR-ALK PLA on each EGFR construct expressed transiently in 3T3 cells stably expressing EML4-ALK. Scale bars shown represent approximately 50 microns. (d) Quantification of experiments shown in (c). Data is expressed as the mean ± the SEM from one experiment.
Figure 48
Figure 48: EGFR-GRB2 signaling does not turn on in the ROS1-3T3, and binding mutations do not disrupt EGFR and ROS1 interactions. (a) Cartoon schematic of direct and indirect GRB2 binding mutations on EGFR. Direct binding sites are shown in gray; indirect sites are shown in yellow. (b) Immunoblot analysis of 4 GRB2 binding mutants demonstrating corresponding phospho-tyrosine disruption from mutation with phospho-specific antibodies. Direct binding mutants are highlighted in gray; indirect mutants are highlighted in yellow. (c) Immunoblot analysis of EV, or WT EGFR expressed transiently in 3T3 cells stably expressing SDC4-ROS1. Cells expressing WT EGFR were treated with either DMSO vehicle control, or 250 nM TAE-684 as the ROS1 FKI for 3 hours. Blots were probed with the indicated antibodies. (d) Representative images of ROS1-GRB2 or EGFR-GRB2 PLA with EV or WT EGFR transiently expressed in 3T3 cells stably expressing SDC4-ROS1. Cells were treated with either DMSO vehicle control 250 nM TAE-684 for 3 hours prior to fixation. Scale bars shown represent approximately 50 microns. (e) Immunoblot analysis of SDC4-ROS1 stably expressing 3T3s expressing EV, WT EGFR, or the GRB2 double binding mutant form of EGFR. Cells were treated with either the DMSO vehicle control or the FKI 250 nM TAE-684 and immunoprecipitated with either ROS1 or EGFR. Blots were probed with the indicated antibodies. (f) Immunoblot analysis of SDC4-ROS1 stably expressing 3T3s expressing EV, WT EGFR (HA tagged), or the GRB2 double binding mutant form of EGFR. Cells were treated with either the DMSO vehicle control or the FKI 250 nM TAE-684 and immunoprecipitated with ROS1. Blots were probed with the indicated antibodies.
Figure 49: FKI and EGFR inhibition may control different downstream RAF effectors (a) Immunoblot analysis of H3122 cells treated with either the DMSO vehicle control, 250 nM crizotinib (FKI) or FKI with 1 μM gefitinib for 3 hours. Blots were probed with the indicated antibodies. (b) STE-1 and (c) CUTO-2 cells were treated with either DMSO control, FKI= 250 nM TAE-684, 1 μM gefitinib, or both for 3 hours and fixed for PLA analysis. RAS-BRAF (purple), RAS CRAF (green), or BRAF-CRAF (yellow) PLAs were performed as indicated. Quantification of at least 2 experiments is shown. Data is expressed as the mean ± the SEM from two experiments.
Figure 50: RAS-CRAF PLAs are a valuable signaling tool in vitro and in situ. (a) H3122 (green) and CAR H3122 (gray) cells were treated with either DMSO vehicle control, or FKi= 250 nM crizotinib, and fixed for RAS CRAF PLA analysis. Quantification of at least 2 experiments is shown. Data is expressed as the mean ± the SEM from two experiments. (b) Representative images are shown from a cohort of ALK+ patients resistant to crizotinib blindly analyzed by RAS-CRAF PLA. Representative images are shown. Scale bars shown represent approximately 50 microns. (c) Graph with scale representing a potential gradient of RAS-CRAF PLA signal under different genotypic circumstances.
Figure 51
Figure 51: EGFR and fusion kinases control different cell fates, possibly through different RAF effector isoforms. (a) Dose response of proliferation assays using KM12 cells treated with a TRKA specific FKI or gefitinib. Data is expressed as the mean ± the SEM from 3 independent experiments performed in triplicate. (b) Apoptosis flow cytometry assays were performed using KM12 cells following treatment with a TRKA specific FKI or gefitinib for 24 hours. Staurosporine was used as a positive control for the induction of apoptosis. Data are expressed as the mean ± the SEM. (c) Cartoon schematic summarizing preliminary major (big arrow) and minor (skinny arrow) cell fate contributions by the fusion kinase and EGFR. (d) Cartoon schematic summarizing the complete model where EGFR may signal primarily through BRAF, and the fusion kinase through CRAF, resulting in control of proliferation or survival, respectively.
CHAPTER V
DISCUSSION

Concept summary

The studies presented in this thesis fall into 2 sections: the identification and characterization of NTRK1 fusion oncogenes in lung cancer and the role for wild-type EGFR as a cooperative signaling partner for ALK, ROS1, RET, and NTRK1 fusion kinase oncogenes in lung cancer. The work presented in chapter 2 has reiterated the importance of identifying dominant oncogenes, like NTRK1 fusions, and targeting them in patients. These patients' responses to targeted therapies are impressive, but incomplete, so it is also important to work towards the identification of rational combination therapies to continue to improve treatment outcomes. Subsequent work in chapter 3 has built on the importance of oncogenes, and demonstrated the need to improve our understanding of how these oncogenes work, and understanding the mechanisms a cell deploys when treated with an oncogene targeted therapy. Two novel roles for wild-type EGFR signaling were identified in this work in the context of oncogene fusion positive lung cancer: transactivation of the 4-different drug-inhibited fusion kinases by EGFR with EGF stimulation and the observation of “adapter switching” between ROS1 or RET fusion kinases and EGFR following drug inhibition. Here, we described a cooperative signaling network between EGFR and the fusion kinases where EGFR can use transactivation of adapter switching to enable cell survival in the presence of a targeted therapy. We were able to build on these initial roles described for EGFR, to identify the potential upstream and downstream signaling mechanisms enabling survival following treatment with a FKI.

We provided a mechanistic explanation for this transactivation, which is dependent on the ectopic addition of the ligand EGF. Moreover, the activation of EGFR by EGF resulted first in re-phosphorylation of the drug-inhibited fusion kinase. We demonstrated phosphorylation of the activation loop tyrosines, which would likely result in a conformational shift of the fusion kinase from an inactive FKI-bound state to an active state. We were able to show that this re-phosphorylation event resulted in de-stabilization of binding of the FKI to ALK and ROS1, using the biotinylated FKIs crizotinib (ALK) and cabozantinib (ROS1). Lastly, the loss of FKI-binding resulted in reactivation of the fusion kinase signaling complexes, and restoration of the signaling capacity of the fusion kinase oncogene. These experiments collectively demonstrate that EGFR’s phosphorylation of fusion kinases can functionally rescue them from targeted inhibition. We were able to show this in vitro, using human-derived lung cancer cell lines, but also conceivable examples in human tumor samples as well, demonstrating the relevance of the transactivation mechanism to human lung cancers. ROS1, ALK, and RET signaling complexes were all detected in drug-resistant human tumors samples that were also positive for EGFR signaling complexes. This suggests that the fusion kinases may have been reactivated by EGFR at the time of disease progression—consistent with our in vitro data when EGF was added ectopically.

Treatment of ROS1 or RET fusions with a FKI resulted in a quantitative increase of EGFR signaling through each of the identified adaptors, GRB2 and SHC1. The rewiring in the HCC78 cells occurred on a rapid timescale, less than 15 minutes (Fig 34). The kinetics of the signaling changes that occurred after drug treatment in these cells is similar to cellular responses powered by full-length kinases following canonical growth factor stimulation. Rewiring in the LC-2/ad cell line occurred at a slower pace, taking approximately 60 minutes for a maximal shift in adaptor binding to occur (Fig. 36). Adaptor switching was not observed in cells that harbor either ALK or TRK, because higher levels of EGFR signaling were present in both cases prior to FKI treatment. This result suggests that in
these particular ALK and TRK cell lines EGFR’s signaling cascade is activated prior to inhibition of the fusion oncogene, but the mechanism for that activation was not determined here. This is consistent with a recent report from a collaborating lab, where they validated EGFR was indeed a member of the ALK-GRB2 signaling interactome in unperturbed cells, but also that pEGFR increased with high doses of crizotinib over time (382). Importantly, the phospho-tyrosine (pTYR) residues on EGFR demonstrating increased phosphorylation in their studies (Y998, Y1110, Y1138, Y1172, and Y1997) are not frequently detected with commonly used pTYR-specific antibodies, such as Y1068 and Y1173.

**Discussion**

One of the reasons the studies conducted in this thesis were able to detect the novel transactivation or other EGFR-induced phosphorylation changes on the fusion kinases was the approach of looking at total phosphorylation changes with the 4G10 pTYR antibody. This was a different approach from evaluating only phosphorylation of sites with clean pTYR specific antibodies available, but that may have potentially limited our results. The most evident flaw with the use of a general pTYR antibody is that it’s more difficult to know with certainty if the pTYRs being recognized are associated with your kinase of interest. The reason is, because this antibody is simultaneously detecting many unspecific phosphorylated tyrosine residues. Without immunoprecipitating with the total antibody of your kinase of interest first, it’s harder to determine what protein they belong to. However, our use of Li-Cor immunoblot imaging system provides more justification to this pTYR immunoblot approach. This system enables us to simultaneously detect two channels (detected with a rabbit and a mouse antibody), then overlay and merge the total and phosphorylation bands from each channel at the apparent molecular weight to identify the kinase of interest. Again, this approach is the primary reason the common transactivation event of ROS1, RET, and ALK was identified in these studies. These fusion kinases are all limited by reliable pTYR specific antibodies that recognize the phosphorylated tyrosine
residues, and for ROS1 and ALK specifically, were missed in my initial experiments. To prove these were indeed the correct bands, RNAi mediated knockdown of the fusion kinase was performed to validate that the phosphorylation bands were correct (data not shown). Indeed, there is one example in the literature where similar treatment conditions were performed on ALK, but the re-phosphorylation event was not visible due to the use of the wrong pTYR specific antibody (they used Y1604 instead of Y1282/83) (302). Fusion kinases protein products are often a different molecular weight compared to the native full-length RTKs from which they are derived, and that size difference can further enable use of this approach with confidence. For example, full length RET is more than 100 kDa larger in size than the CCDC6-RET fusion present in the LC-2/ad cells. Even if the full-length kinase was present with a fusion, that size difference should allow this approach to clearly detect the correctly merged fusion kinase bands (383). In order to improve our understanding of kinase biology in cancer, our studies suggest that it is important to look at total phosphorylation changes, when possible, and not just detect pTYRs based on the antibodies available.

**PLA**

We demonstrated a novel biomarker approach in these studies: signaling PLAs between kinases and adaptors. This approach is unique from other biomarker techniques, such as FISH or sequencing, because it is driven by kinase activity and based on the detection of functional signaling complexes, not limited by the identification of genetic alterations. It can be used to detect oncogenes that are created through genetic alterations, and importantly it can be used to identify wild-type kinases that are activated or signaling in cancer cells. Lung cancer therapy has seen the greatest improvements with the shift in the last decade towards oncogenic-targeted therapy. Part of the success associated with that approach, and why there are such striking patient responses, involves tailoring therapy toward specific, detectable, genetic alterations. The current paradigm is that cancer therapeutic targets often need a detectable genetic alteration (or biomarker) when one is
available, but we are approaching a maximum in the effectiveness of this approach. This maximum is supported with evidence that despite the extensive sequencing efforts and identification of many druggable oncogenes by groups such as the Cancer Genome Atlas or others, there remains a significant number of NSCLC patients and cell line models that have no genetically identifiable alterations that can be targeted with a monotherapy (30,384). Cell line models without identifiable alterations are important, because they are easier to test different hypotheses on potential oncogenic programs. We may continue to identify a few more rare or low frequency driver oncogene events (Fig. 1), but most of the readily identifiable or abundant targets have likely been found after many extensive sequencing efforts (30,384). It would be helpful to start utilizing novel approaches, such as signaling PLAs to address the more difficult, less straightforward mechanisms of oncogenic programs.

As we continue to gain insight into the atypical ways a cancer cell can activate an oncogene, we should recognize that standard diagnostic assays would not be able to detect some of these mechanisms. A typical mechanism of oncogene activation described in this thesis is chromosomal rearrangements resulting directly in a constitutively active oncogenic fusion kinase, but an atypical way may be a similar fusion produced, but this time involving a ligand for a kinase that indirectly results in constitutive activation of that kinase. An example of an atypical and indirect mechanism in creating an oncogene is the CD74-NRG1 fusions observed in lung cancer that can activate ERBB2-ERBB3 heterodimers (27). In this case, the use of a technique unbiased to the mechanism activating oncogenic signaling through these ERBB2 and ERBB3, but that still detects their signaling activation would be preferred.

Looking at protein phosphorylation as the sole indicator of whether or not a tyrosine kinase is functionally relevant to a cancer cell or not may not always be accurate or sufficient. For example, previous work from our lab on ROS1 fusion oncogenes, a known actionable molecular subset in lung adenocarcinoma, demonstrated that wild-type EGFR could mediate acquired resistance in a ROS1 positive cell line (211). The parental ROS1
cell line, HCC78, was made resistant to a ROS1 kinase inhibitor, TAE-684, through the utilization of long-term selective drug pressure, to assess potential mechanisms of therapeutic resistance to ROS1. The TAE-684 resistant cell line had become dependent on EGFR’s signaling cascade, which was determined through pharmacological inhibition and RNAi silencing of EGFR, as well as functional output of EGFR inhibition through downstream signaling changes by western blot, and cellular proliferation assays. However, phosphorylation of EGFR had not changed between the parental cells to the resistant cells (Fig. 35). Our use of the EGFR-GRB2 signaling PLA here between EGFR and the adaptor GRB2 detected a clear and significant change in EGFR’s signaling complexes with GRB2 in the HCC78 parental and resistant cells where detection of pEGFR failed to indicate any visible or quantifiable change. Thus, this evidence supports that the signaling PLA can provide a useful role in identifying activated, and relevant signaling complexes.

The PLA is sensitive, because the procedure allows transient or unstable interactions to be readily detected following fixation, and the amplification step prior to detection (304). Other similar protein interaction techniques, such as förster resonance energy transfer (FRET), can only be used in vitro with cells overexpressing the proteins of interest (305). Another essential aspect of the signaling PLA tool is that this assay works on fresh, frozen, or formalin-fixed, paraffin embedded (FFPE) patient samples. The lab is testing the use of these assays on tissue treated with targeted therapies from different mouse models of lung cancer, similar to what is shown in (Fig. 39). We have been able to use this assay to detect signaling complexes in FFPE tumor samples from murine models and patients. We were also able to use the assay to demonstrate the activation of EGFR’s signaling cascade at the onset of disease progression for a patient treated with a monotherapy against a fusion kinase oncogene, ROS1 (Fig. 43).

Break apart FISH can be helpful to screen patient sample cohorts for rearrangements, but requires pre-existing knowledge of the break-points and provides no
information about the 5’ fusion partner. The probes for this assay can detect where DNA is on a chromosome breaks and when that DNA rearranges, because the fluorescent probe signals will separate from their normal position on the chromosome. FISH cannot determine whether that chromosomal rearrangement forms a functional fusion protein. RNA sequencing technologies are more advantageous here, because you are selecting for a signal that is expressed in the tumor, indicating the fusion protein created a functional, in-frame protein. One of the advantages of the PLA, similar to RNA sequencing, is that it doesn’t limit our knowledge of already identified mutations. There are a few additional examples of how this assay may be helpful in other cancers. Prior work has identified EGFR signaling dependence in cancers without a clear genetically-mediated activation mechanism (211,250,251,315). Also, these EGFR dependencies are not dictated by phosphorylation of EGFR detected by immunoblot analysis, one of the standard detection methods for activated RTKs in cancer. WT EGFR is an FDA-approved drug target in other cancers, including head and neck squamous cell carcinoma (HNSCC) and colorectal cancer (250,251). However, selecting for patients that respond to EGFR inhibitors has been difficult, and an EGFR-adaptor PLA may be a better discriminator of EGFR-dependence than EGFR expression levels. Additionally, AXL and MET kinases have frequently been identified as important in context of acquired resistance, with and without any detectable genetic alterations (213,385,386). PLAs created by our lab for both these kinases may prove to be helpful in future studies to identify when these kinases are functionally important to a tumor (Fig. 34 and AXL data not shown). However, one concern with the PLA is that it is not binary like mutational analysis, and will require a cut-off to determine when the signal is functionally meaningful, or not. Future work using control cell lines or animal models can help determine relevant thresholds of signal. Cells that maintain different magnitudes of normal physiological signaling or disease related signaling complexes, including examples with a pre-determined functional relevance, will help to determine these threshold analyses.
Interactions between EGFR and fusion kinases

Improving our understanding of the nature of the molecular interactions between EGFR and the fusion kinases could provide insight for future studies. Following up on the initial investigation of the heterodimerization model only using PLA will be an important next step. Any differences in co-immunoprecipitation analyses using the L704N receiver and I941R activator mutants (Fig. 47) will help clarify if disrupting the dimer interface is important for these interactions. Regardless of such heterodimerization studies, selective deletion constructs will help identify critical regions for these interactions. It will also help determine if certain regions of the proteins are important functionally for EGFR-mediated phenotypes such as the transactivation (Fig. 28). Alternatively, recent improvements in cryo-electron microscopy technology show it could be used to help capture the detailed interactions and conformational changes between complexes like EGFR and the fusion kinases under different drug treatments with high angstrom resolution (387,388). The detailed molecular interactions between EGFR and fusion kinases may provide critical information for future drug development blocking such interactions. An alternate approach to using a combination of kinase inhibitors that may induce toxicity in patients is to rationally design one drug with the ability to block both proteins. For example, comprehensive structure-function studies from the HER2-HER3 heterodimer was helpful in learning that they can be disrupted using the monoclonal antibody pertuzumab, which is FDA approved for HER2+ metastatic breast cancer (389-392). As the activation of EGFR is allosteric, its protein-protein interactions, or interactions with the plasma membrane and the molecular basis of those interactions may prove informative in developing novel therapeutic strategies.

EGFR signaling

It will be critical in the future to determine mechanisms of activation for EGFR in certain signaling contexts in the studies presented here. For example, the baseline or FKI-induced activation we observed in fusion kinase cell lines through the EGFR-GRB2 PLA
The heterodimerization model proposed in chapter 4 presents one potential explanation for a ligand-independent activation of EGFR, although this model was not thoroughly addressed in these studies. If ALK and TRK fusion kinases are able to heterodimerize with EGFR, this model could explain why EGFR was signaling in these cells under unperturbed conditions. The simplest explanation for the mechanism of EGFR’s signaling activation is ligand-mediated, but EGFR can be activated non-canonical through ionic interactions with the plasma membrane (255). Importantly, EGFR has been shown to signal through different adaptors and pathways when it is activated differently (mutation mediated constitutive activation vs. ligand-mediated activation) (393). These differences could potentially influence different approaches to block its signaling. In our studies, gefitinib was sufficient to block EGFR signaling in vitro and in vivo, but another therapeutic approach may be necessary to avoid toxicity in humans, due to the high doses required to inhibit the wild-type receptor (394). An approach to address the possible role for EGFR ligands in our experiments would be the utilization of a ligand trap, ideally one that recognizes several common ligands, similar in structure to the extracellular binding region of EGFR (395). In (Fig. 31) we showed EGFR-GRB2 signaling complexes in the ALK+ H3122 cell line under control conditions, and in the ROS1+ HCC78 cells EGFR-GRB2 signaling was observed following FKI treatment. This work did not identify the mechanism of EGFR activation here, or rule out if it was through autocrine or paracrine signaling from one of its ligands. The implementation of an EGFR ligand trap in both of these cell models would help to elucidate a potential role for the ligands in mediating the EGFR-GRB2 signaling complexes observed at baseline or induced in these studies.

Designing signaling PLAs between EGFR and its ligands would also be a helpful tool to improve our understanding of which ligands are more relevant in vivo. Evidence strengthening this concept was briefly mentioned in chapter 3, based on a previous observation (Fig. 43c, bottom left panel). A distinct pattern of EGFR PLA signal was
detected, and the pathologist identified the cells inside that pattern as stromal fibrovascular bundles. We hypothesized that these cells may be producing an EGFR ligand, resulting in the enhanced signal immediately adjacent to these stromal cells, and dissipating with increased distance from the source. This could suggest a non-cell autonomous mechanism resulting in activation of EGFR in this particular metastatic patient sample. This technique would require predicting which ligand was being produced or is most relevant in lung cancer patients. Amphiregulin is a promising ligand choice to start with for this assay approach because it is an EGFR-specific ligand (no ERBB family cross-reactivity), and expressed at high levels in lung cancer patients (261,396). Alternatively, having multiple EGFR-ligand PLAs available, and pairing RNA-sequencing from each patient’s tumor would enable the detection of enhanced ligand expression and indicate the most relevant choice. Collectively, this reinforces the potential utility of EGFR-ligand PLAs and the appeal of their use in future studies.

The unexplored roles for other ERBB family members in this thesis leave substantial potential for future studies. Gefitinib, the EGFR inhibitor used in these studies, carries reasonable specificity for mutant or wild-type EGFR over other ERBB family members, but does show activity against wild-type and active ERBB2 at the higher doses (1 \( \mu \text{M} \) was used in these studies) required for WT EGFR (397,398). Our EGFR knockdown data show a more modest effect against cell proliferation compared to treatment with gefitinib (Fig 40). This could be explained by incomplete knock down of EGFR, and the remaining amount of EGFR was enough to provide sufficient accessory signaling. Another possible explanation for our results is cross talk between ERBB family members, and that gefitinib is more efficient at blocking the signaling output than the partial knockdown of EGFR we obtained. EGFR may be activating ERBB3 in these cells, so the loss of EGFR kinase activity would also result in abrogation of HER3 activity. Transactivation of ERBB3 by EGFR has been observed in many different cancer models, including lung cancer cells, and would explain the abrogation
of the signaling contribution with only the EGFR inhibitor (399-403). There are many potential receptor-receptor and ligand-receptor combinations possible from the ERBB family will also be important for the proper execution of future ERBB family ligand traps. If EGFR is dimerizing with one of the other 3 family members, that expands on the need for an EGFR-Amphiregulin PLA and brings into question the relevance of ligands for ERBB3 and ERBB4 and as well (403). An enhanced effect using a pan-ERBB family inhibitor such as dacomitinib instead of gefitinib would also indicate a contribution from other family members. ERBB3 would need to be knocked down using RNAi to determine if it were making a contribution in our cell models.

In order to improve our understanding of adaptor switching, an important next step will be to find a model system to test our GRB2 binding mutant EGFR constructs. Until those mutants are utilized in an appropriate cell model, “adaptor switching” will remain an observation. The failed attempts in the ROS1-3T3 to instigate EGFR-GRB2 signaling complexes are perplexing and draw attention to important points regarding tissue tropism and intrinsic wiring for signaling complexes. NIH-3T3 cells express detectable levels of the adaptor GRB2, and the stable expression of SDC4-ROS1 allows ROS1 to signal through GRB2 in this model (Fig. 48). Our use of kinase-adaptor PLAs is helpful for detecting wild-type kinase signaling complexes, but the inability of EGFR-GRB2 to activate in these cells highlights the question of which adaptor proteins are going to be relevant and how that might change in different cellular contexts. If only EGFR-GRB2 PLAs are assayed, we would not detect a positive result for EGFR signaling in these cells. However, the increase in pROS1 observed with the expression of EGFR in these cells suggests that while EGFR is functionally important and signaling through ROS1, EGFR may be signaling through an alternate adaptor. EGFR has been shown to signal differently when activated constitutively in glioblastoma, suggesting that the mechanism of EGFR activation could affect the adaptors and pathways it engages (393). Our work in the ROS1-3T3 and this work in
glioblastoma suggest that EGFR may signal through different adaptors, depending on the cell context and the mechanism of its activation. This also suggests additional adaptor binding mutants, such as ones for SHC1 or p85, may be necessary to further understand the shuffling of adaptors from dominant oncogenes to accessory pathway components, such as EGFR. Transient overexpression of any component may alter the stoichiometry and concentrations in the cell that we are not realistically measuring expression levels fit for “adaptor switching”. The robust levels of EGFR often obtained with transient overexpression could cause the WT receptor to auto-activate and signal through GRB2 prior to the inhibition of ROS1. Additionally, when expression levels become too high like this, we could hypothesize that the PLA will detect a false positive signal between 2 very highly expressed proteins and not a true interaction or signaling complex. This is the reason we used a fusion: cytokeratin control PLA, to demonstrate we are not just detecting two highly abundant proteins with our interactions between fusion kinases and EGFR (Fig. 33, 37). Stable lentiviral expression may enable the use of an artificial system for these experiments, such as in 293T cells or preferably a cancer cell line. An alternate approach would be to utilize the CRISPR/CAS9 system to edit out endogenous EGFR in actual lung cancer cells, such as the HCC78 cells, and re-express the mutants stably.

Signaling feedback loops, particularly through the MAPK signaling pathway, could also explain the rapid induction of EGFR signaling. Feedback activation of EGFR in response to mutant BRAF inhibition in colon cancer, the mechanistic explanation for the poor efficacy of BRAF inhibition in colon cancer compared to melanoma, is a well-studied example of a positive feedback loop resulting in survival signaling through EGFR (404). These results in colon cancer differ from ours in that there is no change in pEGFR at Y1068 in the fusion kinase cells following FKI treatment, whereas there was a visible increase of pEGFR at Y1068 in the BRAF+ colon cancer study. Blockade of ERK phosphorylation of the phosphatase CDC25A, through BRAF inhibition, rendered CDC25A inactive, abrogating its
de-phosphorylation of EGFR (404). Blockade of the fusion kinase, such as ROS1 and RET in our models, may result in rapid rewiring resulting from a feedback loop from MAPK inhibition that results in the activation of EGFR’s signaling cascade through adaptors such as GRB2 or SHC1. The “switch” of GRB2 or SHC1 from the fusion kinase to EGFR may not be caused by the interactions or proximity of the fusion kinase with EGFR, but simply by a coincidental activation of EGFR through those same signaling branching point(s). GRB2 and SHC1 both have high protein expression levels in these cells, measured by immunoblot analysis (Fig. 34, 36). No phosphorylation changes indicative of a similar feedback loop was observed following a time course of FKI treatment in our studies by western blot in the HCC78 or LC-2/ad cells. The only biochemical difference observed was with the immunoprecipitation of the GRB2 and SHC1 adaptors onto EGFR following FKI treatment (Fig. 34, 36). Another example of a phosphatase in addition to CDC25A that negatively regulates EGFR is MIG6 (405,406). The loss of MIG6 can significantly speed up tumor initiation and progression in the context of constutively active, mutant EGFR, suggesting a tumor suppressive role in lung cancers for MIG6 and that it regulates even oncogenic EGFR (407). Additionally, Sprouty1 and 2 have also been shown to regulate EGFR signaling both positively and negatively (408). CDC25A, MIG6, and Sprouty phosphatase status were not explored in the studies presented here, but may be pursued in future investigations.

Transactivation

The transactivation of drug-inhibited ALK by EGFR shown in these studies was generated in the presence of crizotinib, a first-generation ALK inhibitor. However, there is evidence in the literature that our studies may still be relevant for next generation ALK inhibitors, based on findings in a recent paper regarding ALK acquired resistance to alectinib and ceritinib (226). They reported 2 resistant cell lines that maintained phosphorylation of ALK in the presence of an ALK inhibitor. In their report, they were unable to identify any ALK kinase domain mutations, but ALK maintained phosphorylation in the presence of ALK
inhibitors and both EGFR and ERBB3 had become activated in their resistant cells (226).
Briefly addressed in the discussion of chapter 3, we believe our results may explain the
previously paradoxical result from this article, and that their resistant cell lines may harbor a
chronic state of transactivation of ALK by EGFR. In support of this, both EGF and NRG1
ligand mRNA levels were enhanced, and pEGFR was increased in the resistant cell lines
(226). These resistant cell lines could be tools for future mechanistic studies on kinase
transactivation, such as understanding the role of the different EGFR ligands or ERBB3 in
our studies. These models are unique from ours in that they occurred naturally in an
endogenous cell program and appear to occur stably now, not transiently from ectopic
ligand stimulation. This report is unlike our studies where the ligand was ectopically added
in. Their work also suggests there may be an intrinsic wiring in the H3122 cells that enables
this transactivation phenotype to occur both acutely (under ectopic treatment conditions) as
well as chronically (upon the development of acquired resistance). A similar intrinsic wiring
towards dependence on the EGFR-signaling axis was also observed in drug naïve HCC78
cells in this dissertation, and in a model of acquired resistance against the FKI TAE-684 in
the HCC78 cells already described (Fig. 35) (211). The way that EGFR can mediate intrinsic
and acquired resistance in response ROS1 inhibition or ALK inhibition, demonstrates a bias
towards assistance from EGFR in these cells.

Evidence in the literature from a collaborating lab has already linked the HELP
domain to the activation of RAS-MAPK by EML4-ALK (182). This work has shown that the
HELP domain may enable not only the localization of EML4-ALK near RAS at the
membrane. This may be relevant to our studies, because it also localizes ALK near EGFR
at the membrane. Interestingly, ALK+ cell line, the H2228 cells do not respond to EGF
stimulation in the presence of an FKI with re-phosphorylation (date not shown). Interestingly,
the H2228 harbor the E6:A20 variant of EML4-ALK, whereas the H3122 cells contain the
E13:A20 variant (Table 6). Structural differences between the 2 variants, highlighted by the
additional 7 exons of EML4 and its HELP motif, could explain this phenotypic difference (23,298). HELP motifs are hydrophobic, which is why are important for interactions with and localizing proteins near the inner leaflet of the membrane (409). One could speculate from this that the HELP domain may be important for ALK to be a substrate for EGFR phosphorylation, or for its subcellular localization to be in the right place at the right time. Alternatively, the expression of additional protein(s) downstream of the EGFR signaling axis in the H3122 cells, but not the H2228 cells could also be important for enabling the phosphorylation event or transactivation. The H2228 cells also show the least substantial effect on inhibiting proliferation by adding gefitinib to the FKI (Fig. 39). This data suggests that different fusion variants behave differently, and may be important for future studies determining when co-inhibition of EGFR will be more effective.

MAPK signaling and cell fate

Studies in chapter 4 using specific kinase inhibitors have suggested that EGFR and fusion kinases may be controlling the signaling through BRAF and CRAF, respectively. This further indicates the importance of EGFR in these cells, as BRAF has higher basal kinase activity than CRAF (362). Our studies did not include analysis of ARAF, but it may prove to be important in these contexts, as it can stabilize BRAF-CRAF heterodimers through its role as a scaffold (379). ARAF may be relevant for our understanding of the regulation of downstream signaling by EGFR and the fusion kinases. RNAi knockdown and PLAs between RAS or the other 2 RAF effectors with ARAF will determine if it is an important scaffold to the other RAF isoforms. The differential contributions to signaling by the specific RAS isoforms (H, K, and NRAS) in fusion kinase and/or EGFR remain unclear, because pan-RAS family antibodies were used. In the event that the different RAF effectors are important for regulating different signaling programs, one could hypothesize that the different RAS effectors may be contributing differentially to the MAPK signaling in these cells as well. In order to improve our insight into the roles of different RAS family members in
these fusion kinase positive cells, isoform specific RNAi knockdown of RAS family members should be performed. In order to tie the effects of RAS and RAF specific isoforms to different cellular fates, functional assays should be implemented in conjunction with PLA assays. Additionally, RNA-sequencing analysis following RNAi of the different effectors may help expose the different gene expression programs that are specifically invoked by each one.

The RAS-RAF PLAs used here were also an effective tool for simply evaluating changes in the MAPK signaling pathway. This approach can be utilized for general studies on MAPK signaling changes (Fig. 50), but also in the contexts of different RAS gene status (Fig. 51). In these experiments, we were able to use this tool to identify increased RAS signaling, due to amplification of the RAS gene both \textit{in vitro} in H3122 cells resistant to crizotinib and \textit{in situ} in ALK+ resistant patient samples, some that harbored a RAS amplification. The signaling detected by PLA provided a functional output to the RAS amplification. RAS copy number gain can be detected with FISH, but FISH only detects the genetic change, not the output of that signaling. An interesting approach here would be to pair FISH and RAS-RAF PLA onto each patient sample. This has not been attempted yet, but would be feasible by doing the assays sequentially. The PLA would need to be done first and imaged, followed by FISH and a second set of imaging. The order is important, because FISH has a proteinase K step that would degrade the proteins and epitopes necessary for antibody binding in the PLA. This would allow for the detection of the gene amplification and the functional signaling output of that alteration to be detected at the single cell level. This would account for detecting both intra- and inter-tumor heterogeneity, and may be a powerful tool for future studies with RAS alterations.

The potential roles of EGFR and fusion kinases regulating different downstream effectors and ultimately cell fates would help explain the evolutionary advantage of the cancer maintaining high expression levels of EGFR. There is evidence that EGFR has the
ability to signal through more adaptors and pathways than many other kinases, and that diversification may increase control over additional, pro-tumorigenic cell fates (380). Studies in PC12 cells demonstrate potential differences in cell fate that different kinases can have on the same signaling pathway, MAPK. EGF stimulation results in short-term activation of MAPK signaling due to a negative feedback loop, and results in proliferation, but NGF stimulation induces a positive feedback loop and sustained MAPK activity resulting in neuronal differentiation in PC12 cells (197-199). The different spatio-temporal control of ERK in the PC12 cells is the basis for the different cell fate outcomes. Interestingly, both of the adaptors shown to engage in the adaptor switching observation, GRB2 and SHC1, predominantly signal through the MAPK pathway. The effects of EGFR inhibition on cell fate indicate that it may also be promoting anti-apoptotic signaling in the NTRK1+ KM12 cells, because inhibition of EGFR affects the induction of apoptosis (Fig. 51). Survival signaling may occur through the AKT signaling pathway (410). In the presence of both the fusion kinase and EGFR, the fusion kinase may predominantly engage pro-proliferative signaling and EGFR pro-survival signaling. The physical interactions between EGFR and the fusions may enable a steric preference for certain signaling adaptors to bind to each, driving different downstream pathways and cell fates. Our data indicates that signaling through the fusion kinase TPM3-TRKA may predominantly be controlling proliferation (demonstrated by dose response to the FKI), although it also exhibits effects on survival (single-agent effect of the FKI on apoptosis (Fig. 51). More surprising, while the single-agent EGFR inhibitor had no effect on proliferation, it had a more substantial effect in the apoptosis assay on survival (Fig. 51). This is complicated by the data indicating EGFR can signal through the fusion itself (Fig. 30), or independently through GRB2 (Fig. 31, 32). Our signaling data do not indicate a visible inhibitory effect downstream with gefitinib as a monotherapy, especially on pAKT signaling, but enhanced inhibition of both MAPK and AKT downstream pathways with FKI and gefitinib (Fig. 28, 29). We only look at one site, Serine 473, but others should be
looked at, such Threonine 308. Differences with EGFR inhibition may have been more apparent at Threonine 308 (411). Similarly, our proliferation data indicates a significant anti-proliferative effect with the combination of FKI and gefitinib (Fig. 39), but it is unclear if that is due to blockade of independent signaling from EGFR through GRB2, or its augmentation of TRK signaling through its phosphorylation of pTRKA (Fig. 30). On-going work, such as the utilization of different phospho-specific antibodies in these pathways, like Threonine 308, would be helpful. They may indicate a divergence to different pathway nodes, which will help dissect the contributions of each kinase for various cellular programs. Alternatively, other pathways that may signal in a pro-survival way should be explored, such as the NF-κB or JNK pathways (412). These additional experiments will help determine at the signaling level how EGFR is predominantly controlling survival in these cells.

**Mouse models**

Future studies using mouse models will help improve our understanding of the role for EGFR in the context of fusion kinases *in vivo*. The mouse models used in these studies were a helpful starting point in understanding the effect of combination therapy in a living model, but both the cell-line xenograft (ALK) and the patient-derived xenograft (NTRK1) require the use of flank injections in immunocompromised (Nu: Nu) mice. Nudes lack a functional FOXN1 gene, resulting in athymic mice, and a reduction in functional T cells in this model (413,414). These lung cancer cells are heterotransplanted into the subcutaneous flank of the mouse, not in their true lung microenvironment. The role of the immune system in cancer has become increasingly appreciated in recent years, and the use of an immune-competent mouse in subsequent cancer studies enables researchers to factor in the role the immune system may be playing in response to therapy (373). The fusion kinases studied in this dissertation clearly bear a fundamental role in the cancers that harbor them. Appropriate genetic models that recapitulate that will only improve our understanding of how they operate in their true natural surroundings—which includes an immunocompetent lung tumor
micro-environment, and potentially the genetic diversity or mutational burden that we know exists in human tumors. Recent work has repurposed the genome editing power of the CRISPR/CAS9 system towards creating such relevant mouse models that harbor an endogenous oncogenic fusion: \textit{EML4-ALK} (415). Building off of this technology, our lab can use similar genome editing to build similar mouse models for \textit{ALK}, \textit{ROS1}, \textit{RET}, and \textit{NTRK1}. These immunocompetent mouse models would improve our studies assessing the effects of co-targeting the fusions and EGFR, as well as the role of EGFR signaling in vivo. Indeed, the immunocompetent murine C57Bl/6 strain used in these studies contains high endogenous levels of EGFR in the lung (416). Any additional factors necessary for interactions between EGFR and the fusion kinases should also be present in this environment.

\textbf{Oncogene addiction}

The original model of oncogene addiction states that as a result of its constitutive activation the oncogene is in control and essentially that it is at the peak of the signaling cascade (3). The model indicates that blocking this kinase fusion oncogene should result in apoptosis or differentiation control (3). If this were true, and the cells were 'addicted' to signaling through the kinase fusion, then that addiction paradigm would support the idea that the fusion should be essentially devoid of upstream input. Our work indicates that a modification of the current model of oncogene addiction, one of the central dogmas of cancer biology may be more accurate. This original model suggests a dictatorship driven by the oncogene. However, the work presented here provides evidence against a top-down signaling cascade driven entirely by the dominant oncogene, but rather suggests a slightly more democratic model, where the oncogene can receive input and be influenced by other proteins—in this particular example, EGFR (Fig. 29, 30).
Patients do have dramatic response to oncogene-directed monotherapy, which you would expect if the theory of oncogene addiction were correct. However, the majority of responses are incomplete, inconsistent with the addiction paradigm. This clinical evidence supports a more complicated model in which the majority of oncogene-addicted tumors harbor an innate ability to resist complete tumor ablation with the use of oncogene-directed therapy. Our studies were focused on fusion kinase oncogenes, but this idea may be relevant to other oncogenes as well. This could be a more common theme amongst other oncogenes that are not activated by a “fusion” way as well. It was reported that WT EGFR preferably dimerizes with oncogenic EGFR mutations, and this interaction is functionally important in lung cancers (262). Previous work in lung and pancreatic tumor models has also shown that these KRAS-driven tumors demonstrate increased anti-tumorigenic effects with combined EGFR inhibition (264-266). Indeed, there is clinical evidence of an overall survival advantage in unselected lung cancer patients treated with an EGFR inhibitor (417-419). This clinical data suggests an importance for WT EGFR, based on the overall survival advantage with its inhibition, in unselected NSCLC patients. This survival advantage was the scientific rationale for approving EGFR inhibitors prior to the retroactive identification of drug-sensitizing EGFR mutations (252). The cell context of these fusion kinase oncogenes may also prove to be important, as BCR-ABL fusion kinase+ patients that are treated with imatinib in CML have demonstrated more durable responses than observed against fusion kinases in solid tumors (66). This may indicate that these hematological cells do not harbor cooperative signaling networks, and that is what allows the monotherapy to be more successful at preventing disease reoccurrence over time. However, the success with imatinib as a monotherapy in CML is generally regarded to be an extreme exception, not a standard example for targeted therapies. Collectively, this work demonstrates that certain oncogenes are mechanistically executing their pro-tumorigenic effects with input from other accessory signaling pathways in select cells, and that may explain why monotherapy
responses are transient. The percentage of cells with this input is unclear at this time, but these results suggest EGFR is able to provide accessory signaling to these oncogenes in lung and pancreatic tumors.

**Clinical impact**

**TRK inhibitors in the clinic**

Currently, several small molecule tyrosine kinase inhibitors (TKIs) with activity against the TRK family are being explored in clinical trials. LOXO-101 (Loxo Oncology), previously known as ARRY-470, is a highly selective pan-TRK inhibitor and is currently being investigated in a phase Ia/Ib and phase II trials across multiple tumor types (NCT02122913) (39). RXDX-101 is a pan-TRK inhibitor that also has activity against two other gene fusion targets, ALK and ROS1. A phase I/II study entitled STARTRK-1 of RXDX-101 is currently accruing patients with TRK alterations (Ignyta, NCT02097810). MGCD516 (Mirati, NCT02219711), and DCC-2701 (Deciphera, NCT02288111), multi-kinase inhibitors all have trials for several different molecular sub-types, including TRK kinases. Lestaurtinib (CEP-701; Cephalon) showed promising pre-clinical activity in NTRK1 fusion models, but its future clinical development remains unclear (21). Additionally, reports from four patients with identified NTRK1 fusion genes and one patient with an NTRK3 fusion were recently treated with inhibitors off-label or in other active trials that demonstrated the clinical benefit of targeting this family of oncogenes (21,39,420-423). The first patient was the index lung cancer patient harboring the MPRIP-NTRK1 fusion, who was treated off-label with the multi-kinase inhibitor crizotinib, because at the time no TRK specific inhibitors were available (covered more thoroughly in chapter 2) (21). Crizotinib has only modest activity against TRKA and only produced a proportionally transient, minor radiographic response emphasizing the need for more potent TRK inhibitors in the clinic (424). Two colorectal cancer patients – one with a TPM3-NTRK1 gene fusion and one with an LMNA-NTRK1 gene fusion were treated in the phase I portion of a clinical trial investigating an interrupted
dosing schedule of RXDX-101 (421,423,425). The TRKA+ (TPM3-NTRK1) patient experienced a partial response and provides the first evidence of clinical activity of a TRK inhibitor in a patient with an oncogenic TRK alteration (425). The other response with this inhibitor was also classified as a partial response by RECIST, which lasted 4 months, before the patient developed acquired resistance to the therapy (421). Circulating tumor DNA (ctDNA) analysis from the patient identified 2 novel kinase domain mutations that may have potentially been responsible for disease progression (421). The ETV6-NTRK3 patient with mammary analogue secretory carcinoma (MASC) was also treated on the STARTRK-1 trial, and experienced a dramatic, durable response before developing acquired resistance likely due to a kinase domain mutation (422). Interestingly, the kinase domain mutation for this patient is paralogous to the same one identified in the LMNA-NTRK1 fusion also treated with RXDX-101 (TRKC G623R and TRKA G595R). More recently, a patient diagnosed with metastatic soft-tissue sarcoma was treated on a phase I clinical trial at the University of Colorado with LOXO-101, which was also referred to by its former nomiker as ARRY-470 in this thesis. This patient's tumor harbored an LMNA-NTRK1 fusion, which we were able to validate signaling complexes using the TRK-SHC1 PLA (Fig. 52). The patient experienced a rapid, and substantial regression in tumor burden upon her treatment, deemed a partial response by RECIST 1.1 at 4 weeks, but underwent almost complete tumor regression by 4 months (Fig. 52) (39). LOXO-101 was recently granted breakthrough therapy designation by the FDA in July 2016. Each of these patient responses highlights the advantages of genetic profiling of tumors of all types.

Updates on the current progress of the Loxo Oncology and Ignyta trials were presented earlier this year at the American Association of Cancer Research ( AACR) annual meeting. To date, 5/6 NTRK+ patients treated with LOXO-101 have had confirmed partial responses, an 83% ORR (426). Of the 24 total patients treated with RXDX-101, NTRK+ patients boast a 3/3 or 100% ORR, whereas the ALK+ and ROS1+ group is currently at
16/21 and a 76% ORR (427). Indeed, Ignyta’s STARTRK-1 trial may be a good starting place to instigate clinical testing of the EGFR-fusion kinase co-inhibition strategy. The preclinical work provided in this dissertation supplied evidence that co-targeting this cooperative signaling network up-front may help to improve initial responses. Blocking the early adaptive or survival signaling from EGFR may improve both the depth and durability of responses. In our studies, the use of a clinically available EGFR inhibitor, gefitinib, was sufficient to eliminate the contributing role of EGFR.

**Lung cancer helps speed therapeutic development**

Similar to NTRK1, oncogenic ALK gene rearrangements were found to be important in cancer long before the first ALK inhibitor was FDA approved. The first ALK gene rearrangement was identified in anaplastic large cell lymphoma in 1989 and subsequently unveiled in 1994, but no ALK-targeted therapies were developed in this disease until 22 years later (Fig. 53) (57,428). The critical moment for ALK inhibitor development came in 2007 with the discovery of ALK gene rearrangements in NSCLC (22). The corresponding FDA approval of crizotinib for ALK+ metastatic NSCLC was exceedingly fast, taking only 4 years from the time of first identification of ALK in this patient population (22). A similar trend, or gap, in therapeutic development has been observed for NTRK1 (Fig. 53). This rapid approval highlights the successful strategy of precision oncology by matching targeted therapies with biomarker-selected patients. This approach is particularly highlighted by its success in lung cancers, and the dramatic improvements in the efficient execution of targeted agents into the clinic.

**Conclusions**

In summary, the work presented in this thesis improves our knowledge of fusion kinases in lung cancer. As cancer biologists move away from the novelty of high-throughput sequencing efforts, the rate of identifying novel driver mutations, even rare ones like NTRK1, may have hit a ceiling. In order to continue this momentum of therapeutic
improvement from oncogene-directed therapies in the last ten years, it will become necessary to improve our knowledge of the mechanisms these genetic alterations actually use to drive tumorigenesis, and their accessory signaling networks. Our lab has done this after the identification of NTRK1 by re-focusing subsequent studies on understanding the detailed mechanisms of how it drives lung tumorigenesis in a cooperative signaling network with EGFR. Instead of only using artificial systems like 3T3 or Ba/F3 cells, (neither which expresses endogenous levels of EGFR) we built better models to study this alteration, such as the CUTO-3 lung cancer cell line and the CULC-001 patient derived xenograft mouse model. If we had only studied the NTRK1 fusions in artificial models, we would not have identified the contributing role of EGFR towards this oncogene, or the pattern of its conserved role among other fusion kinases.

We successfully identified NTRK1 fusions in lung cancers, tested the TKI ARRY-470 in our pre-clinical models, helping to push its clinical development under the new identity of LOXO-101 by demonstrating efficacy in multiple tumor models. Based on its rapid successes, LOXO-101 may become an FDA-approved therapy for NTRK1+ alterations in the future. Indeed, LOXO-101 has demonstrated impressive results thus far, but we are already thinking ahead to what should be done as these patients’ tumors start to develop drug resistance. The effective utilization of combination therapies, and targeting “sensitizer” proteins like EGFR in addition to dominant oncogenes, may shift partial responses towards to deeper or complete responses. The elimination of residual tumor cells, through these rational combinations, may decrease residual disease and delay the time to disease progression and acquired resistance. The hope is that over upcoming decades more patients will be treated up front with such rational combinations, and these patients will have a prolonged and lasting response to these drug combinations.
Figure 52: An NTRK1+ sarcoma patient has a rapid and significant response to LOXO-101. (a) The TRK-SHC1 signaling PLA demonstrated robust signaling in the tumor cells, but weak signal in the thick-walled blood vessel. A blood vessel is indicated within the white dotted ellipse. (b) Computed tomography (CT) obtained following pre-operative chemotherapy and primary tumor resection with arrow indicating the presence of an 18mm right lung nodule 4 months prior to starting LOXO-101, baseline imaging just prior to dosing with LOXO-101 on study, and following 1 cycle (28 days), and 4 cycles (4 months) of dosing with LOXO-101. The patient was observed to have metastatic disease only in the lungs and therefore the CT scan images show axial (top) and coronal (bottom) images focusing on the thoracic cavity. The images demonstrate an initial rapid disease progression (13-week interval) followed by a marked tumor response with decreased size and/or resolution of the numerous pulmonary metastases, 4-week and 16-week intervals since baseline.
Figure 53: Fusion kinase identification in lung cancer rapidly improves therapeutic development timelines. (a) Timeline depicting the gap between the identification of ALK fusion kinases in ALCL and the time necessary for the first ALCL patient to be treated with an ALK FKI, compared to the rapid latency period after it was identified in NSCLC. Crizotinib was the fastest FDA approved targeted therapy (less than 4 years from identification in NSCLC to FDA approval). (b) Timeline depicting the lengthier inactivity period of TRK inhibition when it was first identified in colorectal cancer, to the first colorectal patient being treated in the TRK basket trials (NCT02122913, and NCT02097810) that opened in 2014. The time from its discovery in NSCLC to the first patient being treated and FDA granting break-through drug status was almost as quick as ALK.
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