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The Molecular Pathogenesis of EML4-ALK Driven Lung Cancer and Strategies to Overcome Clinical Resistance to ALK Inhibitors

by

Gorjan Hrustanovic

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

This dissertation is dedicated to my mother and father

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Abstract

The Molecular Pathogenesis of EML4-ALK Driven Lung Cancer and Strategies to Overcome Clinical Resistance to ALK Inhibitors.

Gorjan Hrustanovic

A promising strategy to combat cancer drug resistance is to deploy rational upfront polytherapy that suppresses the survival and emergence of resistant tumor cells. The optimal initial combination strategy is unclear in most tumors with oncogenic receptor kinases because they typically engage multiple effector pathways, and which of these individual pathways (if any) is most critical to tumor cell survival is poorly defined. Here, I demonstrate in models of lung adenocarcinoma harboring the oncogenic ALK receptor kinase fusion (EML4-ALK) that the RAS-MAPK pathway, but not other known ALK effectors, is required for tumor cell survival. We reveal that EML4-ALK drives RAS-MAPK activation by engaging all three major RAS isoforms (H, N-, K-RAS), a signaling event that requires the HELP domain of EML4. MAPK pathway reactivation via either genomic amplification of *KRAS*^{WT} or downregulation of the MAPK phosphatase DUSP6 promoted resistance to ALK inhibition. Accordingly, upfront ALK and MEK co-inhibition enhanced both the magnitude and duration of initial response in EML4-ALK lung adenocarcinoma *in vitro* and *in vivo* models. Furthermore, genomic amplification (or gene duplication) of *KRAS*^{WT} or downregulation of DUSP6 was observed in *ALK* fusion positive lung adenocarcinoma patients with acquired ALK inhibitor resistance. Together, my findings provide insight into the function of RAS-MAPK signaling in EML4-ALK lung adenocarcinoma and rationale for upfront ALK-MEK co-inhibition to forestall resistance and improve patient outcomes.

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Introduction

Introduction: EML4-ALK positive lung cancer

Lung cancer is the leading cause of cancer-mortality in the world, and is responsible for approximately 30% of cancer-related deaths in the US per year (more than breast, colon, and prostate combined). The American Cancer Society estimates approximately 225,000 new cases of lung cancer were diagnosed in 2014 in the US, and the 5-year survival rate for stage IV metastatic lung cancer was only 2% in 2013. Lung cancer is traditionally divided into two major histologically defined subtypes: small-cell lung cancer (SCLC, approximately 15%) and non-small cell lung cancer (NSCLC, approximately 85%). NSCLC is then further divided into squamous cell carcinoma (SCC, ~25%), large cell carcinoma (LCC, ~15%), and lung adenocarcinoma (LUAD, ~60%). Therapy for all of these subtypes has historically consisted of cytotoxic chemotherapies such as gemcitabine, cisplatin, and/or pemetrexed, which have had little efficacy. However, since the sequencing of the human genome in 2001, the molecular basis of lung cancer has become more defined. This has led to the identification of distinct genomic drivers (driver oncogenes) and the subsequent development of targeted inhibitors of these hyper-activated and malfunctioning proteins. The subject of my thesis revolves around obtaining a detailed molecular understanding of one of these driver oncogenes, and subsequently uncovering a strategy to improve the survival of this patient population.

The Anaplastic Lymphoma Kinase (ALK) is a human growth factor receptor of the receptor tyrosine kinase (RTK) family. ALK was originally discovered in 1994 as a component of the oncogenic fusion protein nucleophosmin (NPM)-ALK in anaplastic large cell non-Hodgkins lymphoma (ALCL)[1]. Subsequently, ALK genomic aberrations, including activating mutations, amplifications, and gene fusions have been identified in

numerous tumor types, including the discovery of the most common ALK fusion – echinoderm microtubule protein-like 4 (EML4)-ALK fusions in lung cancer[2]. These fusions are constitutively active kinases, hyperactivating multiple tumor promoting effector pathways. The oncogenic potential of EML4-ALK has been validated in numerous studies, including the ability to transform murine fibroblasts and develop lung adenocarcinoma in transgenic mouse models even in the absence of concurrent tumor-suppressor loss[2, 3].

Since the discovery of EML4-ALK positive lung cancer in 2007, numerous ALK inhibitors have entered clinical development for this patient population. Two ALK inhibitors, Crizotinib and Ceritinib, are already approved due to the tremendous initial clinical success in ALK-fusion positive patients[4,5]. The rapid transition from the discovery of EML4-ALK in lung cancer (2007) to the first approved ALK inhibitor (2011) is hailed as an important success story in personalized medicine and clinical development. Despite this initial clinical success of ALK inhibitors, however, our understanding of EML4-ALK biology remains very limited. Moreover, the success of ALK inhibitors in the clinic is limited due to the development of resistance to these targeted ALK therapies, and is the single largest barrier to prolonged patient responses and overall survival. An improved understanding of EML4-ALK biology and uncovering the mechanisms of resistance to ALK inhibitor therapy are critical in order develop novel therapeutic strategies to combat ALK inhibitor resistance and ultimately improve patient survival.

ALK and ALK-fusion signaling (Chapter 1)

Wild-type ALK represents a classic RTK in that it comprises of (1) an extracellular ligand binding domain that receives environmental signals, (2) a transmembrane domain that tethers the receptor to the plasma membrane, and (3) an intracellular kinase domain that dimerizes and activates various mitogenic and survival pathways downstream of the receptor[6]. RTK's (of which there are 58 members) have diverse and often indispensible roles in normal human physiology[7]. However, beyond these general concepts, the details and physiological role of normal ALK signaling remains unclear.

For example, the natural ligand(s) that stimulate and activate ALK remain unknown, and thus ALK is still considered an orphan RTK. More recently, the heparin-binding growth factors pleiotrophin (PTN) and midkine (MK) have been reported as activating ligands for ALK – however contradictory results from other groups have made these findings controversial[8-13].

Moreover, the precise physiological role of ALK in mammals remains unclear; however, evidence thus far seems to point to a role in nervous system development and/or behavior. ALK expression patterns in various animals, including humans, point to highest levels of ALK expression during nervous system development[14,15,16]. However, levels of ALK mRNA and protein appear to be only transiently expressed in normal adult nerve tissue[15]. Similarly, $ALK^{-/-}$ knockout mice are completely viable with no major health problems with the exception of mild behavioral phenotypes, such as lack of response to ethanol exposure, further suggesting ALK's potential role in CNS development[17,18,19]. However, normal ALK function in adult humans remains unclear. It

should be noted that ALK inhibitors have been well tolerated in the clinic, with no consistent adverse events appearing in patients treated with specific ALK inhibitors[20,21].

Much like our knowledge of ALKs normal physiological role, our understanding of ALK signaling remains disjointed and mainly informed by our knowledge of RTK signaling more generally. Because ALK is mainly investigated in the context of its aberrant state in cancer, ALK activation and signaling has thus been studied under the scope of a diverse set of mechanisms (WT activation, overexpression, activating mutations, and fusions with various partners) and in a diverse set of cellular contexts (mouse fibroblasts, non-transformed human cells, and cancer cells of epithelial, myeloid, or mesenchymal lineages), thus it is difficult to conclude what pathways are universally controlled by ALK signaling nor which, if any, are responsible for the molecular pathology of ALK-positive tumors. Nonetheless, ALK activity has been described to activate numerous signaling pathways implicated in tumor growth and survival, including canonical RTK-signaling components such RAS-MAPK, PI3K-AKT, JAK-STAT, mTOR, PLCy, but also sonic hedgehog (SHH), NfKB, CRKL, RAP1, RAC1/CDC42, SHP1/SHP2, SRC, and many other additional potential effector pathways uncovered from numerous proteomics studies[22-32].

Importantly, the signaling adaptor proteins that the kinase domain of ALK is able to recruit upon activation is unique among RTK's, and thus may contribute to a unique and disruptive signaling output when expressed in cell types that have not normally evolved to tolerate ALK expression or activation (such as the cell of origin in ALK-fusion positive tumors, including lung epithelial cells). For example. ALK is the only RTK that binds and interacts with both the adaptors FRS2/FRS3 (typically engaged only

by FGFR family members) and IRS1/IRS2 (typically engaged only by IGF1R), along with its interaction with more universal RTK-adaptor proteins such as GRB2 and SHC[22-26].

In cancer, NPM-ALK in ALCL and EML4-ALK fusions in NSCLC have been the most extensively studied due the prevalence of the patient population and also the availability of patient-derived cell lines. In NSCLC, these studies have led to establishment of MAPK, PI3K-AKT, and JAK-STAT as the primary pathways operating downstream of ALK that lead to the tumor phenotype (**Figure 1**) [22,23,33]. Indeed, hyper-activation of these pathways are more generally considered major players in human cancer, as evidenced by the frequency of activating mutations in these pathways found across human cancers.

Thus, the first major question of my thesis work (Chapter 2) was to establish whether there was an ALK (specifically EML4-ALK in NSCLC) downstream effector pathway that was most critical to EML4-ALK+ tumor cell survival. Because EML4-ALK, like other aberrant RTKs, can activate a diverse set of downstream pathways, it is not traditionally thought that any single one may be responsible for oncogene dependence. Indeed, I found that EML4-ALK positive tumor cells were highly dependent on the RAS-MAPK effector axis, but not other ALK effectors such as PI3K-AKT or JAK-STAT. Moreover, rescue of MAPK signaling at either the level of RAS, RAF, or MEK was sufficient to promote resistance to ALK inhibition, thus establishing MAPK signaling as both necessary and sufficient for growth and survival in EML4-ALK positive tumor cells. The goal of this endeavor was to provide insight into the molecular pathogenesis of the EML4-ALK fusion oncogene and to ultimately uncover a rational

target for a combination therapy approach in the EML4-ALK+ NSCLC patient population.

The biology of ALK fusions (Chapter 2)

Most ALK-fusion positive patients are EML4-ALK positive NSCLC patients (~4-8% of lung adenocarcinomas, or ~40,000 new diagnoses per year worldwide). However, ALK translocations appear in a variety of human tumor types, spanning both solid tumors of epithelial and mesenchymal origin, and also hematopoietic malignancies[33]. Since the discovery of NPM-ALK in ALCL in 1994, the list of ALK translocations and other alterations found in human cancer has expanded dramatically, and continues to expand even today (**Figure 2**)[34]. So far, there have been >30 different ALK fusion partners discovered in >10 tumor types, including lung, breast, renal, colon, and various myeloid malignancies[33,34,]. To add to this complexity, even within the same ALK fusion partner, there are several break-points which thus lead to different variants of the fusion product. This is illustrated most commonly by the EML4-ALK translocations found in NSCLC, where multiple EML4 exon breakpoints (variants 1-8) fuse to exon 20 of ALK[35].

The focus of my studies, the EML4-ALK fusion, occurs in in approximately 4-8% of NSCLC patients, and is by far (>90%) the most common ALK fusion in NSCLC[35]. However, several other ALK fusion partners have been identified in NSCLC at lower frequency, including KIF5B, KLC1, and STRN[36-39]. The NPM-ALK fusion occurs in up to 80% of ALCL cases, however ALK has also, in rare cases, been found to fuse with ring finger protein 213 (RNF213), ATIC, TFG, MSN, TPM3, TPM4, MYH9, and CLTC in ALCL[40-47]. The third highest ALK-fusion patient population is present in

inflammatory myofiborblastic tumors (IMT), where they are found in ~50% of cases and includes a diverse set of fusion partners, including TPM3, TPM4, CLTC, CARS, ATIC, SEC31A, and RANBP2[48-51]. Of note, ALK-fusions have been uncovered in other solid tumors. A study in renal cell carcinoma (RCC) found 2/534 patients with an ALK-translocation[52,53]. Likewise, EML4-ALK fusions have been reported in colon cancer and breast cancer, and fibronectin 1 (FN1)-ALK fusion has been reported in ovarian cancer[54,55].

All fusion partners (and variants thereof) are thought to promote oncogenic signaling of ALK (and indeed other RTK-fusions such as ROS1, RET, and TRKA) via two main features – causing (1) constitutive kinase domain activation and (2) high expression. For example, the fused protein components of EML4 in EML4-ALK fusions always include the BASIC domain of EML4, which is normally required for dimerization of WT EML4. Indeed, functional studies have shown that deletion of the BASIC domain from the EML4 portion of EML4-ALK abrogates the ability of EML4-ALK to dimerize, activate phosphorylation, or transform murine fibroblasts[2]. Secondly, all RTK-fusion partners, including EML4, appear on the N-terminus of the fusion product. Thus, the requisite genomic translocation places the fusion kinase under the control of the fusion partner's regulatory elements– which is often a housekeeping or otherwise highly expressed gene in the cell of origin (such as EML4). This allows for substantially high levels of expression of an already aberrantly activated ALK kinase in cells that normally do not express even the wild-type form.

However, there is a remaining conundrum in our understanding of ALK-fusion and RTK-fusion biology generally, and is the focus of my investigation in Chapter 3.

Namely, how do ALK-fusions gain access to their known effectors, principally MAPK and PI3K-AKT, that are thought to require a lipid-interface to signal? EML4 is invariably fused to exon 20 of ALK across all variants, which omits the trans-membrane (TM) domain of ALK **(Figure 3)**[35]. Moreover, EML4 has no canonical membrane-binding domain, and preliminary studies overexpressing EML4-ALK in murine fibroblasts found no clear plasma membrane association[3]. Thus, there is no obvious explanation as to how EML4-ALK gains access to its known effectors that are traditionally thought to require membrane localization. Moreover, although EML4-ALK has been shown to activate and signal through MAPK (via measurement of ERK phosphorylation), a direct link between EML4-ALK and any of the RAS isoforms has not been demonstrated.

Ras proteins are GTPases that act as binary "on/off" molecular switches and serve as central signaling nodes that regulate numerous cell processes including proliferation, survival, differentiation, mobility, and metabolism **(Figure 4)**. There are three main isoforms of RAS- H, N, and K-Ras. Interestingly, the sole difference between these three isoforms is the C-terminal tail, which regulates post-translational processing and membrane targeting[56]. The importance of RAS and its downstream effectors pathways in tumor biology is highlighted by the frequency of activating mutations/aberrations that occur in either RAS itself, its upstream activators (such as EGFR mutants or ALK fusions) or downstream effectors (RAF-MEK mutations and PI3K-AKT mutations). For example, in lung adenocarcinoma, activating genomic alterations in the RAS pathway components occur in ~70% of patients[57].

Understanding the potentially unique interaction EML4-ALK fusions have with RAS signaling may provide insight into why EML4-ALK fusions are such highly

addictive oncogenes in tumor cells. As such, I hypothesized that the unique biology and localization of EML4-ALK in tumor cells may create therapeutically exploitable dependencies unique to ALK and other RTK-fusions. Indeed, I found that EML4-ALK engages all three major RAS isoforms (H, N, K), an activity that appears to be mediated by the hydrophobic HELP domain of the fusion partner, EML4. Moreover, I found that EML4-ALK resides on intracellular membrane compartments (or aggregates) – a phenomenon which appears to also require the HELP domain. All in all, these findings suggest that there is a third unique and underappreciated component of RTK-fusions – localization, which may explain their unique biology, access to RAS, and ultimately oncogene dependence.

The clinical efficacy of and resistance to ALK inhibitors (Chapter 3)

Approximately 4-8% of NSCLC cases are ALK-fusion positive, the vast majority of which are EML4-ALK variant 1 **(Figure 5a)**[35]. ALK-positive patients are often younger never-smokers, generally harbor minimal mutational burden, and do not reliably co-occur with loss of any one tumor suppressor, including p53[58]. In general, the presence of an ALK-fusion signifies a more aggressive tumor, and is a poor prognostic marker for response to traditional cytotoxic chemotherapy. The first ALK inhibitor tested in ALK-fusion positive patients was the MET/ALK/ROS1 inhibitor crizotinib. The initial Phase I and II (PROFILE 1001 and 1005) studies in ALK-fusion positive patients showed a 60.8% overall response rate (ORR) and a median progression-free survival (mPFS) of 9.7 months, with very mild optic and gastrointestinal side-effects. By comparison, first-line cytotoxic chemotherapy has a ~30% ORR and ~6 month mPFS, and second-line has a

~10% ORR and ~2.5 month mPFS – and with significant toxicity. These trials were the basis for accelerated approval of crizotinib in 2011.

Since then, two confirmatory phase III studies have been completed (PROFILE 1007 and 1014). In PROFILE 1007, crizotinib was compared with chemotherapy in the second-line setting in ALK-fusion positive patients[4]. Crizotinib ORR more than tripled that of chemotherapy (65% vs. 20%), and more than doubled the mPFS of chemotherapy (7.7 vs. 3.0 months). PROFILE 1014 **(Figure 5b)** compared first-line Crizotinib vs. first-line chemotherapy[5]. Likewise, Crizotinib was superior to chemotherapy both in terms of ORR (74% vs. 45%) and mPFS (10.9 vs. 7.0 months).

Despite the fact that most patients will respond to Crizotinib, resistance invariably develops usually within 2 years of treatment. The reasons and mechanisms of resistance to Crizotinib (and other ALK TKI's) has been an active area of clinical and basic science study. Similar to the emergence of "on-target" EGFR-T790M mutations in erlotinib resistance, "on-target" mutations in the ALK-kinase domain that abrogate drug binding occur in approximately 30% of Crizotinib-resistant patient tumors[59]. The most frequently observed kinase mutations are L1196M (which is the analogous gatekeeper mutation to T790M in EGFR) and G1269A, which retain the kinase activity of ALK but prevent binding of Crizotinib in the ATP-pocket[58]. Moreover, amplification of the ALK-fusion gene locus occurs in approximately ~10% of crizotinib-resistant patients, and likely overcomes the ability of patient plasma concentrations of crizotinib to inhibit the kinase at sufficient levels[60]. Indeed, *in vitro* Crizotinib acquired resistance models (generated from H3122 cells, a EML4-ALKv1 positive NSCLC cell line) with ALK amplification

display moderate levels of resistance as compared to models with the L1196M gatekeeper mutation[59].

In the remaining 60% of patients with crizotinib-resistance, the specific mechanisms of resistance remain unclear. However, the proposed explanation to date has been activation of various tyrosine kinases that are able to rescue the downstream signaling effectors of EML4-ALK in the presence of ALK inhibition[58]. These mechanisms of resistance have been termed "bypass" tracks. The first study to implicate bypass signaling identified activated EGFR-signaling as the suspected culprit[60]. Isogenic H3122 cell lines that developed resistance to Crizotinib after continuous exposure to drug showed higher levels of phosphorylated EGFR, and combination treatment with EGFR inhibitors re-sensitized cells to ALK inhibitor treatment. However, of the available patient tissue checked in this study, there was only slight evidence of elevated EGFR phosphorylation by IHC assay in post vs. pre –crizotinib treatment biopsies. Of note, cKIT amplification was identified in 2/18 patients in this cohort, and KIT overexpression caused resistance to crizotinib in H3122 cells *in vitro,* providing evidence for another potential bypass track. In a similar study, up-regulation of insulin-like growth factor 1 receptor (IGF1R) was unveiled as a bypass resistance mechanism to ALK TKI therapy[61]. Similar to the EGFR study, Crizotinib-resistant cell lines that presented upregulation of phosphorylated IGF1R were re-sensitized to ALK inhibitors upon combination treatment with an IGF1R inhibitor. Moreover, a recent study unveiled SRC activation in patient-derived cell-lines that were recovered from patients after Crizotinib resistance[62]. In this study, the SRC inhibitor dasatinib was able to resensitize SRC-activated cells to ceritinib.

Heat shock protein 90 (HSP90) inhibitors have also gained attention as a potential therapeutic option in patients with ALK-positive, ALK TKI resistant disease. This has emerged from the discovery of ALK-fusions as HSP90 clients. Disruption of the HSP90-EML4-ALK complex via HSP90 inhibition leads to rapid degradation of EML4-ALK[63]. Moreover, many of the bypass track components found to play a role in TKI resistance, such as EGFR, are also obligate HSP90 client proteins[63,64]. Indeed, *in vitro* studies have shown that ALK-positive cell lines are indeed highly sensitive to HSP90 inhibition, both in the context of an ALK-TKI naïve and ALK-TKI resistant state[65]. However, HSP90 inhibitors have had significant toxicity to date, even as monotherapy, which limits their therapeutic index and potential for combination therapy[64,66]. For example, in a recent phase III trial (GALAXY-1) investigating the HSP90 inhibitor ganetespib in NSCLC, 41% of patients obtained grade 3-4 neutropenia. Moreover, the response rates seen in the ALK-positive population have been modest. Thus, identifying novel therapeutic approaches and targets outside of ALK itself are the next major step in improving patient outcomes.

Despite the advances made in understanding resistance to ALK TKI's, our knowledge remains limited. To date, there has been no clinical validation that any of the bypass tracks found *in vitro* are actually mechanisms of resistance to ALK TKI's in patients. Thus, there are no clinical therapeutic strategies to combat off-target ALK TKI resistance to date. Chapter 4 of my thesis is focused on the question of resistance – specifically aimed at identifying novel mechanisms of resistance to ALK TKI therapy. To this end, I have discovered that MAPK re-activation is a hallmark of ALK-TKI resistance. In patients, I have uncovered that this can occur via $KRAS^{WT}$ amplification

(~20% of crizotonib resistant patients), or DUSP6 down-regulation, which I found to be generally in ALK TKI resistant patient tumor samples.

Combating ALK TKI Resistance (Chapter 4)

Next-generation ALK inhibitors, such as ceritinib (approved in 2014), alectinib, entrectinib, AP26113, ASP3026, X-396, and PF0646 are currently under clinical-development and have demonstrated impressive clinical efficacy in patients that have developed resistance to crizotinib[20,21].

Ceritinib has a 20-fold lower enzymatic IC50 for ALK compared to crizotinib. Aside from being a more potent and selective inhibitor of ALK, ceritinib is also efficacious against the crizotinib-resistant ALK mutations L1196M, G1269A, I1171T, and S1206Y[67]. A phase I clinical trial of ceritinib in ALK-positive NSCLC showed a 58% ORR and a 7.0 month mPFS[20]. Among patients who had previously received crizotinib, the ORR was 56% (45/80). Interestingly, this study concluded that ceritinib had efficacy in patients that did *not* have any on-target crizotinib resistance mutations as well as those that did. The reason for ceritinib's efficacy in patients that developed crizotinib resistance, but did not have an on-target mutation, remain unclear. It is speculated that this may be due to either 1). ceritinibs off-target inhibition of IGF1R (a potential bypass track), 2). simply more potent and complete inhibition of ALK due to ceritinibs potency over crizotinib, or 3). crizotinib wash-out prior to initiation of ceritinib therapy allowed ALK to re-emerge as the dominant driver. Nonetheless, resistance to ceritinib therapy invariably develops, similar to crizotinib. So far, 4/10 patients biopsied

post-ceritinib developed a secondary G1202R or F1174C/V mutation, which was confirmed to abrogate ceritinib binding to ALK *in vitro*[67].

Alectenib is an even more potent and selective third-generation ALK inhibitor and also has significant CNS penetration due to its lack of interaction with the ABCB1 transporter (which crizotinib and ceritinib both do)[68]. Like ceritinib, alectinib can inhibit the L1196M, G1269A, C1156Y, and F1174L crizotinib-resistance secondary mutations[69]. In a Phase I/II study, alectinib showed an impressive 93.5% ORR and an mPFS of >27 months in ALK-TKI naïve patients, which led to alectinibs approval in Japan in 2014[21]. In the second-line, crizotinib-resistant population, a phase I/II clinical trial in the US showed a 54% ORR[70]. Although these trials have shown some of the best responses seen to date in advanced NSCLC, resistance to alectinib invariably develops as well. So far, the only known resistance mutations to alectinib are ALK V1180L, I1171T (which can be hit by ceritinib), and G1202R (which is refractory to crizotinib, ceritinib, and alectinib)[58,71].

Thus, combating ALK TKI resistance to date has focused on two strategies. 1) treating patients with ALK inhibitors that are able to bind to and inhibit secondary mutations that confer resistance to the previous ALK inhibitor, or 2). moving more potent ALK inhibitors to the front-line setting, such as alectenib (~27 month front-line mPFS vs. crizotinibs ~10 month front-line mPFS). However, on-target secondary mutations occur in only ~30% of ALK TKI resistant patients, and even the potent available ALK inhibitors develop off-target mechanisms of resistance eventually.

One proposed strategy to combat eventual ALK TKI resistance is to employ combination therapies targeted against both ALK and any one of the “bypass pathways”

mediating ALK TKI resistance. This however is a challenge exacerbated by the fact that it is currently impossible to predict which bypass track will emerge after resistance develops in a particular patient. There is clinical evidence to suggest that targeting resistance mechanisms - after resistance has already developed - is not efficacious. In BRAF-mutant melanoma, for example, resistance to BRAF inhibition often occurs through re-activation of the MAPK pathway, however targeting MEK after resistance has already developed in patients has no added benefit to cytotoxic chemotherapy[73]. However, targeting BRAF+MEK in the upfront setting shows significantly higher response rates and duration of response compared to BRAF inhibition alone[72]. Furthermore, recent evidence suggests that there is significant heterogeneity in resistance mechanisms amongst different tumor population within the same tumor, further delineating the need to improve upfront therapy and pre-empt resistance[74]. However, finding a common, therapeutically exploitable, thread in mechanisms of resistance has generally been challenging in RTK-oncogene driven cancer.

It is interesting to note, however, that all of the identified and proposed mechanisms of resistance to ALK TKI's so far have converged on re-activation of the RAS-MAPK pathway, perhaps delineating its importance for maintaining growth and survival in ALK-fusion transformed cells. On-target mutations of ALK re-activate ALK signaling and therefore MAPK signaling, and EGFR, IGF1R, KIT, MET, and SRC activation are all upstream activators of MAPK as well. Moreover, a KRAS-G12V mutation has been found in a crizotinib-resistant tumor sample, and a MAP2K1 K57N-activating mutation has been reported in a ceritinib-resistant tumor sample.

Chapter 5 of my thesis is thus aimed at testing a novel combination strategy of ALK + submaximal MEK inhibition that was informed by the work presented in Chapters 2-4. Importantly, this was tested in the upfront treatment-naïve setting. I hypothesized this would enhance initial response and preemptively target all known mechanisms of resistance (including my own discovery of $KRAS^{WT}$ amplification and DUSP6 downregulation in Chapter 4) and thus also prevent the onset of resistance in EML4-ALK positive tumors.

Figure 1

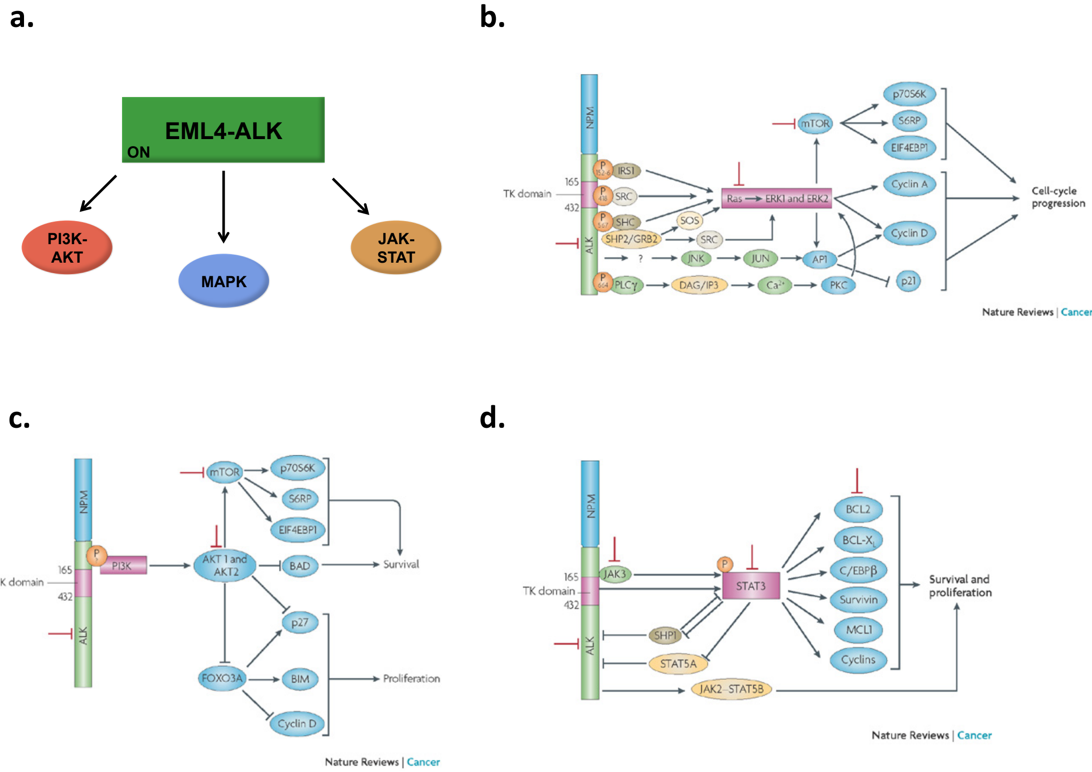


Figure 1. ALK-fusion signaling

a) The three main known effector pathways of EML4-ALK in NSCLC. b-d) detailed schematics of each individual pathway from A, as known in NPM-ALK in ALCL. In ALCL, NPM-ALK signals to many known signaling components via MAPK, JAK-STAT, or the PI3K signaling pathways. This same amount of detail is not confirmed in EML4-ALK in NSCLC. Panels b,c, and d are taken from Chiarle *et al.* The anaplastic lymphoma kinase in the pathogenesis of cancer. *Nat Rev Cancer 2008.*

Figure 2

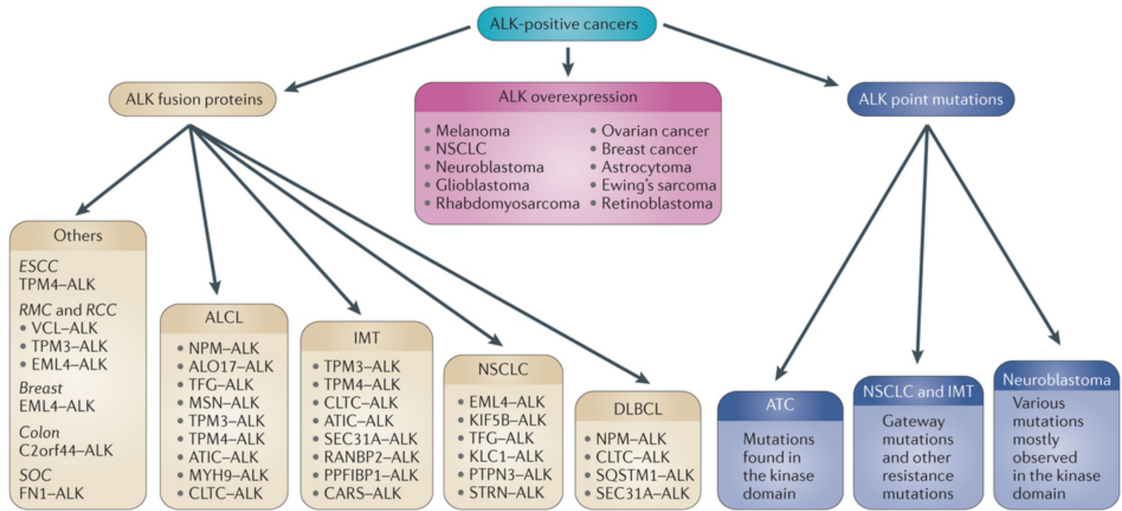
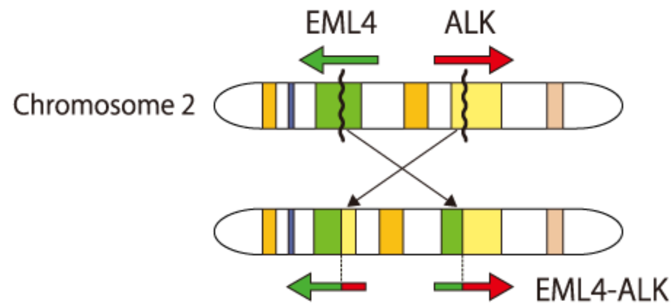


Figure 2. ALK alterations in human cancer
Summary of ALK alterations (fusions, point mutations, or overexpression) in human cancer. ALK fusion proteins (beige) consist of the ALK kinase domain fused to the amino-terminal side of various fusion partners. The largest patient population is NSCLC, where approximately 4-8% of patients harbor an ALK-fusion, most commonly EML4-ALK variant 1. The list of identified ALK-fusions in various cancers continues to expand to this day. ALK overexpression (pink) is observed in many human tumor types, but the functional consequences have not been explored. ALK activating mutations are found primarily in neuroblastoma, NSCLC, and anaplastic thyroid cancer (ATC). Figure from Hallberg *et al*. Mechanistic insight into ALK receptor tyrosine kinase in human cancer biology. *Nat Rev Cancer* (2013).

Figure 3

a.



b.

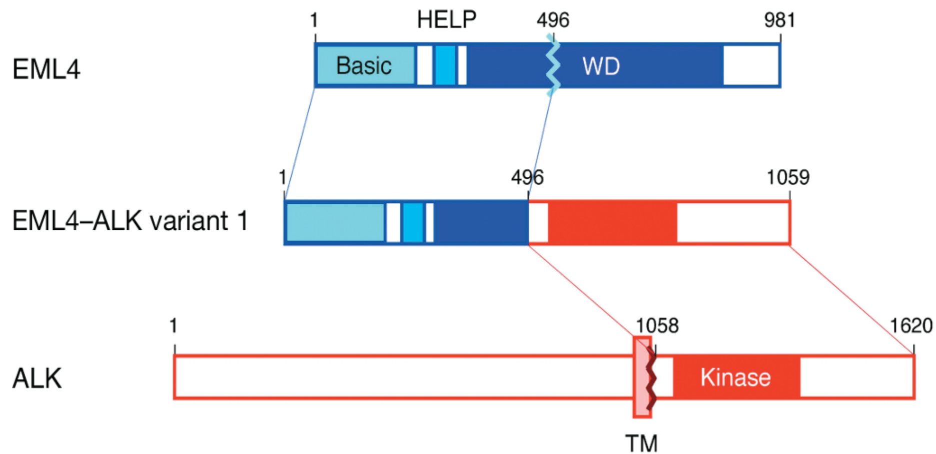


Figure 3. The EML4-ALK (variant 1) fusion in NSCLC

a). The genetic event that causes EML4-ALK fusions. An intrachromosomal inversion on chromosome 2 results in a fusion of exon 13 of EML4 (5') to exon 20 of ALK (3'), resulting in an EML4-ALK (E13:A20) gene fusion. b). The product of the gene fusion. The EML4-ALK variant 1 fusion consists of exons 20-26 of ALK which includes the kinase domain, but omits the ALK exracellular and transmembrane (TM) domains. The fusion partner, EML4, retains the Basic, HELP, and a portion of the WD domains in the EML4-ALK variant fusion product. The Basic domain is required to dimerization and constitutive activation of the fused ALK kinase.

Figure 4

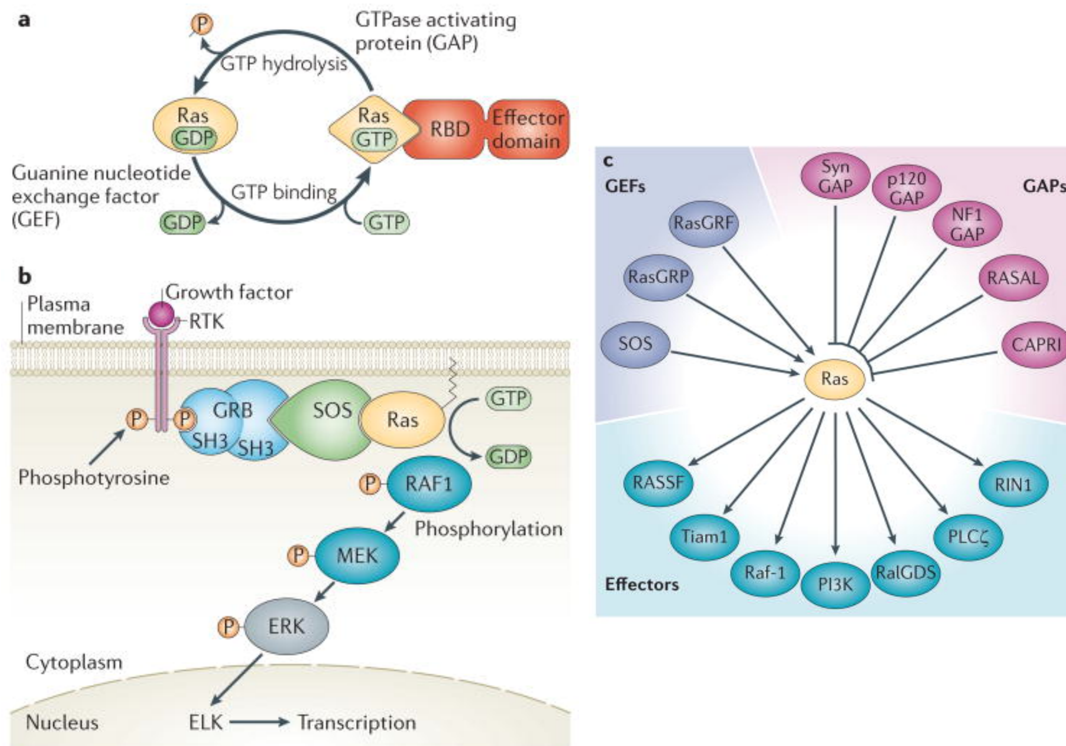


Figure 4. RAS signaling
a) The "on/off" or GTP/GDP cycle of Ras. GDP-bound (off) Ras is activated by a GEF, which catalyzes the release of GDP into the cytosol and thus permits binding of GTP (on). Proteins with Ras-binding domains (RBDs) can then bind to RAS-GTP and initiate downstream signaling. Finally, GAPs increase the intrinsic GTPase activity of Ras and catalyze the GTP-GDP conversion reaction, reverting Ras to the "off", GDP-bound state. b) the Ras-Raf-Mek-Erk pathway. The adaptor GRB2 binds to activated/phosphorylated RTKs and then brings SOS (a RasGEF) to the membrane, where Ras is localized via its C-terminal tail. SOS enhances GTP-loading of Ras, and therefore activates Ras-Raf-Mek-Erk signaling. c) other Ras effector pathways. Ras can signal to many other downstream pathways outside of MAPK, including PI3K. Similarly, there are numerous Ras GAPs and GEFs. Figure taken from Ahearn, I.M. *et al*. Regulating the regulator: post-translational modification of Ras. *Nat Rev Mol Cell Biol* (2011).

Figure 5

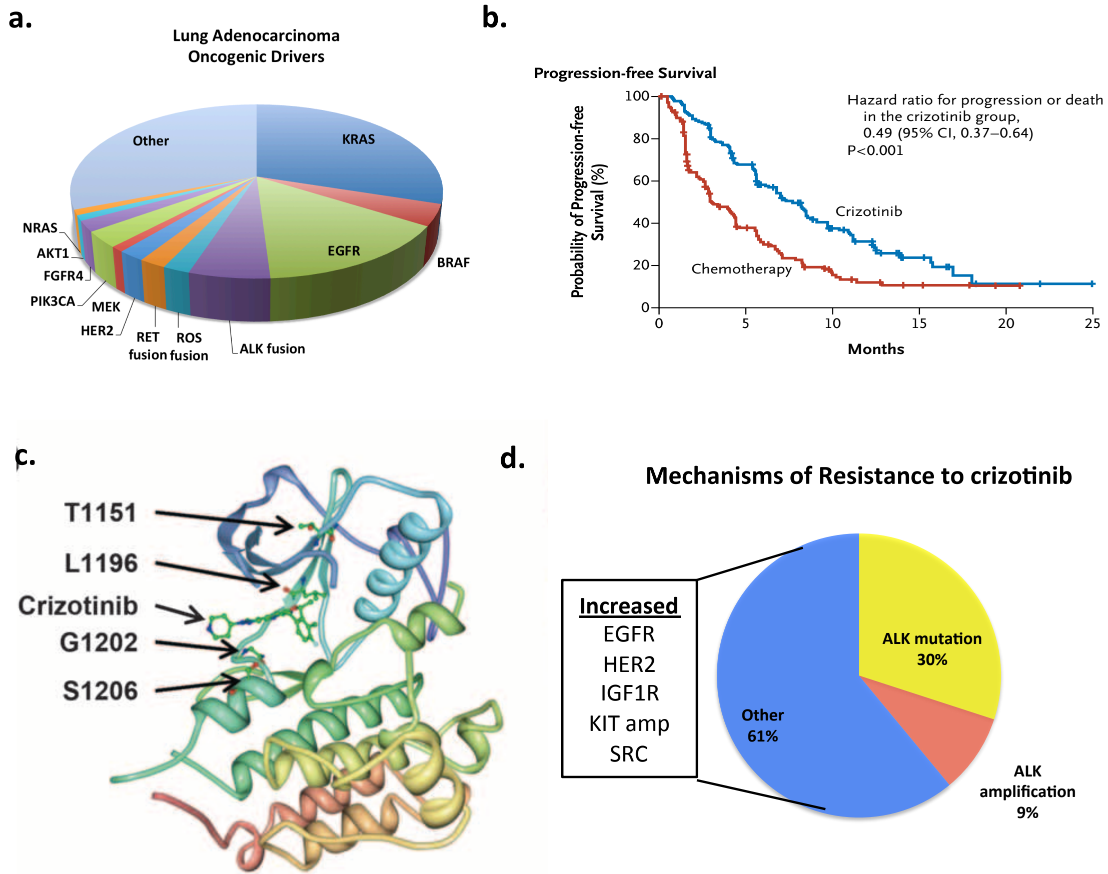


Figure 5. Clinical efficacy and resistance to crizotinib
a) Pie chart depicting the population of patients with known, targetable, oncogenic drivers in lung adenocarcinoma. ALK-fusions appear in approximately 4-8% of lung adenocarcinoma patients, which represents ~40,000 patients/yr worldwide. b). Progression-free survival benefit of crizotinib over conventional chemotherapy in first-line ALK-positive patients. Figure taken from Shaw, A.T. *et al*. (NEJM 2013, ref#5) c). The most common ALK on-target secondary mutations found after crizotinib resistance. These mutations abrogate binding of the drug, as shown, and cause resistance to crizotinib. Next-generation ALK inhibitors such as ceritinib and alectinib can overcome these mutations. Figure from Katayama *et al*. (Clin Cancer Res 2015, ref#58) d). Frequency of resistance mechanisms to crizotinib. Approximately 30% of patients develop secondary mutations as shown in c). ~9% amplify the EML4-ALK locus. The remaining 60% remain unclear, but “bypass signaling” by EGFR, IGF1R, KIT, SRC, or HER2 have all been implicated.

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Chapter 1

Characterizing the Effector Pathways of EML4-ALK Fusions

Chapter 1: The effector pathways of the EML4-ALK oncoprotein

In most oncogene-driven cancers and ALK-fusion positive lung cancers specifically, most efforts to date aimed at combating ALK inhibitor resistance have focused on characterizing and treating acquired resistance after it has already emerged in the clinic[1]. One alternative emerging strategy to both enhance initial response and combat the development of acquired resistance to targeted therapy is to define and deploy rational upfront co-therapies that target not only primary oncoprotein (such as EML4-ALK) but also additionally a critical effector of the primary oncoprotein. This strategy has been successful in *BRAF*^{V600E} mutant melanoma patients, where upfront inhibition of the primary driver BRAF^{V600E} plus its established primary effectors, MEK1/2, results in substantial clinical activity that is superior to either RAF or MEK inhibitor monotherapy[2,3,4]. In contrast, combined RAF and MEK inhibitor treatment was largely ineffective in melanoma patients with acquired BRAF inhibitor resistance[5]. Together, these clinical data show the importance of upfront rational combination therapy to minimize tumor cell survival and evolution during initial treatment, thereby enhancing initial response and forestalling the emergence of resistance in patients.

In contrast to BRAF^{V600E} melanomas that rely predominantly on MAPK pathway signaling, the optimal upfront co-targeting strategy has been less clear in tumors harboring oncogenes that engage multiple effector pathways, such as receptor tyrosine kinases (RTKs). Mutant *EGFR* and *ALK*, *ROS1*, or *RET* gene rearrangements are examples of prominent oncogenic RTKs in lung adenocarcinoma. Of these, EGFR mutations have been the most extensively studied, and there has been no consistent single pathway dependency uncovered.

A rational co-targeting strategy requires an understanding of the signaling events that are most critical for survival in tumor cells with a particular oncogenic RTK, enabling a context-specific therapeutic strategy to minimize tumor cell survival. To address this knowledge gap, we explored the molecular basis of EML4-ALK oncogene dependence in lung adenocarcinoma to both improve the fundamental understanding of ALK oncogene function and identify a rational upfront polytherapy strategy to enhance response to ALK inhibition in patients.

Results

EML4-ALK can signal via the engagement of PI3K-AKT, MAPK, and JAK-STAT signaling[6]. However, which effector pathway, if any, is most critical for EML4-ALK driven cell survival has not been defined. Therefore, we first investigated downstream pathway dependencies in EML4-ALK lung adenocarcinoma cells, focusing on the most common *ALK* fusion variant in lung adenocarcinoma (*EML4-ALK* E13:A20, variant 1). As expected, the ALK inhibitors crizotinib or ceritinib significantly decreased cell growth and levels of phosphorylated ALK, ERK, AKT, and STAT3 in two distinct patient-derived EML4-ALK (E13:A20) cell lines, H3122 and the recently-derived STE-1[7] (**Fig. 1**). To define the functional importance of each of the three major effector pathways (PI3K-AKT, MAPK, JAK-STAT) in EML4-ALK lung adenocarcinoma cells, we assessed the impact of selective pharmacologic inhibition of each of them on growth and survival. Strikingly, we found that inhibition of MAPK (via MEK inhibition), but not of PI3K-AKT or JAK-STAT, signaling was sufficient to suppress cell growth to a degree that was similar to ALK inhibitor treatment (**Fig. 2a-b**). Conversely, constitutive genetic activation of MAPK signaling at the level of either the GTPase RAS

(*KRAS*$^{G12V/C/D}$), RAF (*BRAF*^{V600E}), or MEK (*MEK*^{DD})[8] was sufficient to rescue EML4-ALK lung adenocarcinoma cells from ALK inhibitor treatment, whereas genetic activation of AKT signaling (via myristoylated-AKT) was not (**Fig. 3**). We noted that KRAS^{G12V} expressing cells rescued MAPK signaling, but not AKT signaling, in the presence of ALK inhibition. Genetic activation of STAT3 partially rescued EML4-ALK lung adenocarcinoma cells from ALK inhibitor treatment, consistent with prior work[9], although the effect was modest when compared to genetic activation of the MAPK pathway in both H3122 and STE-1 cells (**Fig. 4**). Together, these data reveal a specific and primary requirement for MAPK signaling in EML4-ALK lung adenocarcinoma cells, and establish that MAPK signaling specifically is both necessary and sufficient to rescue EML4-ALK lung adenocarcinoma cells from ALK oncogene inhibition.

Based on these findings, we explored whether this MAPK dependence and MEK inhibitor sensitivity found in EML4-ALK lung adenocarcinoma cells was present more generally in oncogene-driven lung cancer. Most lung adenocarinomas harbor a genetic lesion capable of hyperactivating MAPK signaling, including oncogenic *KRAS*, *BRAF*, *EGFR*, *MEK*, *ERBB2*, *MET*, *ALK*, *RET*, and *ROS1* variants, among other events on the MAPK pathway[10]. However, MEK inhibitors have had limited clinical efficacy generally in lung adenocarcinoma patients to date, indicating the need to better define subsets of lung adenocarcinoma that are most dependent on MEK activity[11]. By examining a panel of human lung adenocarcinoma cell lines representing many clinically-relevant genetic subsets of lung adenocarcinoma and that exhibit MAPK pathway activation[10], we found that the EML4-ALK models were amongst the most sensitive to MEK inhibition as measured by both cell viability and apoptosis assays (**Fig. 5**). This MEK inhibitor

sensitivity in the EML4-ALK models was similar to that observed in lung adenocarcinoma cells with oncogenic KRAS or BRAF but not those with oncogenic EGFR, which had little response to trametinib monotherapy (**Fig 5**). These findings suggest that EML4-ALK lung adenocarcinoma cells may depend upon MAPK signaling, similar to cells with oncogenic variants in MAPK pathway genes (i.e. KRAS, BRAF). Furthermore, the data reveal the specificity of MAPK dependence and MEK inhibitor sensitivity in lung adenocarcinoma cells with oncogenic *ALK* but not those with other oncogenic RTKs, most notably including mutant *EGFR*.

Discussion

We have found that MAPK signaling is both necessary and sufficient for growth and survival in EML4-ALK variant 1 (E13:A20) positive tumor cells. Although ultimately limited by the scarcity of available EML4-ALK positive cells lines, our studies suggest that EML4-ALK oncogene dependence may be a function of MAPK hyper-activation, similar to BRAF-mutant tumor cells. In this setting, co-targeting ALK and MAPK may be a logical and effective strategy in patient tumors.

Unexpectedly, this appears to be a phenomenon specific to ALK-fusion positive tumor cells, as we do not see this same MAPK dependency in EGFR-mutant tumor cells. Indeed, prior studies have shown that EGFR mutant tumors, although activating MAPK, are not dependent on MAPK signaling. Conversely, it has been suggested that the ERBB receptors (EGFR, HER2) perhaps rely on the PI3K/AKT axis, and as such EGFR/HER2 targeted therapy is currently being investigated in combination with PI3K inhibitors in lung and breast cancer, among others.

The lack of significant response to PI3K/AKT inhibitors, and the lack of growth rescue with Myr-AKT suggest that this axis is indeed relatively dispensable in ALK-fusion positive tumor cells. A likely explanation for this is that MAPK signaling has been shown to regulate pathways traditionally found downstream of PI3K-AKT, such as mTOR signaling and regulation of the apoptotic machinery. Thus, it is possible that the abnormally high MAPK flux obtained from ALK activation is able to usurp these effectors from the PI3K-axis, ultimately rendering it dispensable.

Interestingly, we found that introduction of KRAS-G12V into H3122 and STE-1 cells did not appear to rescue AKT signaling (as measured by phosphorylation of S473), suggesting that RAS may indeed be uncoupled from PI3K in this cellular context. Moreover, in kinetic experiments I have performed (not shown), AKT phosphorylation is rescued ~4h after ALK inhibitor treatment, whereas MAPK signaling remains inhibited for >24h.

Prior work has shown that genetic hyperactivation of STAT3 signaling can partially rescue H3122 cells from ALK inhibition, via stabilization of the anti-apoptotic protein survivin[9]. Indeed, we have confirmed those results and expanded them to STE-1 cells. In both cases, STAT3 hyperactivation promotes a subtle resistance phenotype, however, it is significantly less robust than hyperactivation of MAPK components. Moreover, treatment with JAK inhibitors has no effect on H3122 or STE-1 viability. Feedback activation of STAT3 signaling has also been implicated in TKI resistance generally. In our cell line models (H3122 and STE1), we observe very high levels of STAT3 re-activation at ~24h post ALK TKI treatment (data not shown), however treatment with a JAK-inhibitor does not have any additional effect on cell viability -

suggesting that feedback re-activation of STAT3 signaling is likely not responsible for maintaining survival in our models. Thus, we conclude that STAT3 signaling is not necessary for ALK-fusion positive cell survival, but may be (slightly) sufficient at high levels. Because of this intermediate role (as compared to MAPK and PI3K) in ALK cell survival, we have explored the JAK-STAT axis as a potential co-target in later chapters.

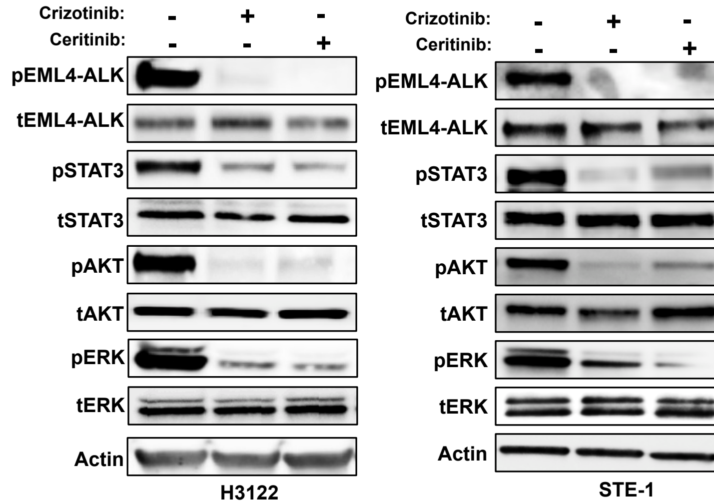
The three pathways investigated in my work (MAPK, PI3K/AKT, and JAK-STAT) are far away from being the only pathways activated or influenced by hyper-activated ALK signaling. Outside of likely activating canonical RTK effectors such as PLCy/PKC, SRC, and Rho/Rac signaling, ALK has been shown to control other biological pathways relevant to cancer, including NFKB, sonic hedgehog, CRKL, and others[12]. I have myself performed proteomic and transcriptome analysis on H3122 cells (+/- ALK inhibitor treatment) and thus potentially uncovered several additional novel effector components downstream of EML4-ALK. This data is included in the appendix. Despite the range of potential ALK effector pathways, the MAPK, PI3K, and JAK-STAT pathways are the three that have clinically approved inhibitors that have been safely administered to patients already – both alone and in combination. Thus, my decision to focus on these three was in light of the hope that any insight into the role of these three pathways would allow for immediate clinical investigation.

Interestingly, ALK and EGFR are both hyperactivated RTK's, and thus would have been predicted to have similar effector dependencies. This is thus our first clue that ALK-fusions, (and perhaps RTK-fusions more generally) are biologically distinct and behave differently than other, more classic, hyper-activated RTK's in human cancer. The

potential molecular basis (and explanation) for ALK-fusions' MAPK dependency is explored in the next chapter.

Figure 1

a.



b.

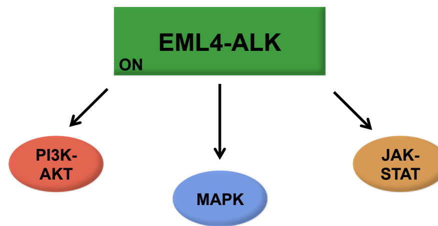


Figure 1. EML4-ALK controls MAPK, AKT, and STAT signaling in EML4-ALK variant 1 expressing NSCLC cell lines

a). H3122 and STE-1 cells treated with either crizotinib (1uM) or ceritinib (200nM) for 6 hours. Upon ALK inhibition, levels of phosphorylated ERK, AKT, and STAT3 are diminished. The H3122 (E13:A20) cell line is the only widely available EML4-ALKv1 cell line. The STE-1 (E13:A20) cell line was recently derived from a patient of Christine Lovly and colleagues (Vanderbilt). The crizotinib IC50 (72-hour cell-titer glo)for these cell lines are H3122:~300nM and STE-1:~250nM (data not shown). b). Schematic representation of EML4-ALKv1 effector pathways.

Figure 2

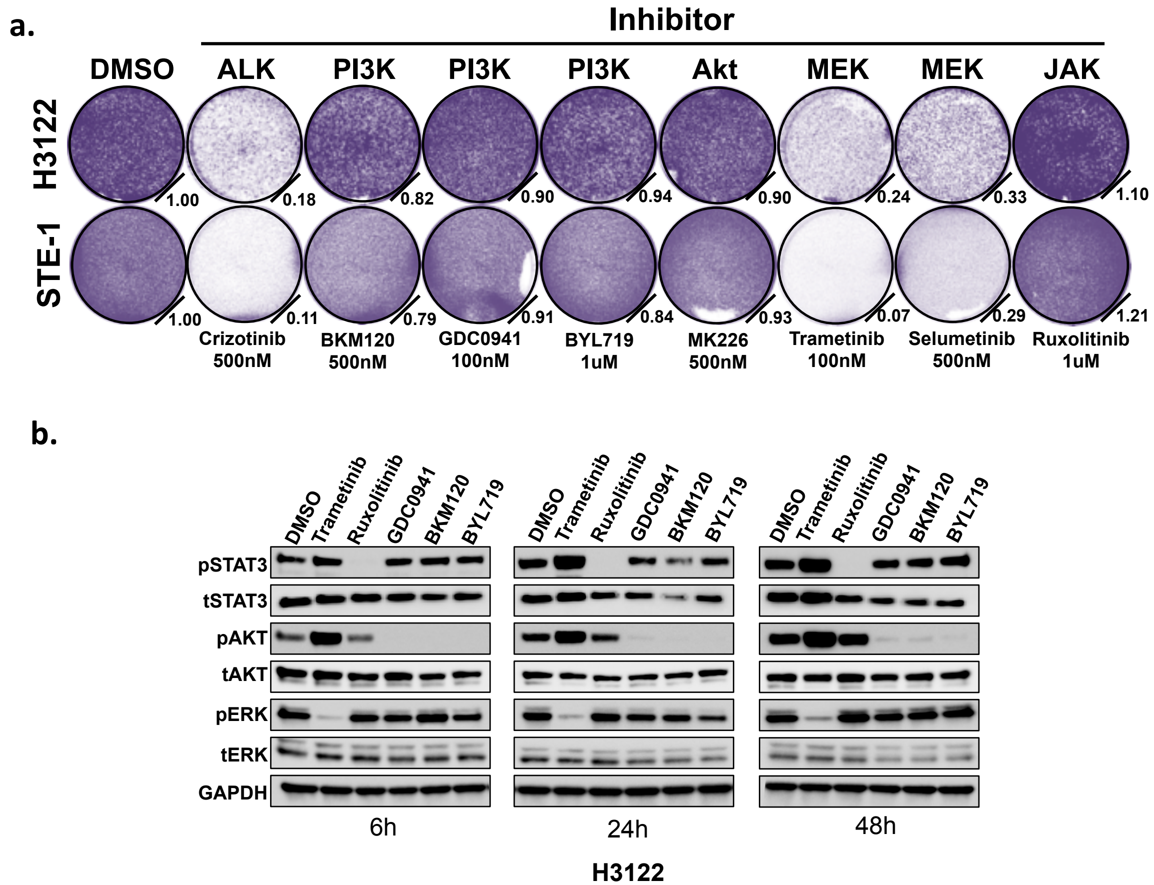


Figure 2. EML4-ALKv1 positive cells are sensitive to MEK inhibitors

a). H3122 and STE-1 cells were exposed to indicated drugs at indicated concentrations for 5 days, and then stained by crystal violet. Of note, both MEK inhibitors (trametinib, selumetinib) inhibited growth to a degree similar to the ALK inhibitor crizotinib, whereas PI3K or JAK pathway inhibitors had little effect. Fresh drug was replaced every 48h. Additional MEK (binimetinib, PD032) and JAK inhibitors (tofecitinib, momalotinib, baricitinib) were tested as well, yielding similar results (data not shown). b). immunoblot analysis with indicated antibodies on lysates taken from H3122 cells treated with the indicated pathway inhibitors at 6, 24, and 48h showing target inhibition up until time of media change in a). Of note, MEK inhibition induces feedback activation of STAT3 and AKT.

Figure 3

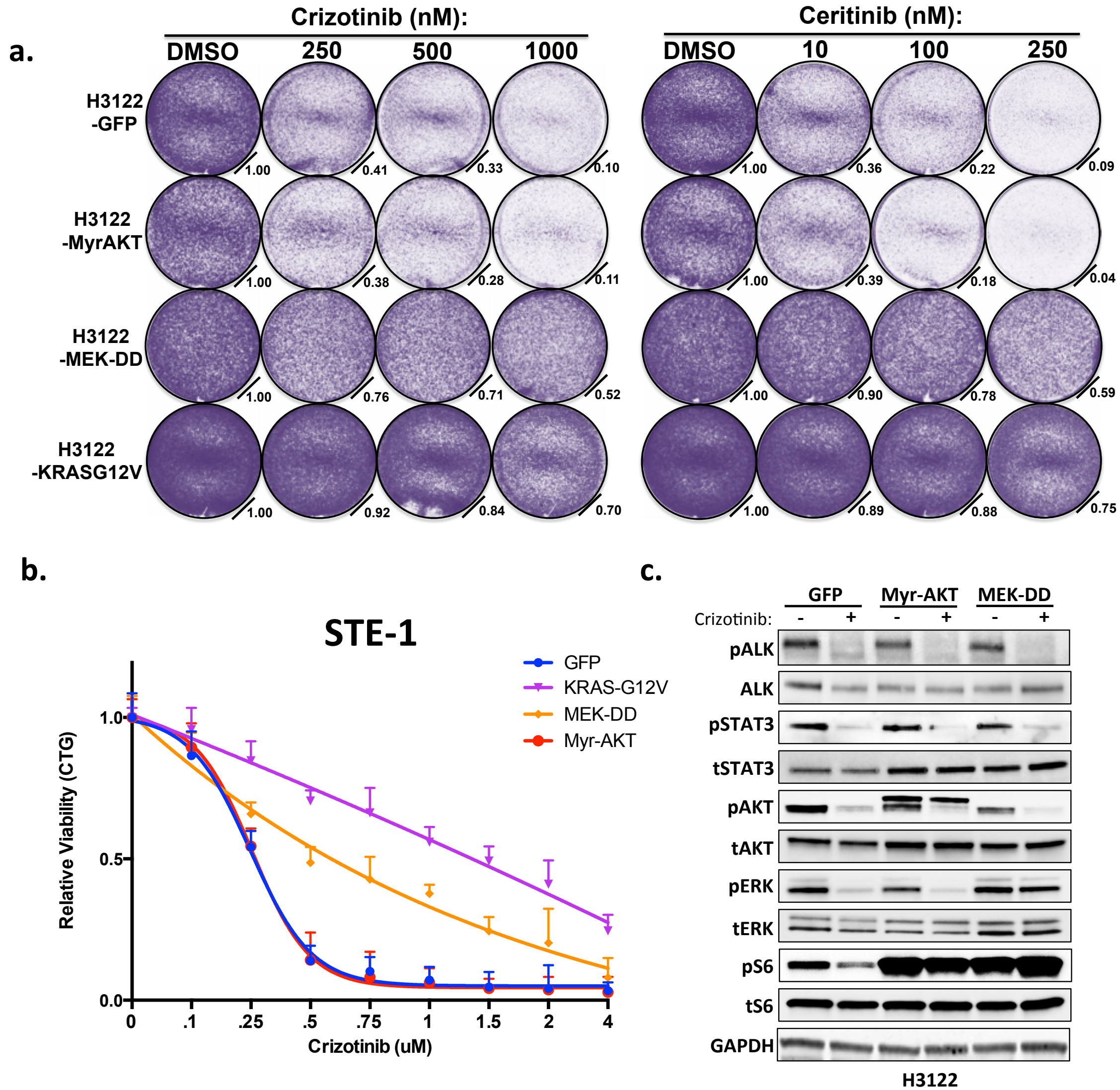


Figure 3. MAPK signaling is sufficient for survival in EML4-ALKv1 expressing NSCLC cells

a-b). H3122 and STE-1 cells expressing either GFP (control), or genetically activated AKT (MyrAkt), MEK (MEK-DD), or Ras (KRAS-G12V) were a). exposed to either crizotinib and/or ceritinib over 5 days and stained with crystal violet or b). measured via 72hr cell-titer glo assay. Hyperactivating MAPK pathway components rescued cells from ALK inhibition, whereas rescue of AKT had no effect. H3122-BRAF^{V600E} also rescued cells from ALK inhibition (data not shown) c). Immunoblot analysis with indicated antibodies of H3122 cells in A, showing rescue of pERK, but not pAKT or pSTAT3, in MEK-DD expressing H3122 cells. Despite pAKT and pSTAT3 remaining inhibited upon ALK inhibition in H3122-MEK-DD cells, the cells still have a robust resistance phenotype indicating the sufficiency of MAPK signaling in EML4-ALKv1 positive cells.

Figure 4

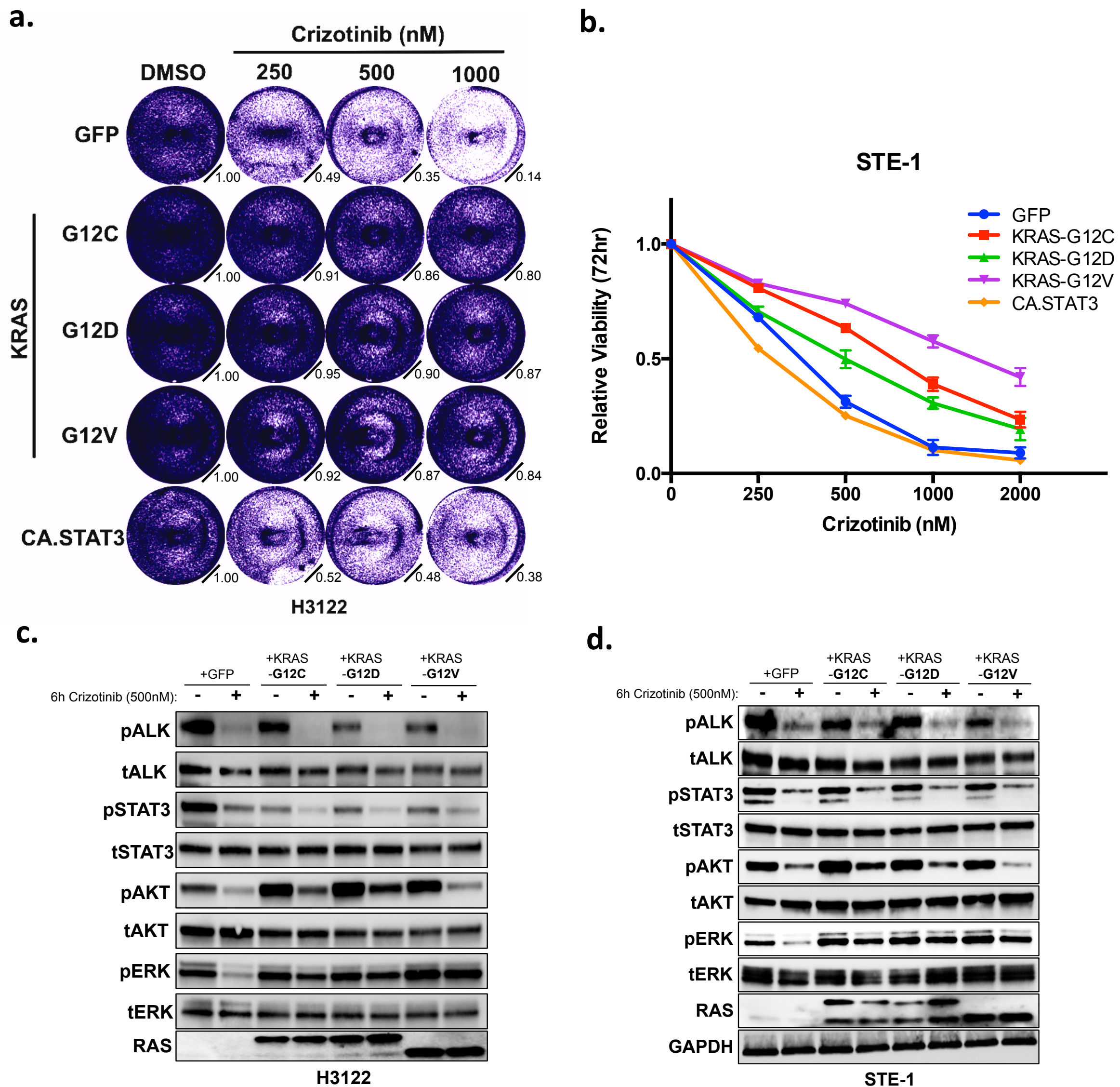


Figure 4. Rescue of RAS signaling overcomes ALK dependence

a-b). H3122 and STE-1 cells expressing either GFP (control), or genetically activated KRAS contructs (G12C, G12D, G12V) and constitutively active STAT3 (CA.STAT3) were exposed to crizotinib via either a). 5d crystal violet assay or b). 72hr cell-titer glo assay. In H3122, there is a slight rescue phenotypes with CA.STAT3, but which is much less pronounced than activation of any of the KRAS-activating mutants. However, in STE-1 cells, CA.STAT3 has no effect on ALK sensitivity. c-d). Immunoblot analysis of H3122 and STE-1 cells from A and B. In all cases, ERK phosphorylation is rescued from ALK inhibition with the presence of each mutant KRAS.

Figure 5

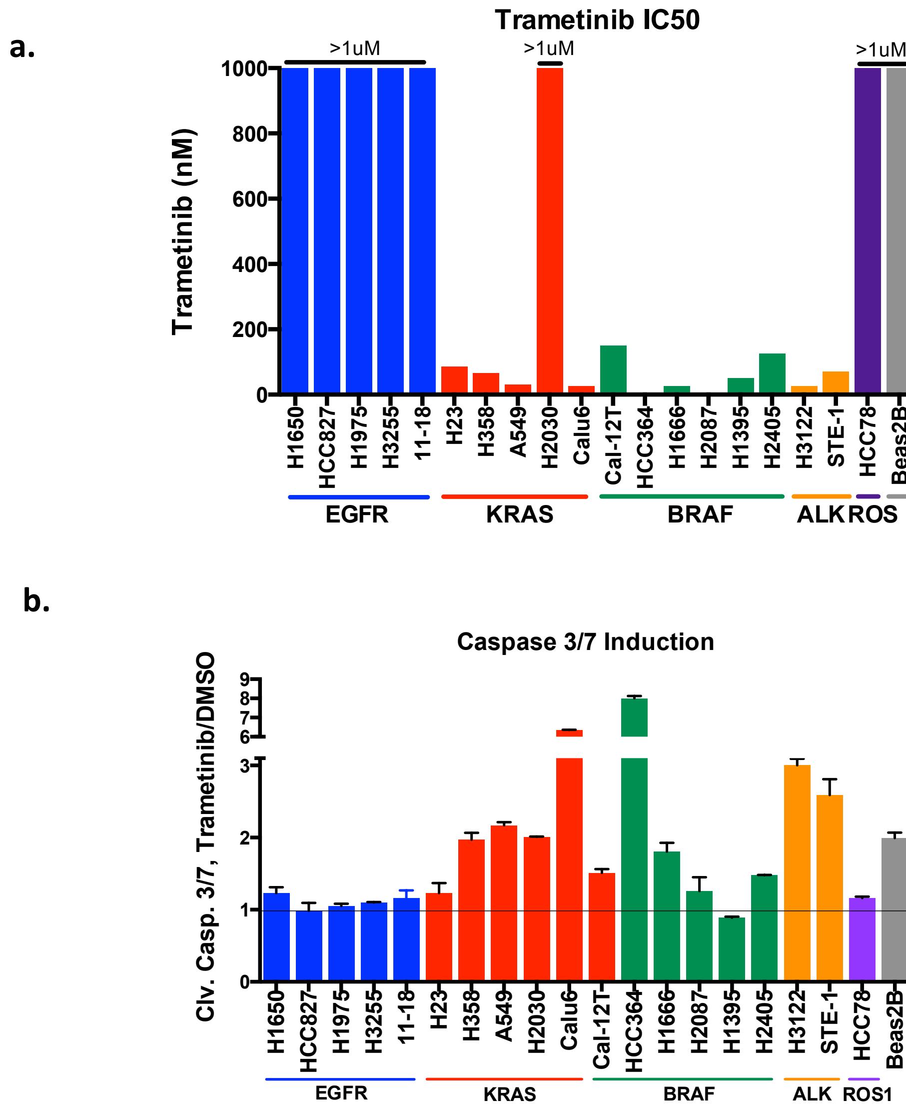


Figure 5. Sensitivity to MEK inhibition across oncogenic drivers in NSCLC
a). NSCLC cell lines representing the most common oncogenic drivers in NSCLC (KRAS, EGFR, ALK, BRAF, and ROS1 genetic alterations) and the normal lung epithelial cell line Beas2B were treated with several doses (1, 10, 100, 1000nM) of trametinib for 72h (cell-titer glo), and estimated IC50 values are plotted. EML4-ALKv1 lines (orange) are as sensitive to MEK inhibition as cell lines with pathway alterations directly on the MAPK pathway (RAS, BRAF). Of note, all EGFR-mutant cell lines tested are highly resistant to MAPK inhibition. b). Cell lines from A were exposed to either 100nM trametinib or DMSO for 24h, and caspase 3/7 cleavage was measured via caspase-glo. EGFR-mutant cell lines (blue) show no evidence of apoptosis in response to MEK inhibitors, whereas EML4-ALK (orange) induce significant levels of cleaved caspase 3/7 as compared to DMSO treated controls.

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Chapter 2

The Molecular Pathogenesis and Biology of EML4-ALK Fusions

Chapter 3: EML4-ALK fusion biology

Receptor tyrosine kinases (RTKs) activate downstream effector pathways in response to extracellular signals (ligands)[1]. This canonical function of RTKs requires a transmembrane domain on the RTK that allows the receptor to span the plasma membrane. This allows the receptor to bind ligands on the extra-cellular surface, while simultaneously accessing intracellular signaling components such as phosphoinositol-3-kinase (PI3K) and RAS. However, all known ALK-fusions (including variants 1-8 of EML4-ALK) found in human cancer consist of a fusion partner linked to exon 20-27 of ALK, which contains the kinase domain, but not the transmembrane domain (TM), of full-length ALK[2].

The class I PI3K family of proteins are heterodimeric molecules composed of regulatory (p85) and catalytic (p110) subunits and are implicated in a diverse set of cellular activities, including cell profileration and survival[3]. The regulatory subunit of PI3K family members can either directly interact with RTKs, or interact with various RTK adaptor proteins, which mediate its inhibitory role on the catalytic subunit (p110)[3-5]. The catalytic subunit of PI3K(s) functions by phosphorylating inositide lipid groups into 3-phosphorylated phosphoinositides (PI), which serve as docking sites for proteins with PI-binding domains, such as plextrin homology domains. Of these proteins, phosphoinsotide-dependent kinase-1 (PDK1) binds direcly to PtdIns(3,4,5)P3 and PtdIns(3,4)P2 via its plextrin homology domain along with AKT, allowing interaction of PDK1-AKT and therefore activating phosphorylation of AKT on threonine 308[3-5]. Because the initiating step in PI3K-mediated AKT activation requires a membrane-interacting lipid group, PI3K-AKT activity requires membrane localization for activity.

Similar, the RAS family of guanosine tri-phosphate (GTP) interacting proteins (GTPases) play a prominent role in mitogenic and survival signaling mediated by RTKs[6]. The Ras family of proteins are GTPases which normally function to catalyze the conversion of GTP to GDP. When RAS is GTP-loaded, it serves as a docking site for protein components that lead to their activation and subsequent downstream signaling. The serine/threonine kinase RAF1, which phosphorylates and activates MEK1/2, contains a RAS-GTP binding domain (RBD)[7]. Upon binding to GTP-loaded RAS ("activated" RAS), RAF1 forms a complex with RAS and KSR1, a scaffolding protein that recruits RAF's effector MEK and allows for its phosphorylation and activation[8,9]. Because RAS proteins are GTP-ases, they intrinsically turn themselves "off" by catalyzing their bound GTP into GDP. Indeed, most "activating" mutations of RAS, such as KRAS G12D, V, or C, impair the intrinsic GTP-ases activity of RAS, thereby promoting the GTP-loaded or "on" state[10]. The ability and efficiency of RAS proteins to catalyze GTP-GDP (or on/off states) normally is mediated by guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs)[11,14,15]. GAPs bind to and stabilize the weak intrinsic catalytic activity of the RAS-GTPases by providing additional catalytic residues that allow H_20 molecules to more efficiently provide a nucleophilic attack on the gamma-phosphate of GTP – thus in effect increasing RAS-GTPase activity and therefore promoting the "off"-state of RAS[12]. Conversely, GEFs (such as a SOS, RAS-GRP, and cdc25 proteins) interfere with the binding of GDP to RAS, and thus allowing its release into the cytoplasm[15]. Because intracellular concentration of GTP are much higher than GDP, this open pocket is preferentially

replaced by GTP[17]. Thus, the interplay/activation/expression of GEFs and GAPs are key mediators of RAS on/off activity.

Because the proteins thought to be required for RAS-activation (RAS-GTP loading) are GEFs, RAS activation in cells is largely dependent on RAS's access to these GEFs[13]. Activated RTK's provide binding sites for the adaptor GRB2, which binds to SOS. Thus, activated RTKs, which reside locally concentrated on plasma membranes due to their trans-membrane domains, provide a large local concentration of scaffold and adaptor proteins that promote RAS-signaling[18,19]. RAS proteins themselves are targeted to and bind to these membrane components via post-translational modifications of the C-terminal tail, such as prenylation, farnesylation, and palmitoylation (though the KRAS-4B isoform can not be palmitoylated)[20]. The lipidated tails of RAS proteins allow them to anchor to membrane compartments, which consequently bring them into contact with upstream signaling components such as membrane bound RTKs and their associated scaffolds, adaptors, and GEFs[13]. Importantly, however, *in vitro* studies have shown that RAS does not *require* membrane binding for their biochemical GTPase activity, thus it may be that the lipid-processing of the RAS proteins may only be necessary for proximity to RTKs and/or a mechanism to bring GEFs, RAS proteins, and their effectors (such as Raf1) to the same local environment[21-23].

Nonetheless, it is thus traditionally assumed that RAS and PI3K both 1). reside on membrane compartments and 2). may require lipid association to function. Yet, EML4-ALK contains no canonical membrane spanning domain and yet we know can signal through MAPK and PI3K. This presents a conundrum – how does an activated tyrosine kinase with no membrane-spanning domain access its known effectors? Answering this

question may begin to explain not only ALK and RTK-fusion biology generally, but also why EML4-ALK has such a unique dependence on MAPK signaling. Thus, we next investigated the molecular basis of MAPK pathway dependence in EML4-ALK lung adenocarcinoma cells.

Results

The signaling components linking EML4-ALK to MAPK pathway activation remain poorly defined. Indeed, although RAS GTPases canonically link receptor kinases to MAPK signaling, whether oncogenic ALK fusion proteins activate RAS has not been demonstrated (to our knowledge). Therefore, we investigated whether RAS was activated downstream of EML4-ALK in lung adenocarcinoma cells. Using GST-RBD affinity capture[24], we found that RAS activation was indeed coupled to ALK signaling in both H3122 and STE-1 cells (**Fig. 1**). As three major RAS isoforms (H-, N-, and K-RAS) typically regulate growth and survival in cancer cells, we further explored which isoform(s) was coupled to EML4-ALK in lung adenocarcinoma cells. Using RAS isoform specific antibodies[25], we found that all three major RAS isoforms (H-, N-, K-RAS) were both expressed and activated by EML4-ALK in lung adenocarcinoma cells (**Fig. 1**). We next studied the functional consequences of RAS isoform activation on downstream signaling and survival in EML4-ALK lung adenocarcinoma cells. In contrast to ALK inhibitor treatment, genetic silencing of each RAS isoform individually had no substantial effect on downstream signaling or on cell viability (**Fig. 2**). However, simultaneous knockdown of all three RAS isoforms was sufficient to significantly suppress cell growth and phosphorylated ERK levels, but not phosphorylated STAT3 nor phosphorylated AKT levels (**Fig 2**). These results suggest that H-, N-, and K-RAS

cooperate to control MAPK signaling downstream of EML4-ALK in lung adenocarcinoma cells, and further indicate that AKT and STAT3 activation are uncoupled from H-, N-, and K-RAS in these cells. These observations establish a previously unappreciated direct molecular link between EML4-ALK and H-, N-, and K-RAS, and further indicate the critical role of RAS-MAPK signaling in the survival of EML4-ALK lung adenocarcinoma cells. The data also provide a potential explanation as to why EML4-ALK lung adenocarcinoma cells may rely predominantly on MAPK signaling.

Given this link between EML4-ALK and the three major RAS isoforms (some of which can signal from multiple subcellular membrane compartments), we investigated how EML4-ALK might engage RAS. RAS signaling to its downstream effector pathways typically occurs on a cellular membrane compartment (either the plasma membrane or intracellular membranes)[26,27]. However, all described ALK fusions found in lung adenocarcinoma contain the kinase domain of ALK but not the native ALK transmembrane domain that enables membrane anchoring[28,29]. Moreover, the most common ALK fusion partner, EML4 variant 1, also does not harbor an established membrane-binding domain. Therefore, we first investigated the cellular distribution of EML4-ALK using immunofluorescence staining of endogenous ALK in both H3122 and STE-1 lung adenocarcinoma cell lines. Intriguingly, we found that endogenous EML4-ALK resided on an intracellular vesicular compartment and not on the plasma membrane, where many native receptor kinases often engage RAS (**Fig. 3**). Based on these findings, we sought to understand how a fusion protein with no known membrane-anchoring

domain might engage effectors that require a lipid interface to signal, such as RAS, potentially from an intracellular locale.

The EML4 portion of EML4-ALKv1 contains a Basic, HELP, and WD repeat domain (**Fig. 4**). Prior studies have shown that the Basic domain in EML4 is necessary for EML4-ALK dimerization and activation[30]. However, the functional importance of the other domains in EML4 has not been fully explored. We noted that the HELP domain of EML4 contains approximately 50% hydrophobic residues, suggesting that it may mediate peripheral or transient cellular membrane association and thereby enable access to effectors such as RAS. Although the function of the HELP domain in EML4 and other proteins (including EML4-ALK fusions) remains incompletely understood, previous studies have shown that removal of the HELP domain may impair the transforming capacity of EML4-ALK[30]. Moreover, recent studies suggest that the HELP domain in EML4 may regulate EML4 subcellular localization[31,32]. We therefore hypothesized that the HELP domain in the EML4 component of the EML4-ALK fusion might be required for proper EML4-ALK localization and downstream RAS and MAPK signaling in cells. To address this hypothesis, we introduced wild-type (WT) EML4-ALK or a mutant form lacking the HELP domain (ΔHELP) into immortalized but non-transformed lung epithelial (Beas2B) cells and examined EML4-ALK localization and signaling. We found that introduction of $EML4\text{-}ALK^{WT}$ into Beas2B cells resulted in distinct localization on intracellular vesicular structures, and not the plasma membrane, by immunofluorescence staining (**Fig. 4**). In contrast, the ΔHELP EML4-ALK mutant did not display this discrete intracellular localization but instead exhibited diffuse cytoplasmic expression in this system (**Fig. 4**).

We further found that EML4-ALK^{WT} expression activated ERK and also STAT3, but not AKT in both Beas2B and 293T cells (**Fig. 5**). Moreover, EML4-ALK^{WT} expression enhanced GTP-loading of all three major RAS isoforms (H-, N-, and K-RAS) (**Fig. 5**), consistent with our previous findings. In contrast, deletion of the HELP domain severely impaired the ability of EML4-ALK to activate ERK and any of the RAS isoforms in both Beas2B and 293T cells (**Fig. 5**). Together, these data suggest that the HELP domain of EML4 in EML4-ALK may regulate EML4-ALK cellular localization and be critical for the engagement and activation of RAS isoforms and MAPK signaling by EML4-ALK. Consistent with these findings, we found that EML4-ALK activation was uncoupled from RAS activation and MAPK signaling in H2228 human lung adenocarcinoma cells that endogenously express a rarer EML4-ALK variant (3b) in which EML4 lacks the HELP domain (**Fig. 6**)[33]. In these H2228 cells, ALK inhibition failed to significantly suppress RAS-GTP and phosphorylated ERK levels, and cell viability (compared to H3122 cells that express EML4-ALK variant 1 containing the HELP domain in EML4) (**Fig. 6**). These findings provide additional evidence linking both the EML4 HELP domain in EML4-ALK with RAS-MAPK signaling and also ALK inhibitor efficacy with MAPK pathway suppression. Altogether, these data provide insight into the molecular basis of RAS-MAPK activation and dependence in EML4-ALK lung adenocarcinoma cells.

Discussion

These studies reveal that EML4-ALK variant 1 activates H, N, and K-RAS signaling, a phenomenon that appears to be influenced by the presence of the HELP domain of the EML4. This is the first time that the fusion partner of an RTK-fusion

protein has been implicated in regulating signaling of the kinase independent of its effects on dimerization. Moreover, this uncovers a new field of study in RTK-fusion biology exploring the roles and consequences of the various fusion partners found in human cancer.

Although the engagement of EML4-ALKv1 was impaired by the deletion of the HELP domain, these studies also revealed that STAT3 was still activated to some degree upon expression of the ΔHELP EML4-ALK mutant (**Fig 5**), potentially due to RAS-independent activation of STAT3 by EML4-ALK. This finding may explain the oncogenic activity of EML4-ALKv1-ΔHELP (albeit modestly impaired compared to EML4-ALK^{WT}) observed previously[30, 34]. This observation also indicates that the ΔHELP EML4-ALK mutant is not generally deficient in downstream signaling, indicating the specificity of the effect of deletion of the HELP domain on RAS-MAPK signaling.

We were unable to identify the structures that EML4-ALKv1 interacted with, in neither the endogenous systems (H3122 and STE1), nor the Beas2B overexpression system. Our initial hypothesis was that the HELP domain was allowing for at least peripheral or transient interaction with intracellular membrane compartments, such as endosomes, however we were unable to confirm this with co-staining of several endosomal markers (Rab7 and EEA1), mitochondrial markers, and golgi markers (data not shown). However, the relatively low frequency of these structures in HELP-deficient EML4-ALK Beas2B cells suggest that the localization of these structures (given the subsequent signaling changes) may be required for, or at least promote, engagement with RAS proteins. It is possible that the structures we observe are not localized on membrane structures at all, but instead are aggregates or oligomers of EML4-ALK in the cytosol. If

so, then RAS may be interacting with these aggregates independent of membrane-docking. Indeed, adaptors and GEFs should still be able to interact with, bind to, and become activated by a soluble activated RTK such as EML4-ALK. In theory, perhaps the only reason GEFs and RAS are thought to "require" a lipid interface to function is because in most systems, the structures that bring these components together in high local concentrations on indeed membrane bound RTKs or GPCRs. However, it may be that ALK fusions present a unique system in which the local concentration of activated RTK/adaptors/GEFs can indeed occur in the cytoplasm. Indeed, a pharmacological screen performed on an isogenic crizotinib-resistant cell line derived from H3122 looking for re-sensitizers to crizotinib showed that the farnsesyl-transferase inhibitor Tipifarnib was the most growth *promoting* molecule in the screen (Appendix B). This would suggest a model where FTase inhibition prevents RAS-binding to membranes, in effect increasing the amount of cellular RAS that would be available for the cytosolic aggregation of EML4-ALK. This is also consistent with the ability of EML4-ALK to access all three major RAS isoforms (H. N, K), which have been proposed to signal from diverse membrane compartments such as the plasma membrane, golgi, endosomes, and the ER. The major difference between H. N, and K RAS isoforms is the C-terminal processing, which is proposed to "tag" the different isoforms to different compartments – however, in the scope of cytosolic EML4-ALK aggregates, all RAS isoforms would be created equal[13]. Nonetheless, the non-lipid mediated access of RAS specifically in the context of ALK or other RTK-fusions remains speculative and thus represents a major future direction for further study.

Figure 1

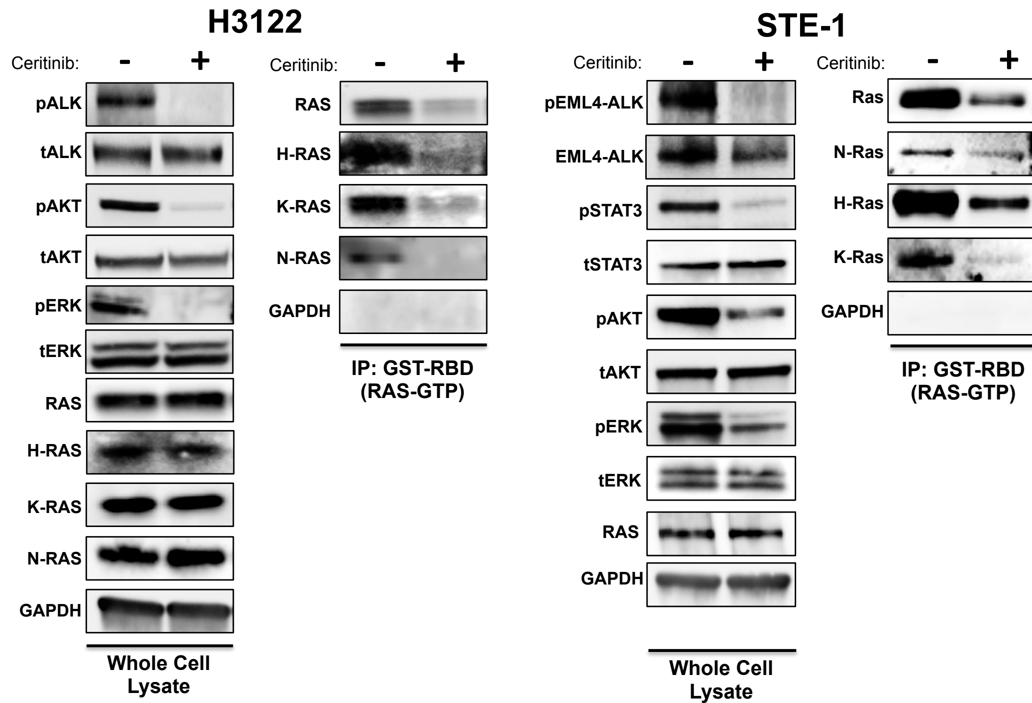

Figure 1. EML4-ALKv1 engages and signals via H-, N-, and K-Ras

H3122 and STE-1 cells were treated with 200nM ceritinib (30m) and lysates (whole cell lysates, WCL) were collected. An additional GST-RBD pull-down (immunoprecipitation, IP) was performed on the lysates (to capture GTP-loaded RAS). Immunoblot analysis with the indicated antibodies was performed on both WCL and IP:GST:RBD samples. The Ras isoform-specific antibodies have been validated previously (ref#25). In both H3122 and STE-1, ALK inhibition resulted in a decrease of H, N, and K-Ras GTP-loading, indicating ALK is engaging and activating all three isoforms.

Figure 2

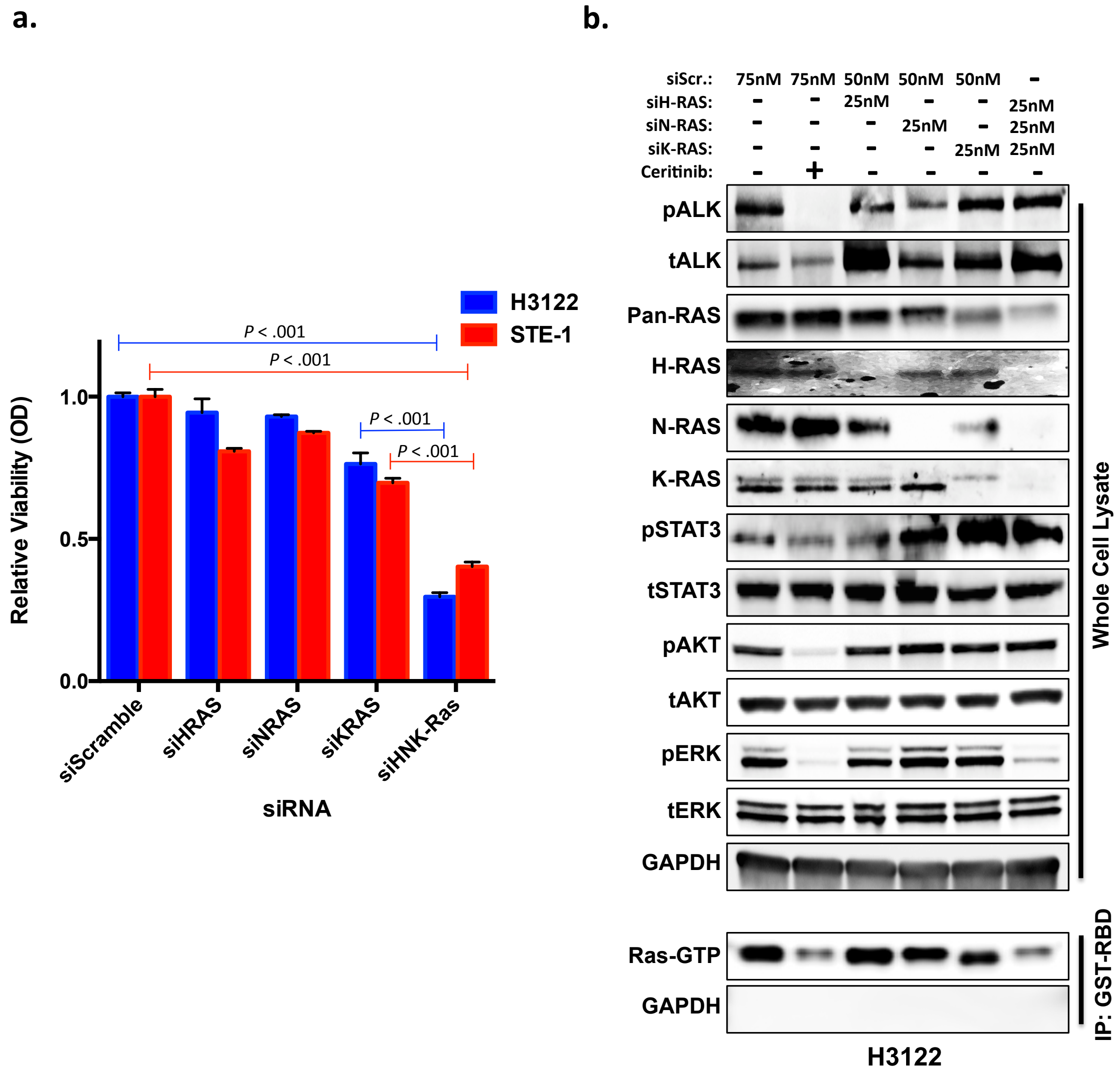

Figure 2. EML4-ALKv1 expressing cells are not dependent on any single Ras isoform
a). H3122 and STE-1 cells were transfected with siRNA's individually targeting H-, N, and K-Ras (or the triple combination). Cells plated into 6-well plates 24h post transfection, and a 5d crystal violet growth assay was performed. Bar graphs represent quantified absorbance values of crystal violet stain at day 5. b). H3122 cells from A were subjected to immunoblot analysis with indicated antibodies 72h post siRNA transfection. Individual knockdown of any Ras isoform did not affect downstream signaling (pAKT, pERK) negatively. However, triple knockdown (H.N,K) led to substantial pERK inhibition, but no effect on pAKT, and increased pSTAT3. GST-RBD immunoprecipitations (IP) are included to show total RAS-GTP loading. Only triple knockdown is able to decrease cellular RAS-GTP levels to a similar level as ALK inhibiton (lane 2). These results were repeated in STE-1 cells (data not shown).

Figure 3

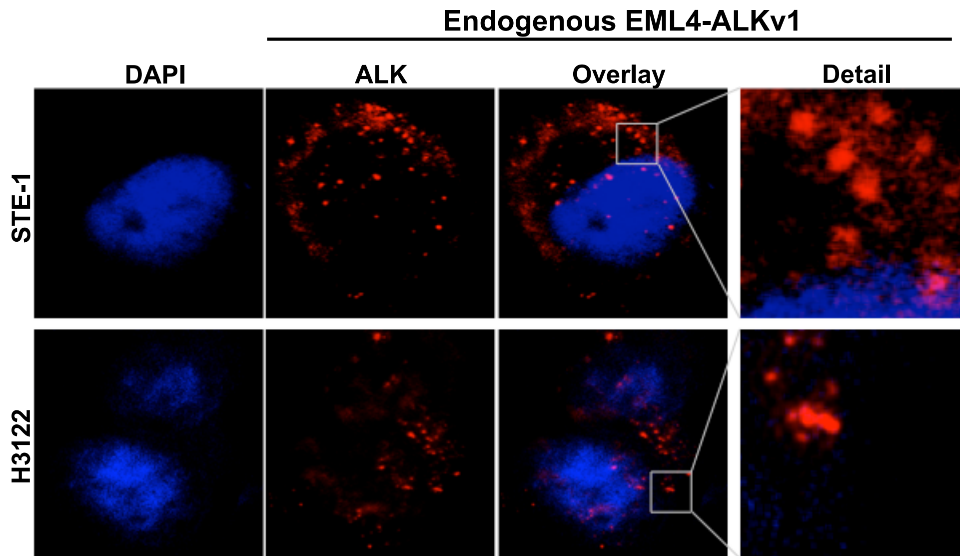

Figure 3. EML4-ALKv1 localizes on intracellular structures.
Immunofluorescence staining on endogenous EML4-ALKv1 in H3122 (E13:A20) and STE-1 (E13:A20) using an ALK monoclonal antibody. There is no obvious staining of ALK on the plasma membrane, but instead on intracellular structures and/or cytosolic aggregates. The identity of these structures remains an area of further study.

Figure 4

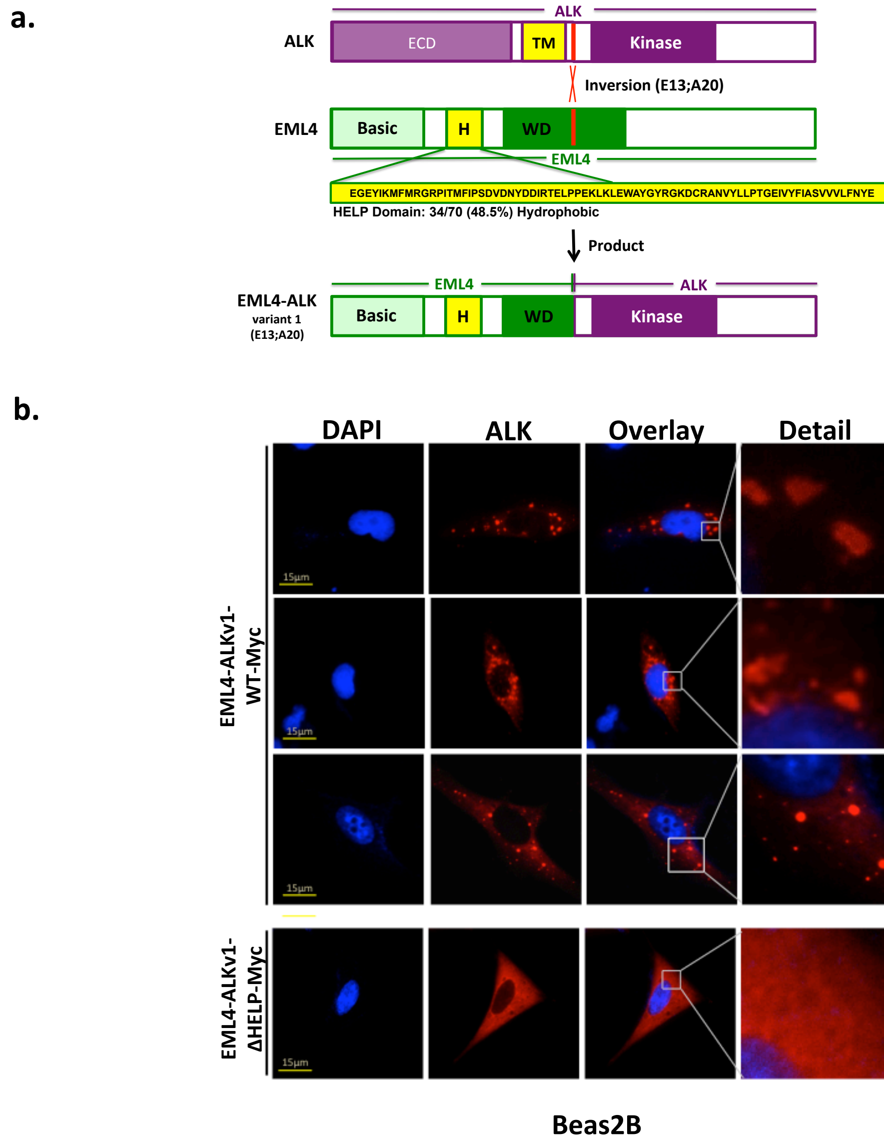

Figure 4. The HELP domain of EML4 mediates localization of EML4-ALKv1
a). Schematic representation of EML4-ALK variant 1 (v1) and its domains. The transmembrane (TM) domain of ALK is absent from the EML4-ALK fusion product. The HELP (H) domain of EML4 contains ~50% hydrophobic amino acids, as shown. The ΔHELP mutant of EML4-ALKv1 deletes this domain. b). Myc-tagged EML4-ALKv1 and EML4-ALKv1-ΔHELP were transfected into immortalized lung epithelial cells (Beas2B) and immunofluorescence was performed with an anti-Myc antibody. WT EML4-ALKv1 (top three panels) displays distinct intracellular staining on membrane compartments and/or cytosolic aggregates. Conversely, deletion of the HELP domain (EML4-ALKv1-ΔHELP) leads to more dispersed cytosolic staining. Thus, the HELP domain is required for the localization pattern seen with EML4-ALKv1.

Figure 5

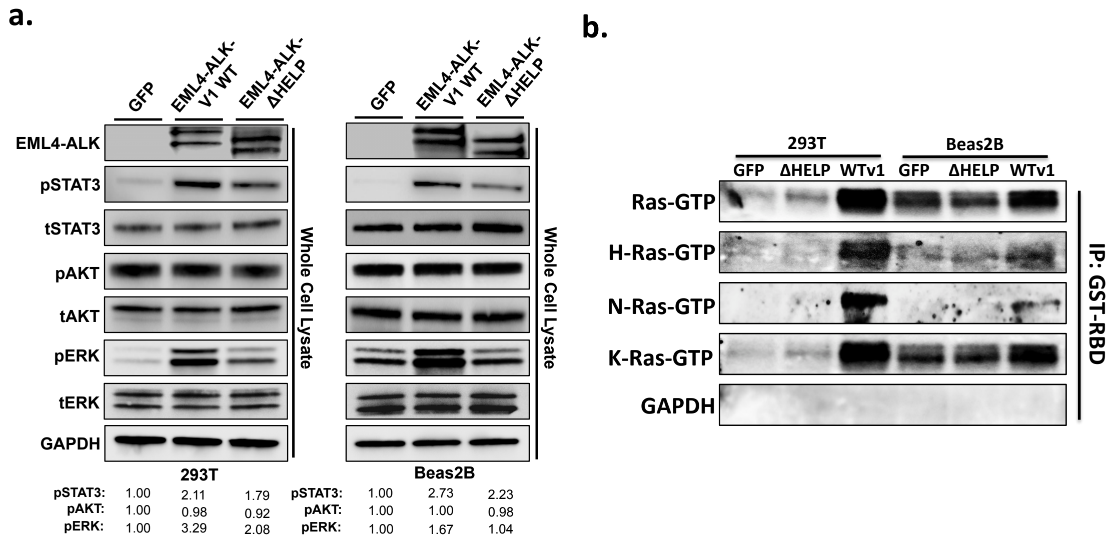

Figure 5. The HELP domain of EML4 mediates engagement of RAS-MAPK signaling
a). Beas2B and 293T cells were transfected with either WT EML4-ALKv1 or EML4-ALKv1-ΔHELP (HELP deletion mutant), and cells were lysed after 72h and immunoblot analysis was performed using the indicated antibodies. EML4-ALKv1 increases levels of phosphorylated (active) STAT3 and ERK, but not AKT. The ΔHELP mutant, however, is impaired in its ability to activated ERK. Quantifications were calculated in ImageJ. b). GST-RBD RAS-GTP pulldowns of lysates from A. EML4-ALKv1 is able to activated all three Ras isoforms (H, N, K – Ras) as measured by GTP-loading. However, deletion of the HELP domain (ΔHELP) impairs the ability of EML4-ALK to activate the Ras isoforms.

Figure 6

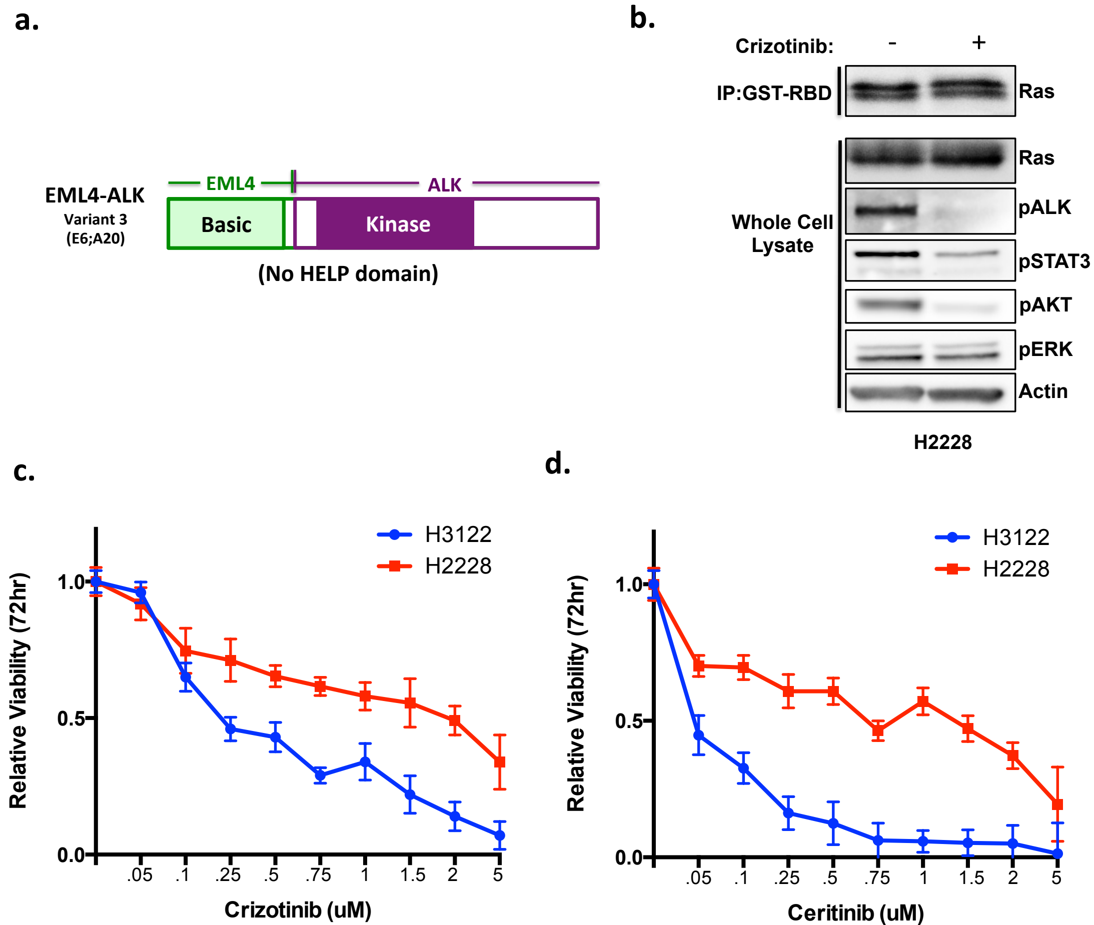

Figure 6. EML4-ALKv3 expressing cells do not engage RAS-MAPK.
a). Schematic representation of EML4-ALK variant 3 (E6:A20), which is expressed in the H2228 NSCLC cell line. Exon 20 of ALK is fused to exon 6 of EML4, which only includes the Basic domain and omits the HELP and WD domains. b). H2228 cells were treated with crizotinib for 6h, and whole cell lysates and GST-RBD pulldowns were performed. Upon ALK inhibition, pSTAT3 and pAKT are diminished, but pERK and RAS-GTP levels remain unaffected. c-d). H2228 cells were treated with increasing doses of b). Crizotinib or c). Ceritinib. Viability was measured by 72hr Cell-titer glo assay. As shown, H2228 (E6:A20) cells are more resistant to ALK inhibitors than H3122 (E13:A20) cells.

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Chapter 3

Novel Mechanisms of Resistance to ALK Targeted Therapy

Chapter 3: Mechanisms of Resistance to ALK TKI therapy

Although the clinical benefit of ALK inhibitors is substantial in the ALK-fusion positive NSCLC population, resistance to these therapies is by enlarge inevitable and is thus the greatest barrier to prolonged patient survival. Crizotinib is the current front-line standard-of-care for ALK-positive patients, with a median duration of response of approximately 10 months[1]. The second generation ALK inhibitor ceritinib, which is more potent and selective for ALK, is approved in the second-line setting and has a similar duration of response[2]. The third generation ALK inhibitor, alectinib, which has superior potency against ALK compared to both crizotinib and ceritinib, and also crosses the blood-brain barrier readily, has recently shown 27 month median PFS in early-stage trials[3].

Because crizotinib was approved in 2007 and is the primary ALK inhibitor used in the clinic today, most of our knowledge of clinical resistance to ALK inhibitors stems from our experience with crizotinib. However, even this knowledge is limited. Approximately 30% of crizotinib-relapsed patients develop an on-target, secondary ALK mutation that abrogates binding of the drug[4]. This is a significantly lesser proportion of patients that develop resistance to erlotinib via secondary on-target mutations, in which approximately 50% of patients will develop EGFR T790M. The remaining ~70% of crizotinib-resistant tumors are thought to develop upregulation of other RTK's which serve as bypass tracks for ALK signaling – these include EGFR, IGF1R, and HER2 upregulation[5]. Overexpression of these RTK's is sufficient to promote resistance to ALK inhibitors *in vitro* (in H3122 cells), and isogenic resistance-derived cell lines have been shown to up-regulate either EGFR, IGF1R, or HER2 phosphorylation. In these models,

treatment with the relevant RTK-inhibitor (EGFR, IGF1R, or HER2) was able to partially re-sensitize these cells to ALK inhibitor treatment[6-8]. The only study to check EGFR phosphorylation changes (by IHC) in pre- vs. post- crizotinib patient samples found that 4/9 had at least some evidence of higher pEGFR signal in the post-treatment sample[6]. However, whether simply up-regulation of these RTKs is a mechanism of resistance in patients has yet to be clinically validated. Moreover, it has been shown that overexpression of many RTK's or growth factors is sufficient to promote resistance to TKI's generally *in vitro,* making it more difficult to elucidate a concrete mechanism in an individual patient that could be potentially therapeutically exploitable[8,9].

The clinical experience with the next-generation ALK inhibitors ceritinib and alectinib is still in its infancy, thus there have been few studies examining ceritinib and alectinib resistance to date. Of these, additional secondary mutations in ALK have been identified. In one study, 4/10 crizotinib-refractory patients developed a ALK G1202R or F1174C/V mutation after ceritinib relapse[10]. Another study implicated Src activation as a potential bypass mechanism of resistance to ceritinib, and also identified an activating MEK (MAPK2K1 K57N) mutation in ceritinib-refractory patient[11]. For alectinib, the V1180L and I1171T/N/S ALK kinase domain mutations appear to confer resistance[12,13,14]. Interestingly, many of the secondary resistance mutations found to one ALK inhibitor are often responsive to another inhibitor – with the exception of G1202R, which appears to be refractory to all three of the most clinically advanced ALK inhibitors (crizotinib, ceritinib, and alectinib)[13]. Interestingly, one alectinib-reistant patient possessed a MET-gene amplification, and was subsequently treated with crizotinib (which hits MET and ALK) and responded[16].

Thus, the vast majority of our insight into ALK TKI resistance stems from secondary, on-target, mutations that abrogate drug binding. The identification and clarification of the remaining (off-target) mechanisms of resistance (~70% of patients) to ALK TKI therapy largely remains a black box and area of ongoing investigation.

Results

Because my previous findings indicated that RAS-MAPK signaling may be the critical pathway controlling tumor growth and survival downstream of EML4-ALK, we hypothesized that the development of acquired ALK inhibitor resistance might consistently require RAS-MAPK pathway re-activation. Accordingly, we developed multiple *in vitro* models of acquired ALK inhibitor resistance by continuously exposing H3122 cells to either crizotinib (Crizotinib Acquired Resistance, CAR, n=3) or ceritinib (LDK378 Acquired Resistance, LAR, n=3) and explored the molecular basis of resistance in the derived sub-lines. First, we found that each resistant sub-line was cross-resistant to each ALK inhibitor (data not shown). Further, by DNA sequencing analysis of the coding regions of ALK we did not detect on-target mutations or a copy number alteration that could explain ALK inhibitor resistance in these sub-lines (data not shown). Importantly, we observed that each *in vitro* model of ALK inhibitor resistance harbored MAPK pathway re-activation during ALK inhibitor treatment, as measured by the levels of phosphorylated ERK (**Fig. 1a**). To test whether these resistant sub-lines remained dependent on this MAPK signaling (similar to the parental cells), we treated each sub-line with single agent inhibitors of EML4-ALK effector pathways (**Fig. 1b**). We discovered that all of the resistant models retained significant MAPK signaling (MEK) dependence, whereas by contrast suppression of JAK-STAT or PI3K-AKT signaling had

less impact on tumor cell survival. These data suggest that MAPK signaling is not only consistently rescued in lung adenocarcinoma cells with acquired ALK inhibitor resistance but also necessary for tumor cell survival and ALK inhibitor resistance *in vitro*. The data provide further support that the MAPK signaling axis is a key determinant of growth and survival in EML4-ALK lung adenocarcinoma cells.

Next, we sought to identify the mechanisms by which the ALK inhibitor resistant models rescued MAPK signaling to drive resistance, focusing on the crizotinib-resistant models because this ALK inhibitor is currently most widely used. To this end, we performed whole exome sequencing analysis in our resistant sub-lines, the analysis of which did not reveal canonical mutations in *ALK*, *K/N/HRAS*, *A/B/CRAF*, *MEK1/2*, *ERK1/2*, or upstream RTKs that could explain MAPK pathway activation or resistance (data not shown). However, strikingly, this exome sequencing analysis instead revealed focal amplification of $KRAS^{WT}$ in CAR1 cells (confirmed by KRAS fluorescence-in-situ-hybridization, FISH), but not in CAR2 or CAR3 cells (**Fig. 2a, b**). Accordingly, we found that CAR1 cells had significantly higher levels of KRAS4A and 4B transcript, KRAS protein, and RAS-GTP levels as compared to parental H3122 cells (**Fig 2c**). Knockdown of KRAS decreased viability and the levels of phosphorylated ERK and increased apoptosis and sensitivity to crizotinib in CAR1 cells (**Fig 3a, b**). Conversely, overexpression of $KRAS^{WT}$ was sufficient to promote crizotinib resistance in both STE-1 and H3122 cell lines (**Fig 3c-e**). These data uncover amplification (or upregulation) of $\textit{KRAS}^{WT}$ as a novel mechanism of ALK inhibitor resistance.

To examine the potential clinical relevance of these findings, we analyzed tumor biopsies from a cohort of patients (n=15) that had developed acquired resistance to ALK

inhibitor therapy for evidence of *KRAS* copy number gain (**Fig. 4a**). We observed focal amplification of *KRAS* in three patients (3/15, 20%) with acquired ALK inhibitor resistance, as determined by *KRAS* FISH analysis conducted in both the pre-treatment and post-resistance tumor re-biopsy (**Fig. 4b**). The pre-treatment tumor biopsy showed no evidence of *KRAS* amplification in each of these three patients (patients #2, #13, #14), suggesting that this event emerged during the onset of resistance to crizotinib. These resistant patient tumors with *KRAS* amplification also had no evidence of *ALK*, *RAS*, or *RAF* mutations. This finding further supports a potential primary role for $KRAS^{WT}$ genomic amplification in the development of ALK inhibitor resistance, likely via re-activation of MAPK signaling as we observed in the EML4-ALK lung adenocarcinoma *in vitro* models.

Intriguingly, in seven other resistant patient tumor samples (7/15, 47%) we discovered $KRAS^{WT}$ gene duplication events in tumor cell sub-populations (appearing as KRAS gene doublet signals by KRAS FISH, **Fig. 4a**). Although the functional impact of this $KRAS^{WT}$ gene duplication is unknown, gene duplication of $BRAF^{WT}$ or $PTPN11^{WT}$ can drive both MAPK pathway activation and tumor growth[16,17]. We explored the potential functional impact of $KRAS^{WT}$ gene duplication, which might produce lower levels of KRAS than genomic amplification, using a genetically controlled system to assess the relationship between relatively lower or higher levels of $KRAS^{WT}$ expression and ALK inhibitor sensitivity. We found that expression of $KRAS^{WT}$ over a dose response increased p-ERK levels and promoted resistance to ALK inhibitor treatment, even at relatively lower levels of $KRAS^{WT}$ expression, in H3122 cells (**Fig. 3d, e**). Together, our data suggest that KRAS gene duplication may be an additional mechanism

promoting MAPK signaling and ALK inhibitor resistance, potentially in cooperation with other co-occurring molecular events. These findings provide rationale for additional studies to fully explore the potential role of KRAS gene duplication in signaling and resistance in cancer. Altogether, our findings reveal $KRAS^{WT}$ copy number gain as a novel mechanism of ALK inhibitor resistance in lung adenocarcinoma.

Because $KRAS^{WT}$ copy number gain was not found in CAR2 and CAR3, we sought to uncover the mechanism by which these cells rescued MAPK signaling and therefore explain the ALK inhibitor resistance phenotype. We noted that CAR2 and CAR3 cells had slightly lower basal levels of RAS-GTP than H3122 parental cells despite having significant basal levels of phosphorylated ERK (**Fig 5a, Fig 1a**). This finding suggested that MAPK pathway re-activation might have occurred, in part, via deregulation of components acting downstream of RAS in these cells.

Dual Specificity Phosphatase (DUSP) phosphatases are critical regulators of MEK-ERK signaling and typically regulate MAPK signaling at a level below RAS[18,19]. To determine if a specific DUSP may play a role in MAPK pathway activation and ALK inhibitor resistance in EML4-ALK lung adenocarcinoma cells, we examined the levels of all expressed DUSP family members in transcriptome datasets we generated from H3122 parental and ALK inhibitor resistant cells. We found that DUSP6 exhibited higher expression compared to other DUSP family members in parental H3122 cells, suggesting that DUSP6 may be a key regulator of MAPK signaling in this system (**Fig 5b**). Moreover, DUSP6 was significantly downregulated in both CAR2 and CAR3 sub-lines (**Fig. 5b**, >10 fold reduction in RNA levels in **Fig 5b**). Thus, we hypothesized that DUSP6 downregulation may promote MAPK reactivation and contribute to ALK

inhibitor resistance in EML4-ALK lung adenocarcinoma cells. To test whether DUSP6 downregulation is required for resistance in this system, we stably reconstituted DUSP6 expression in H3122 CAR2/3 cells. Reconstitution of DUSP6 expression restored sensitivity to crizotinib and the ability of crizotinib to suppress MAPK (p-ERK) signaling in this system (**Fig. 5c, d**). Additionally, we found that knockdown of DUSP6 was sufficient to promote crizotinib resistance in parental H3122 cells, an effect accompanied by rescue of ERK phosphorylation in the presence of ALK inhibition (**Fig. 5e, f**). Together, these findings indicate a causal role for decreased DUSP6 in ALK inhibitor resistance in EML4-ALK lung adenocarcinoma cells. Although it is possible that other inputs (such as RTK activation) may contribute along with DUSP6 downregulation to promote MAPK reactivation in CAR2 and CAR3 cells, our collective data provide further support that reliance on MAPK signaling for survival is a critical feature of EML4-ALK lung adenocarcinoma cells.

We sought to clinically validate our preclinical findings uncovering DUSP6 downregulation as a potential driver of ALK inhibitor resistance. We established and validated an immunohistochemistry (IHC) assay to measure DUSP6 levels in patient tumors, and used it to conduct a comparative analysis of DUSP6 expression in an additional cohort of treatment naive and post-ALK inhibitor resistance patient tumor samples (n=25; prioritizing DUSP6 analysis over other molecular testing given the limited amount of sample available for analysis in these small tumor biopsies). We found that DUSP6 levels were generally decreased in samples obtained upon ALK inhibitor resistance, compared to the pre-treatment tumor specimens in this cohort (**Fig. 6a**). This patient tumor analysis included six cases in which the pre-treatment and post-resistance

specimens were matched within an individual patient. In five out of six (80%) of these matched cases, DUSP6 levels were decreased in the resistant tumor compared to the matched pre-treatment tumor, whereas in one case DUSP6 levels remained similar (**Fig. 6a, b**). Sufficient material was not available for dual analysis of both DUSP6 levels and KRAS amplification in the majority of cases. However, three resistant tumor samples that lacked DUSP6 expression and had sufficient material were examined for the concurrent presence of KRAS copy number gain by FISH analysis and showed no evidence of KRAS copy number alteration (**Fig. 6c**). This finding supports a model in which DUSP6 downregulation might be sufficient to promote ALK inhibitor resistance in some patient tumors, consistent with our preclinical findings in EML4-ALK lung adenocarcinoma cells. Altogether, the clinical data support our preclinical findings uncovering a critical role for MAPK signaling in EML4-ALK lung adenocarcinoma and unveil $KRAS^{WT}$ copy number gain and downregulation of DUSP6 as two novel biomarkers and mechanisms of ALK inhibitor resistance. Interestingly, five patients with crizotinib resistance in our cohort (#13 –KRAS amp +, #14 –KRAS amp+, #15 –DUSP6 score 0, #18 –DUSP6 score 2, #20 –DUSP6 score 0) subsequently received either an investigational ALK inhibitor (ASP3026) or ceritinib and each experienced relatively rapid disease progression (within 2-4 months) (**Fig. 6c**), with the exception of patient #18 (whose resistant tumor had moderate DUSP6 levels) who responded for 8 months before progressing. This observation offers support for a potentially important role for increased KRAS and decreased DUSP6 levels in limiting ALK inhibitor response in patients.

Discussion

Our findings highlight reactivation of MAPK signaling as a previously underappreciated hallmark feature of acquired ALK inhibitor resistance in lung adenocarcinoma cells, and unveil *KRAS*^{WT} copy number gain (via either gene amplification or potentially duplication) and downregulation of DUSP6 as new biomarkers and mechanisms of ALK inhibitor resistance. These mechanisms of resistance to ALK inhibitor treatment and those reported previously (including copy number gain or secondary mutations in ALK that restore ALK activation, upregulation of EGFR or IGF1R RTKs, a MEK activating mutation) can activate MAPK signaling[6-8, 20-23]. Thus, we propose a new model in which MAPK pathway re-activation (potentially via diverse mechanisms) might be a necessary feature for the development of ALK inhibitor resistance more broadly.

Wild-type KRAS would typically be thought to require an upstream activator for GTP-loading, so why EML4-ALK appears to not be relevant in the context of more KRAS protein remains unclear. However, we postulate that massive amounts of KRAS protein (as seen in CAR1 and presumably in patient tumor cells with KRAS amplification) circumvents the need for an upstream activator. Because intracellular GTP concentrations are significantly higher than GDP, we hypothesize that the amount of intrinsic KRAS-GTP that would result from more KRAS protein may overcome the ability of GAPs to catalyze the hydrolysis reaction. Alternatively, we cannot exclude the presence of other RTKs or mitogenic signals acting as RAS activators. Wild-type KRAS amplification has been described as mechanism of resistance to cetuximab in colorectal cancer[24]. Moreover, wild-type KRAS amplifications occur in a small proportion of NSCLC patients with no co-occurring

oncogene mutation (such as EGFR, ALK, ROS1, or BRAF alterations)[25]. Thus, it is possible that higher levels of RAS protein may be oncogenic or lead to an increased "on" state generally. The effect of KRAS gene duplication remains unclear, as it is difficult to model gene duplication in an *in vitro* setting. Moreover, we do not know how KRAS gene dosage correlates with gene product (protein), thus it is unclear if KRAS gene duplication produces sufficient KRAS protein to promote resistance to ALK TKI. However, it is likely that it does produce more, and therefore can be thought to at least contribute to the resistant phenotype in some cells, perhaps in conjunction with other mechanisms of resistance such as RTK bypass. Interestingly, gene duplication events, such as PTPN11 and BRAF, have been shown to be oncogenic[16,17].

Likewise, although we show the potential contribution of DUSP6 down-regulation in MAPK reactivation and therefore ALK TKI resistance, we cannot conclude this is solely responsible for ALK TKI resistance. In fact, re-activation of MAPK signaling almost certainly would require activation of some upstream signal (such as an RTK), in which case DUSP6 downregulation would either 1) amplify the MAPK signaling output it provides and/or 2) maintain it for a longer duration. However, it will likely be difficult to pinpoint one sole culprit for upstream bypass activation in patients. Nonetheless, the role of DUSP6 in ALK TKI resistance serves as a proof-of-concept of the importance of MAPK re-activation in ALK-fusion positive tumor cells.

Figure 1

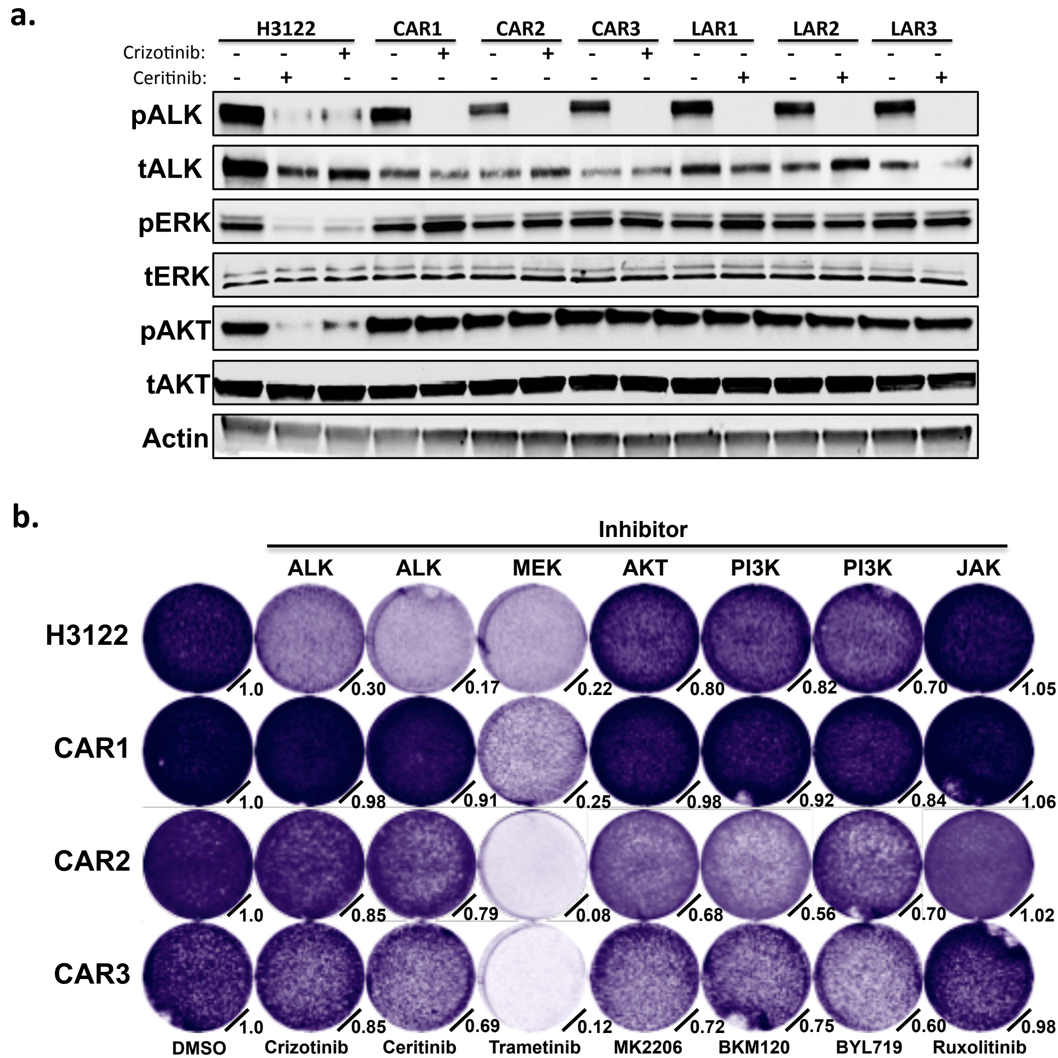


Figure 1. Crizotinib and ceritinib acquired resistance lines re-activate MAPK signaling

a). Isogenic resistant lines were derived from H3122 cells via continuous exposure to crizotinib (CAR) or ceritinib (LDK378, LAR) for ~4 months. Immunoblot analysis was performed on these isogenic lines +/- ALK inhibitor treatment. In all cases, MAPK signaling as measured by ERK phosphorylation remains unaffected. b). Resistant models in A were treated with individual pathway inhibitors and a 5day crystal violet viability assay was performed using the indicated inhibitors (with fresh drug added every 48h). Quantifications were performed by solubilizing the stained cells at day 5 with 1% SDS, and absorbance values (OD) were measured using a Spectramax luminometer and compared to the DMSO treated controls. Doses were crizotinib (500nM), ceritinib (100nM), trametinib (100nM), MK2206 (500nM), BKM120 (500nM), BYL719 (1uM), Ruxolitinib (1uM).

Figure 2

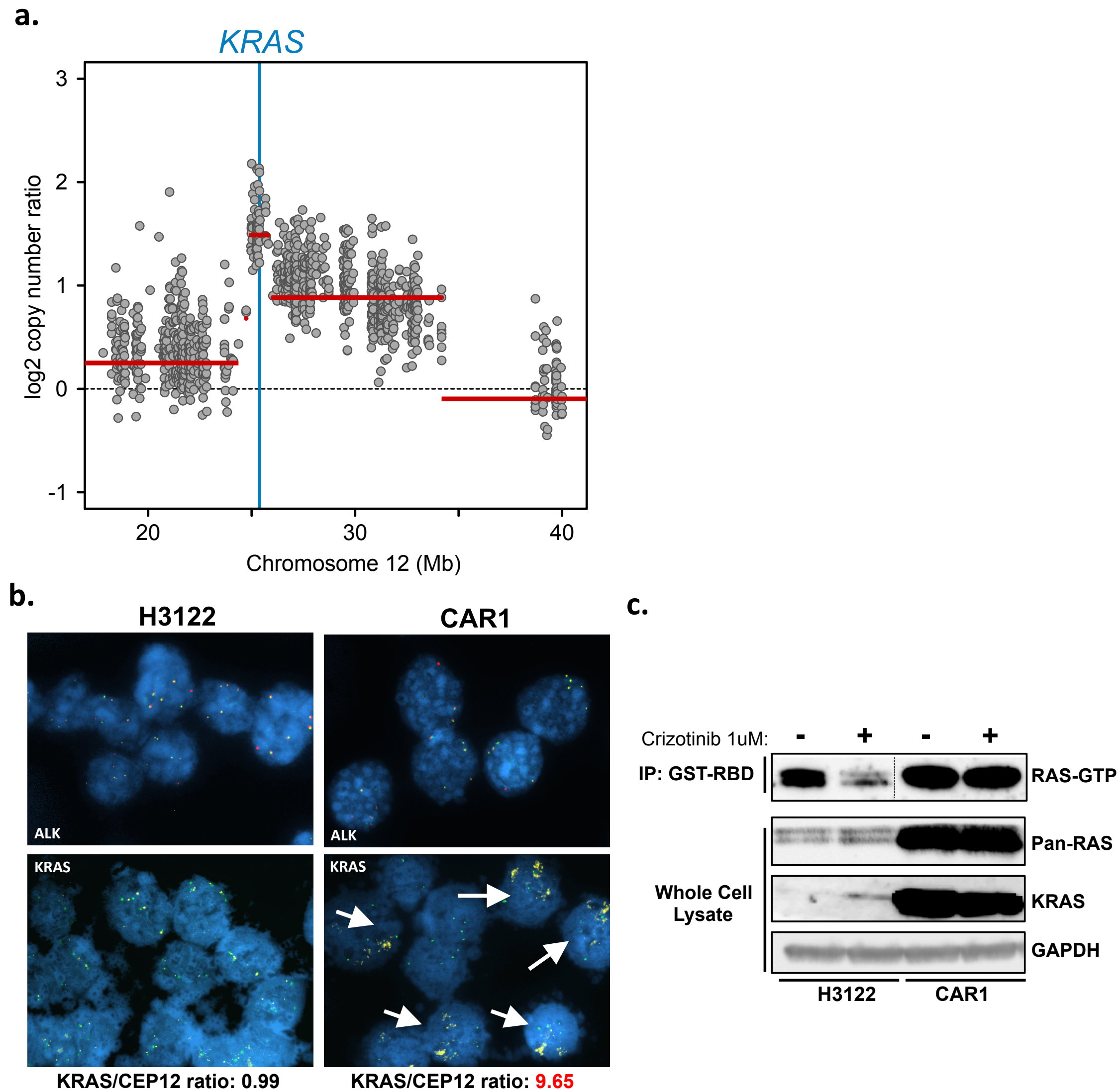


Figure 2. CAR1 harbors a focal KRAS^{WT} amplification

a). Exome sequencing was performed on CAR1 and H3122, and a focal copy number gain event was identified on the KRAS locus on chromosome 12 (shown here). b). KRAS amplification was confirmed by FISH. KRAS probes (yellow) and CEP12 (control, green) are shown in the bottom panels. Top panels show ALK-break apart FISH probes confirming presence of ALK-fusion in both H3122 and H3122-CAR1. c). Immunoblot analysis was performed with indicated antibodies on H3122 and CAR1. GST-RBD pulldowns were also performed to check for RAS-GTP levels. As shown, CAR1 harbors significantly more KRAS protein than H3122. Additionally, RAS-GTP levels are unaffected by ALK TKI treatment in CAR1.

Figure 3

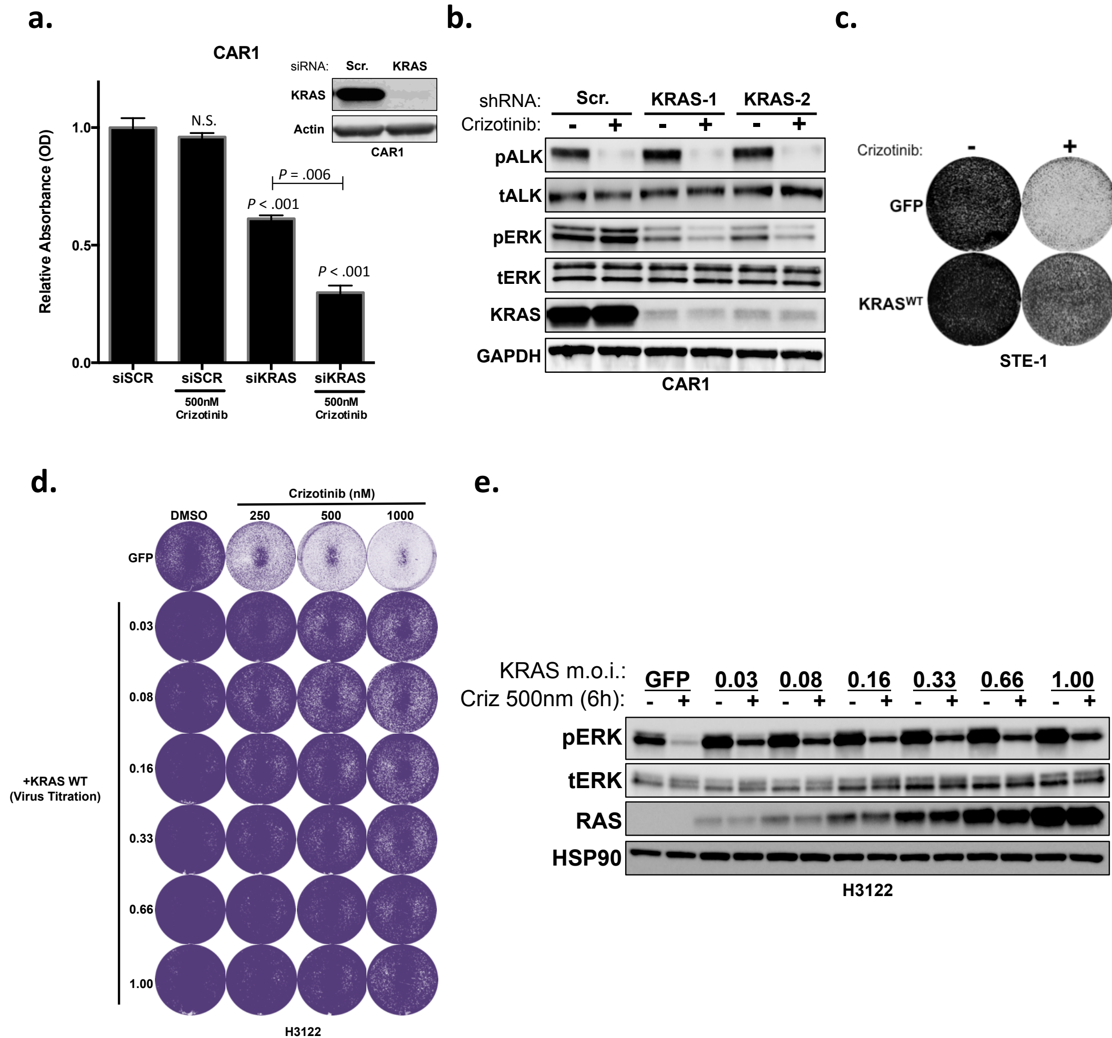


Figure 3. KRAS mediates resistance to ALK TKI.
a-b). KRAS is necessary for growth and ALK TKI resistance in CAR1. CAR1 cells were subjected to either KRAS siRNA (a) or shRNA (b) knockdown and viability, sensitivity to crizotinib, and signaling was assayed. In (a), siKRAS reduced viability and also re-sensitized cells to 500nM crizotinib. This effect tracked with a reduction in ERK phosphorylation in (b). c-e). Overexpression of wild-type KRAS is sufficient to promote resistance to ALK TKI in STE-1 (c) and H3122 cells (d-e). In the case of H3122 cells, different viral titers were used to achieved differential levels of KRAS overexpression (as shown in (d)). Even relatively lower levels of KRAS were sufficient to promote resistance to ALK TKI in H3122.

Figure 4

a.

Patient	Age	Sex	ALK inhibitor	ALK resistance mutation	RAS/RAF/MEK mutation	KRAS Copy Number Alteration
1	27	F	Ceritinib	ALK F1174C	None[a]	Negative
2	70	M	Crizotinib	None	None[b]	Amplification[d]
3	49	F	Crizotinib	ALK L1196M	None[b]	Duplication[c]
4	42	F	Crizotinib	ALK CNG	None[a]	Duplication[c]
5	40	M	Crizotinib, AP26113	None	Not tested	Duplication[c]
6	54	M	Crizotinib	None	None[a]	Duplication[c]
7	62	F	Crizotinib	ALK G1269A	None[a]	Duplication[c]
8	41	M	Crizotinib	None	None[a]	Negative
9	41	F	Crizotinib	None	None[a]	Negative
10	75	M	Crizotinib	EGFRdel19	None[a]	Duplication[c]
11	52	F	Crizotinib, Ceritinib	None	None[a]	Negative
12	51	M	Crizotinib, AP26113	ALK CNG	None[a]	Negative
13	53	F	Crizotinib	None	None[e]	Amplification[d]
14	41	F	Crizotinib	None	None[e]	Amplification[d]
15	44	M	Crizotinib	None	None[b]	Duplication[c]

[a]KRAS, NRAS, BRAF, MEK by SNaPshot
[b]by direct sequencing for KRAS, NRAS, BRAF, MEK1/2
[c]atypical KRAS FISH pattern including doublets in post-treatment biopsy
[d]KRAS/CEP12 ratio > 2.2 in post-treatment tumor cells
[e]by direct sequencing for NRAS, BRAF, MEK1/2

b.

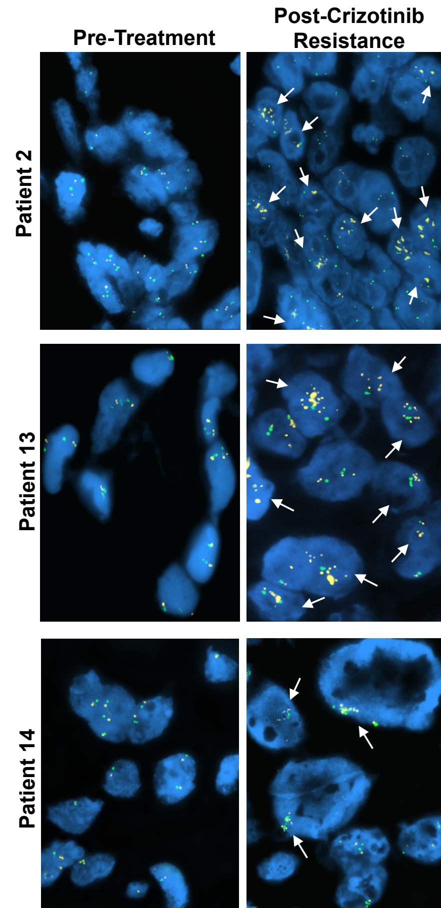


Figure 4. KRAS copy number gain is acquired in ALK TKI resistant patient tumors. a). 15 crizotinib-resistant patient tumors were assayed for presence of KRAS copy number gain (via FISH), RAS pathway alterations, and other known ALK resistance mechanisms (ALK secondary mutations and ALK CNG). 3/15 (20%) had KRAS copy-number gain appear in their resistant tumor (KRAS/CEP12 ratio > 2.2) but was not present in their pre-treatment tumor. Several patients (7/15, 46%) had evidence of KRAS duplication/doublet events. b). FISH analysis of the pre-treatment and post-treatment tumor biopsies from KRAS amplification positive patients (#2, #13, #14). KRAS probes = yellow, CEP12 (control) probes = green.

Figure 5

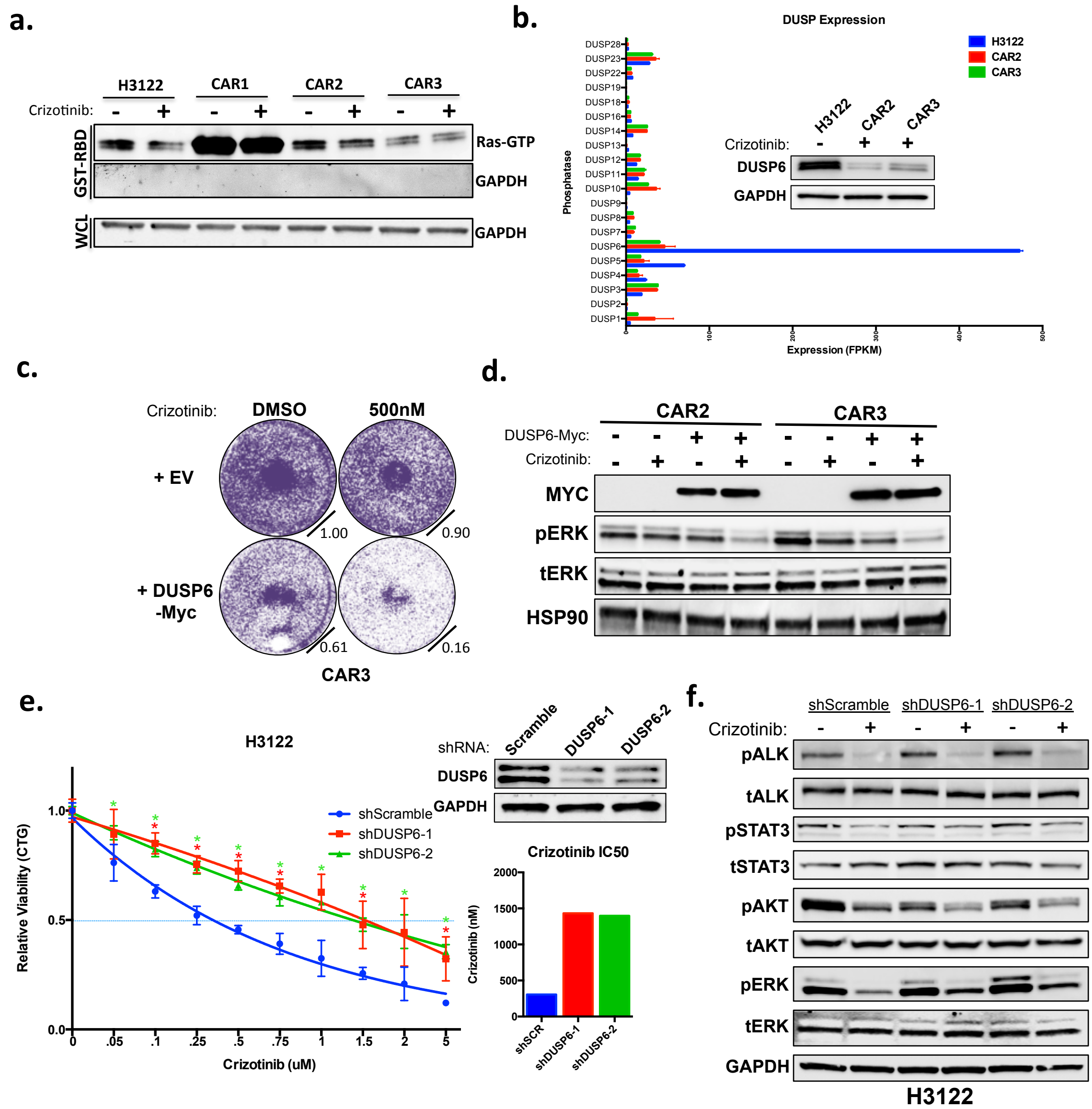
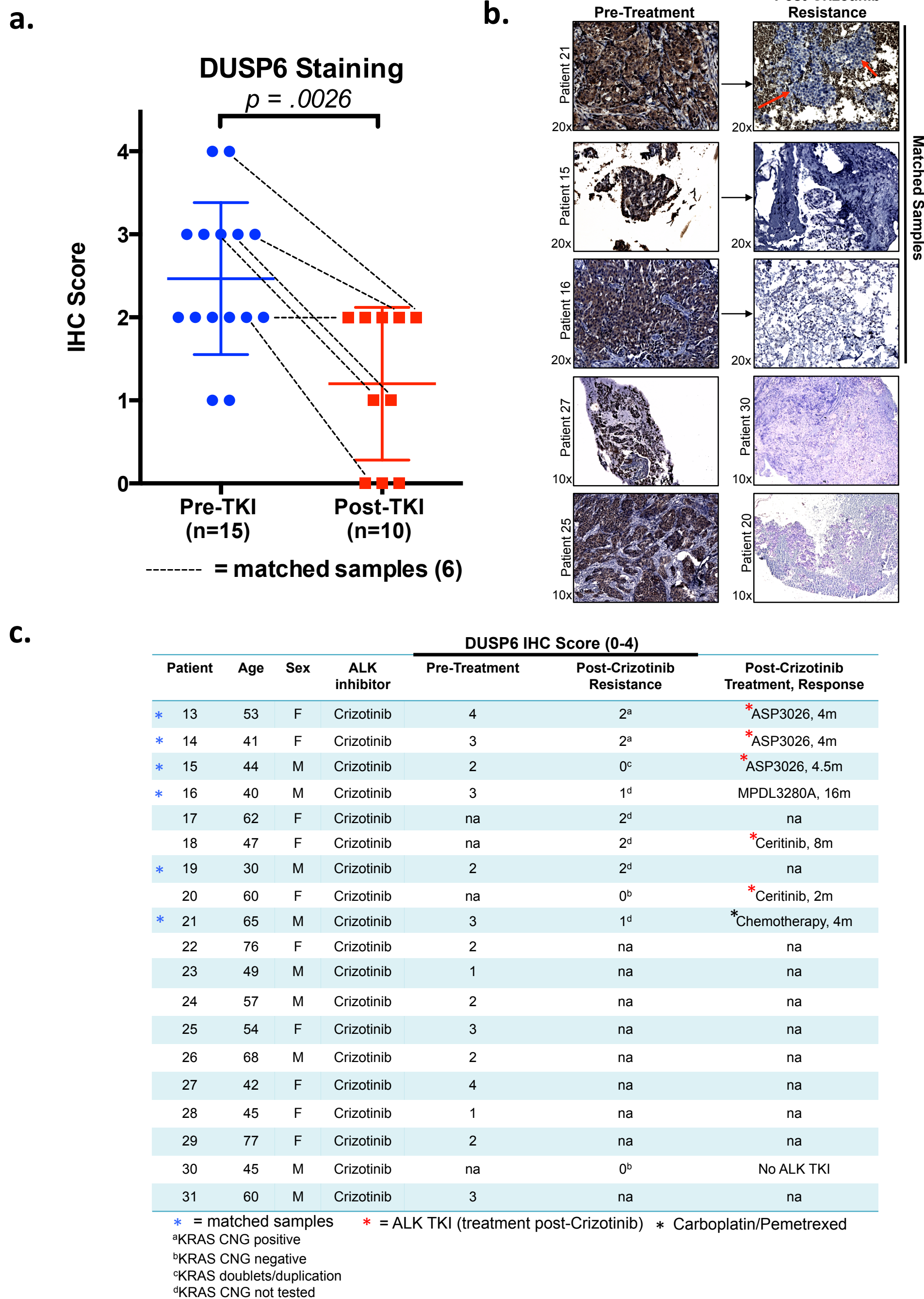


Figure 5. DUSP6 downregulation in models of ALK TKI resistance.
a). GST-RBD pulldowns of H3122 and CAR1-3 show no increase in RAS-GTP levels in CAR2 and 3. b). RNAseq analysis depicting high levels of DUSP6 mRNA in H3122, and downregulation thereof in CAR2/3. Inset shows immunoblot confirming downregulation of DUSP6 protein level in CAR2/3. c-d). DUSP6 reconstitution reduces growth and re-sensitizes CAR3 cells to crizotinib. Immunoblot analysis in (d) shows the effects of DUSP6 reconstitution and crizotinib treatment on ERK phosphorylation. e-f). H3122 cells were transduced with two independent shRNA's targeting DUSP6, and cells were subjected to a 72hr cell-titer glo assay to measure crizotinib sensitivity. Insets in (e) show knockdown efficiency and crizotinib IC50 shift. Immunoblot analysis in (f) depict pERK rescue in the presence of ALK inhibition upon DUSP6 knockdown.

Figure 6



Patient	Age	Sex	ALK inhibitor	DUSP6 IHC Score (0-4) Pre-Treatment	DUSP6 IHC Score (0-4) Post-Crizotinib Resistance	Post-Crizotinib Treatment, Response
* 13	53	F	Crizotinib	4	2[a]	*ASP3026, 4m
* 14	41	F	Crizotinib	3	2[a]	*ASP3026, 4m
* 15	44	M	Crizotinib	2	0[c]	*ASP3026, 4.5m
* 16	40	M	Crizotinib	3	1[d]	MPDL3280A, 16m
17	62	F	Crizotinib	na	2[d]	na
18	47	F	Crizotinib	na	2[d]	*Ceritinib, 8m
* 19	30	M	Crizotinib	2	2[d]	na
20	60	F	Crizotinib	na	0[b]	*Ceritinib, 2m
* 21	65	M	Crizotinib	3	1[d]	*Chemotherapy, 4m
22	76	F	Crizotinib	2	na	na
23	49	M	Crizotinib	1	na	na
24	57	M	Crizotinib	2	na	na
25	54	F	Crizotinib	3	na	na
26	68	M	Crizotinib	2	na	na
27	42	F	Crizotinib	4	na	na
28	45	F	Crizotinib	1	na	na
29	77	F	Crizotinib	2	na	na
30	45	M	Crizotinib	na	0[b]	No ALK TKI
31	60	M	Crizotinib	3	na	na

* = matched samples * = ALK TKI (treatment post-Crizotinib) * Carboplatin/Pemetrexed
[a]KRAS CNG positive
[b]KRAS CNG negative
[c]KRAS doublets/duplication
[d]KRAS CNG not tested

Figure 6. DUSP6 is down-regulated crizotinib resistant patient tumors.
a-b). DUSP6 expression in patient tumors (either pre- or post- crizotinib, of which 6 were matched pairs from the same patient) as measured by IHC assay using a DUSP6 antibody. Representative images are shown in (b). c). Complete patient table of all patient samples stained for DUSP6. Additional available information is listed (KRAS copy number status and subsequent treatment) where available.

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Chapter 4

Combining ALK and MEK Inhibitors in EML4-ALK Positive Lung Cancer

Chapter 4: Combining ALK inhibitors with sub-maximal MEK inhibition enhances response and prevents resistance in EML4-ALK positive lung cancer models.

In most oncogene-driven cancers and ALK-positive lung cancers specifically, most efforts to date aimed at combating ALK inhibitor resistance have focused on characterizing and treating acquired resistance after it has already emerged in the clinic[1]. One alternative emerging strategy to both enhance initial response and combat the development of acquired resistance to targeted therapy is to define and deploy rational upfront polytherapies that target not only primary oncoprotein (such as EML4-ALK) but also additionally a critical effector of the primary oncoprotein. This strategy has been successful in $BRAF^{V600E}$ mutant melanoma patients, where upfront inhibition of the primary driver $\mathrm{BRAF}^{\mathrm{V600E}}$ plus its established primary effectors, MEK1/2, results in substantial clinical activity that is superior to either RAF or MEK inhibitor monotherapy[2,3,4]. In contrast, combined RAF and MEK inhibitor treatment was largely ineffective in melanoma patients with acquired BRAF inhibitor resistance[5]. Together, these clinical data show the importance of upfront rational combination therapy to minimize tumor cell survival and evolution during initial treatment, thereby enhancing initial response and forestalling the emergence of resistance in patients.

In contrast to $\mathrm{BRAF}^{\mathrm{V600E}}$ melanomas that rely predominantly on MAPK pathway signaling, the optimal upfront co-targeting strategy has been less clear in tumors harboring oncogenes that engage multiple effector pathways, such as receptor tyrosine kinases (RTKs). Mutant *EGFR* and *ALK*, *ROS1*, or *RET* gene rearrangements are examples of prominent oncogenic RTKs in lung adenocarcinoma. A rational co-targeting strategy requires an understanding of the signaling events that are most critical for

survival in tumor cells with a particular oncogenic RTK, enabling a context-specific therapeutic strategy to minimize tumor cell survival. To address this knowledge gap, we have previously explored the molecular basis of EML4-ALK oncogene dependence in lung adenocarcinoma (Chapters 1-4) to both improve the fundamental understanding of ALK oncogene function and identify a rational upfront polytherapy strategy to enhance response to ALK inhibition in patients.

Due to the frequency of RAS-MAPK alteration in various cancers, there are over 200 MEK-inhibitor trials ongoing currently in over 16 cancer types[6,7]. Despite the extensive clinical investigation of MEK inhibitors (largely as mono-therapy) since 2000, only trametinib is currently approved for BRAF-V600E mutant melanoma, however binemetinib (MEK162), selumetinib, and cobimetinib are all in late stage trials. Additionally, although ALK inhibitors have been well tolerated in patients thus far, MEK inhibitors have been less so due to the importance of MAPK signaling in numerous normal cell processes[8,9]. MEK inhibitors generally exhibit dermatologic problems (such as rash, dermatitis, and dry skin) ocular toxicities such as central serous retinopathy, low-grade diarrhea, and vascular disorders such as hypertension[9]. Thus, combining MEK inhibitors with other therapies requires careful consideration.

Results

We next explored the potential therapeutic implications of our findings, namely exploiting the MAPK dependency we've uncovered previously. Interestingly, we consistently found that ALK inhibition alone was insufficient to fully abrogate MAPK signaling (**Chapter 1, Fig 1**), likely due to inputs from other RTKs or feedback suppression of MAPK phosphatases[10-12]. Based on this observation and the MAPK

dependency we uncovered, we hypothesized that elimination of this residual MAPK signaling by treatment with sub-maximal doses of MEK inhibitor in combination with an ALK inhibitor may enhance response in EML4-ALK lung adenocarcinoma cells. The use of low dose MEK inhibitor is attractive given the toxicity observed in patients treated with MEK inhibitor monotherapy at doses that completely abrogate MAPK pathway output, particularly when combined with other agents[7,13]. Indeed, we found that the addition of low-dose trametinib (1 nM) was sufficient to sensitize both H3122 and STE-1 cells to ALK inhibition, with polytherapy eliciting markedly greater induction of apoptosis than either monotherapy as measured by PARP cleavage (**Fig 1**). We similarly found that treatment with a distinct MEK inhibitor, selumetinib, or the ERK inhibitor SCH772984, suppressed cell growth and enhanced response to the investigational ALK inhibitor alectinib in H3122 cells (with the combination effect less pronounced with SCH772984 given the marked single-agent effect of this ERK inhibitor, data not shown). In contrast, trametinib (at an even higher dose of 100 nM) did not sensitize EGFR-mutant lung adenocarcinoma cells to the EGFR inhibitor erlotinib (data not shown). The superior efficacy of combined ALK plus MAPK pathway inhibition we observed *in vitro* was further extended and confirmed *in vivo* using H3122 tumor xenografts, in which substantial tumor regressions occurred only in the cohort treated with an ALK inhibitor plus low-dose trametinib (**Fig. 2a, b**). Consistent with our *in vitro* findings, residual MAPK activity was observed *in vivo* in the tumor xenografts treated with ALK inhibitor monotherapy (ceritinib, 25mg/kg) and this residual MAPK signaling was suppressed by the addition of low-dose trametinib (1 mg/kg, **Fig. 2c**). Importantly, whereas mice treated with the maximal tolerated dose (MTD) of trametinib alone (3 mg/kg) exhibited

significant systemic toxicity, the combination of the ALK inhibitor and low-dose trametinib (1 mg/kg) did not cause significant toxicity (**Fig 2d**). We similarly observed superior *in vivo* tumor responses and lack of overt toxicity with combination treatment in mice harboring STE-1 xenografts upon combination therapy with crizotinib and (sub-maximal) trametinib, when compared to each monotherapy (**Fig 3a-c**). Because we noted that introduction of activated STAT3 could modestly decrease ALK inhibitor sensitivity (Chapter 1, Fig 4), we tested whether JAK inhibitor treatment impacted ALK inhibitor response in ALK+ lung adenocarcinoma cells. We found that JAK inhibitor treatment did not impact tumor growth or ALK inhibitor response in EML4-ALK cell lines and tumor xenografts (**Fig 4a, b),** suggesting specificity in the *in vitro* and *in vivo* effects of MEK inhibition on ALK inhibitor response. Together, these findings indicate the potential utility, feasibility, and specificity of combined ALK inhibitor and sub-maximal MEK inhibitor polytherapy to enhance initial response in EML4-ALK lung adenocarcinoma.

In EML4-ALK lung adenocarcinoma cells, MAPK re-activation can potentially occur via a diverse set of mechanisms, including $KRAS^{WT}$ amplification and DUSP6 downregulation (which we uncovered), growth factor and RTK signaling (established previously in ALK+ tumor cells[14-18]), or RAS mutations or NF1 loss (more generally)[19]. Thus, we hypothesized that blocking MAPK signaling upfront would prevent or delay the emergence of resistance in ALK inhibitor-naïve lung adenocarcinoma cells. Indeed, the addition of low doses of trametinib (1 nM) to crizotinib treatment was sufficient to significantly suppress the development of acquired resistance, and the use of a modestly higher dose of trametinib (10 nM) prevented resistance altogether in both H3122 and STE-1 cells *in vitro* (**Fig. 5**). We confirmed these findings *in vivo*, further establishing

that combination therapy with ceritinib and sub-maximal trametinib both enhances the magnitude of initial response and forestalls the development of acquired resistance to ceritinib monotherapy *in vivo* using the STE-1 tumor model (**Fig. 6,** where a higher dose of ceritinib was used to enable the acquired resistance assay *in vivo*). Taken together, the data provide rationale for upfront combination therapy with an ALK and a MEK inhibitor (the latter potentially at a sub-maximal dose) to both enhance initial response and to minimize or eliminate the onset of acquired ALK inhibitor resistance in ALK+ lung adenocarcinoma.

Discussion

Here we demonstrate, in *in vitro* and *in vivo* models of EML4-ALK lung cancer that ALK plus sub-maximal MEK inhibitor combination therapy is well tolerated, enhances initial responses, and prevents resistance to ALK TKI monotherapy. Because of the toxicity historically observed with MEK inhibitors, the sufficiency of low-dose MEK inhibitor to enhance a given ALK inhibitor response is key for clinical translation.

Prior work using a single patient-derived model of ALK inhibitor resistance showed that MEK inhibition might be effective in treating ALK inhibitor resistance after it has already occurred in some patients with an activating MEK mutation (as second-line therapy), or in settings where an ALK inhibitor alone is ineffective[18, 20]. Our study provides new insight by unveiling the unanticipated innate dependence upon RAS-MAPK signaling in EML4-ALK lung adenocarcinoma and its underlying mechanistic basis, and by offering *in vitro* and *in vivo* evidence for an alternative new approach to treating ALK fusion lung adenocarcinoma patients: upfront, first-line ALK and MEK inhibitor combination therapy to induce complete initial anti-tumor responses and

potentially prevent the emergence of resistance to ALK inhibition alone. This upfront combination therapy strategy might convert the generally incomplete and temporary responses observed with crizotinib, and even more potent ALK inhibitors such as ceritinib and alectinib, into complete, sustained remissions in patients.

Because wild-type ALK is not significantly expressed in any normal adult tissue (and moreover has no known function) [21,22], ALK-fusion positive tumors provide a unique opportunity for combination therapies. Since ALK may not have an important physiological role in adult humans, ALK inhibitors theoretically spare broad systemic on-target toxicities. In other words, the primary target in ALK-fusion positive tumors seems to be only actually expressed in the tumor itself. Indeed, selective ALK inhibitors have been tremendously safe to-date, with relatively minor toxicity profiles[23]. This is especially important and beneficial when considering combination with MEK inhibitors, as the MAPK pathway is broadly activated in normal adult cells.

As mentioned previously, our study is ultimately limited by the scarcity of available ALK-fusion positive cell lines. Establishing more ALK-fusion positive cell lines, or patient-derived xenograft (PDX) models are critical in order to expand the utility of our findings more broadly. Recently, a group has reported a protocol and establishment of several ceritinib-resistant cell lines derived from patients[18]. Testing ALK and sub-maximal MEK combination therapy in these models would be helpful in future studies.

Figure 1

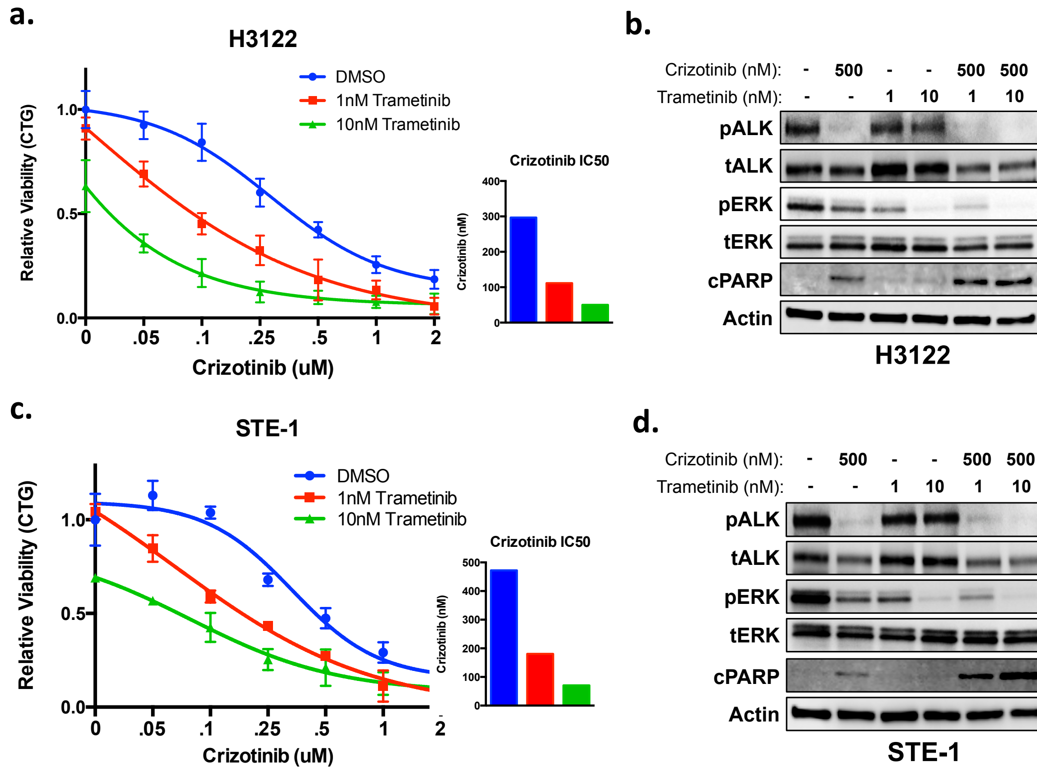

Figure 1. Low-dose MEK inhibitor enhances the efficacy of crizotinib *in vitro*. (**a-d**) Cell titer glo and immunoblot assays in H3122 (**a, b**) and STE-1 (**c, d**) cells treated with crizotinib +/- trametinib (1 nM or 10 nM). Viability assays were performed with a 72-hour Cell-titer glo assay. Insets display crizotinib IC50 values. Immunoblot analysis was performed with indicated antibodies on cells treated with crizotinib (500nM), trametinib (1nM or 10nM), or the combination for 24h.

Figure 2

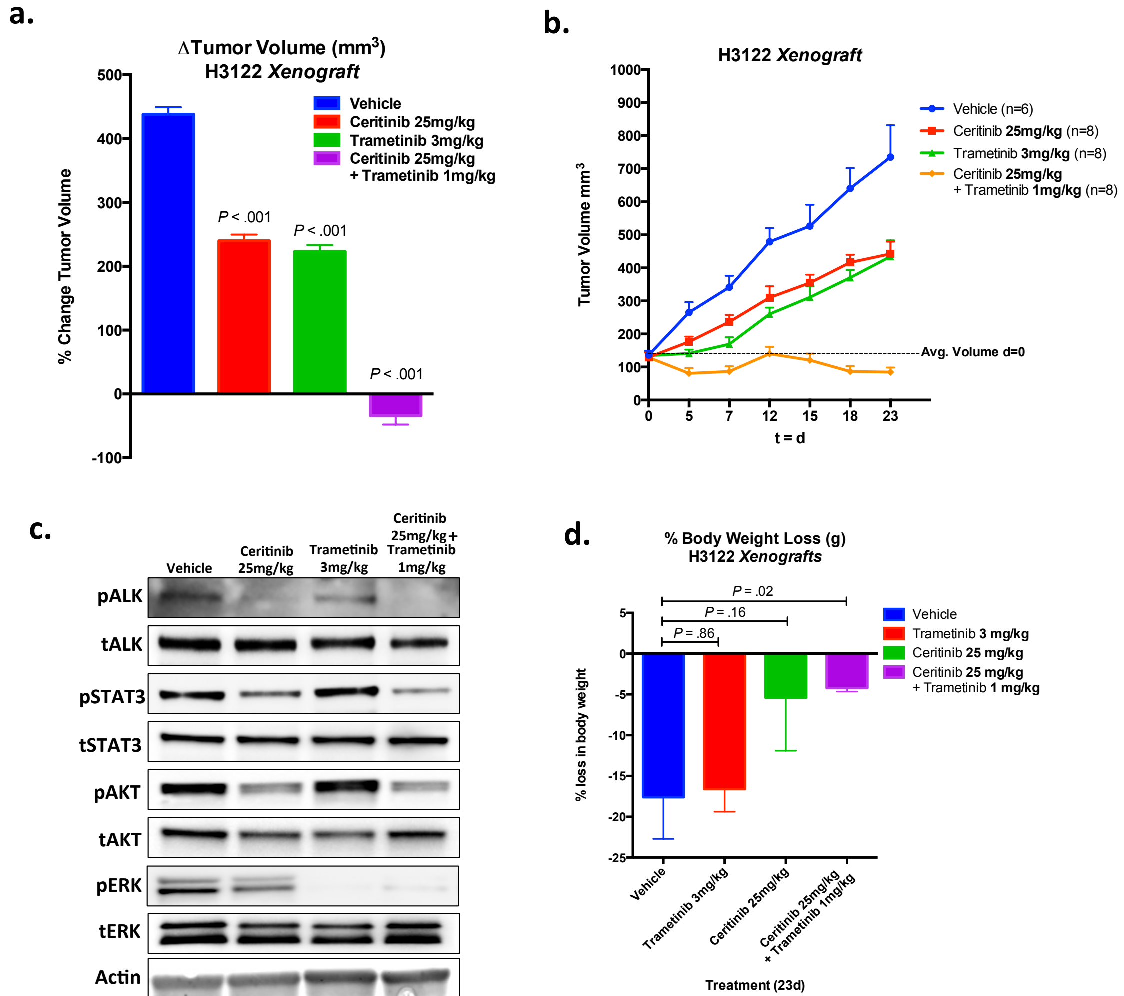

Figure 2. Low-dose MEK inhibitor enhances the efficacy of ceritinib *in vivo*.
(**a-b**). H3122 xenograft tumors were grown on the flanks of mice (n=8 per treatment group) up to ~150mm^3 and then treated with the indicated drugs by oral gavage. Tumor volume and mouse body-weight was measured every 3-5 days. Final tumor change at endpoint from baseline is shown in (a), and measurements from entire experiment are shown in (b). **(c)** Immunoblot analysis with the indicated antibodies in lysates from H3122 xenograft tumors 48h after each treatment. **(d)** Percent body weight loss of mice from (a-c) given indicated treatments. Values represent percent body weight loss from onset of drug treatment.

Figure 3

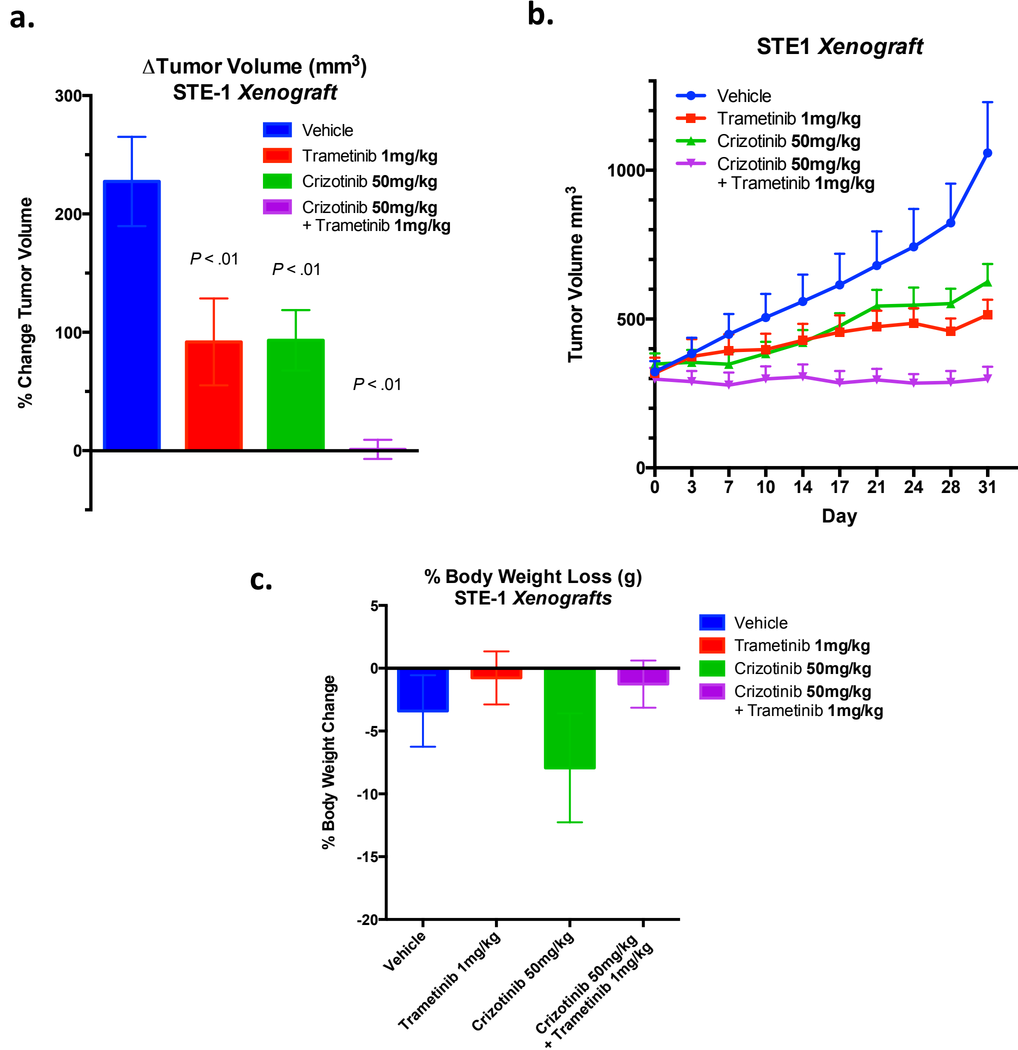

Figure 3. Low-dose MEK inhibitor enhances the efficacy of crizotinib in a novel *in vivo* ALK-positive tumor model.

(**a-c**). STE-1 xenograft tumors were grown on the flanks of mice up to ~300mm^3 and then treated with the indicated drugs by oral gavage. Tumor volume and mouse body-weight was measured every 3-5 days. Final tumor change at endpoint from baseline is shown in (a), and measurements from entire experiment are shown in (b). **(c)** Percent body weight loss of mice from (a-b) given indicated treatments. Values represent percent body weight loss from onset of drug treatment.

Figure 4

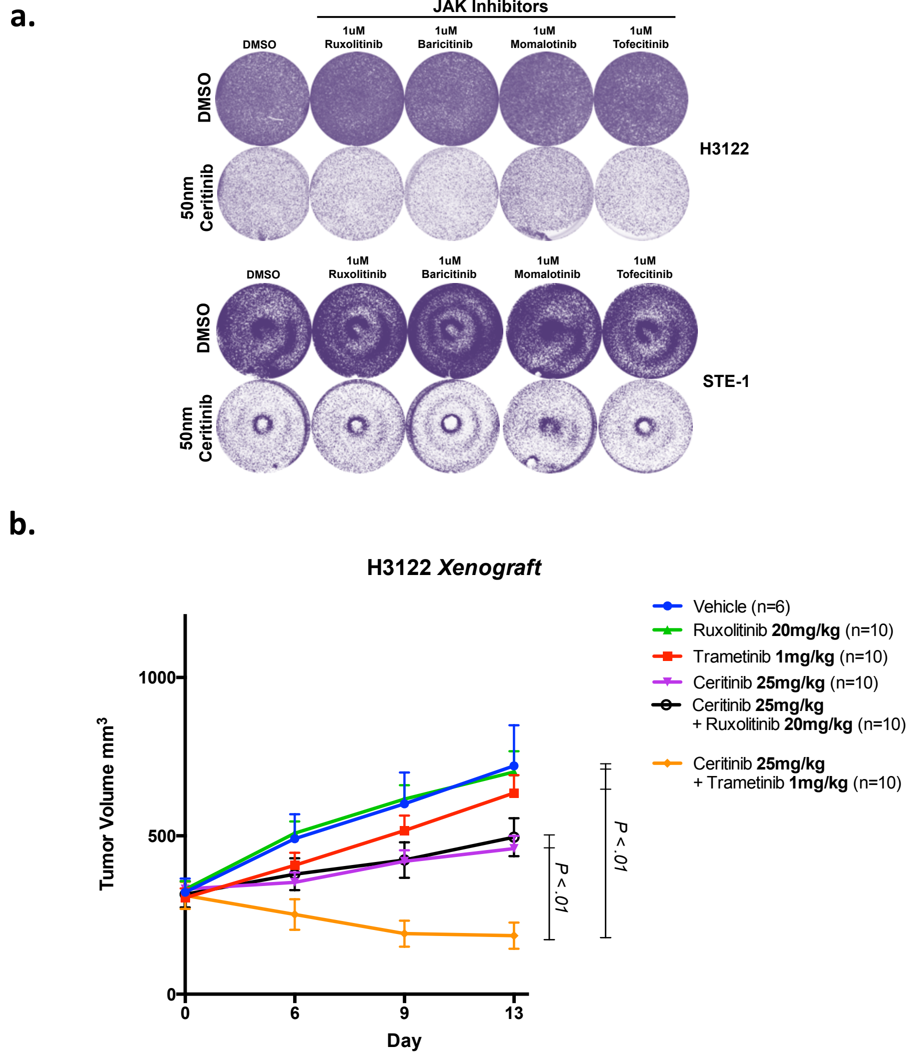

Figure 4. JAK inhibitors do not enhance the effect of ALK inhibitors *in vitro* or *in vivo*. (**a**) Crystal violet assay (5-day) of H3122 and STE-1 cells treat with 50nM ceritinib +/- various JAK inhibitors (indicated). **(b)** H3122 xenografts were grown to ~300mm^3 and then treated with indicated drug combinations by oral gavage, and tumor measurements were taken on indicated days.

Figure 5

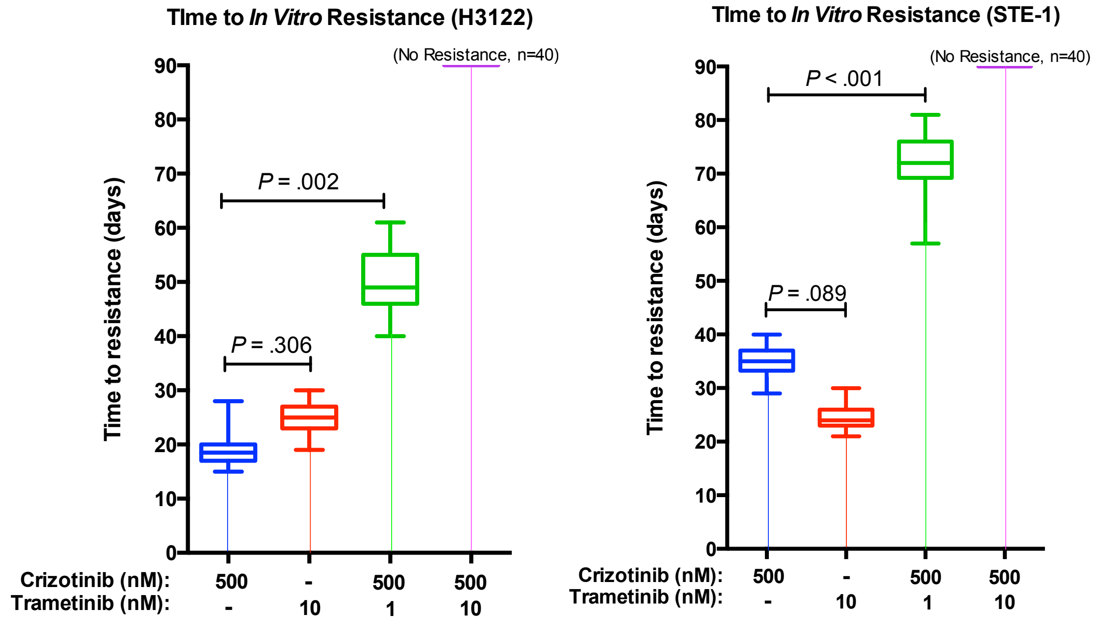

Figure 5. Addition of low-doses of MEK inhibitors forestall resistance to ALK inhibitors *in vitro*.

H3122 (left panel) and STE-1 cells (right panel) were plated in 96-well plates and exposed to the indicated drugs. Fresh media (with DMSO or drug) was replaced every 72-hours. Time (days) until resistance to therapy was observed and recorded. Resistance was defined as cells reaching >90% confluency in their well.

Figure 6

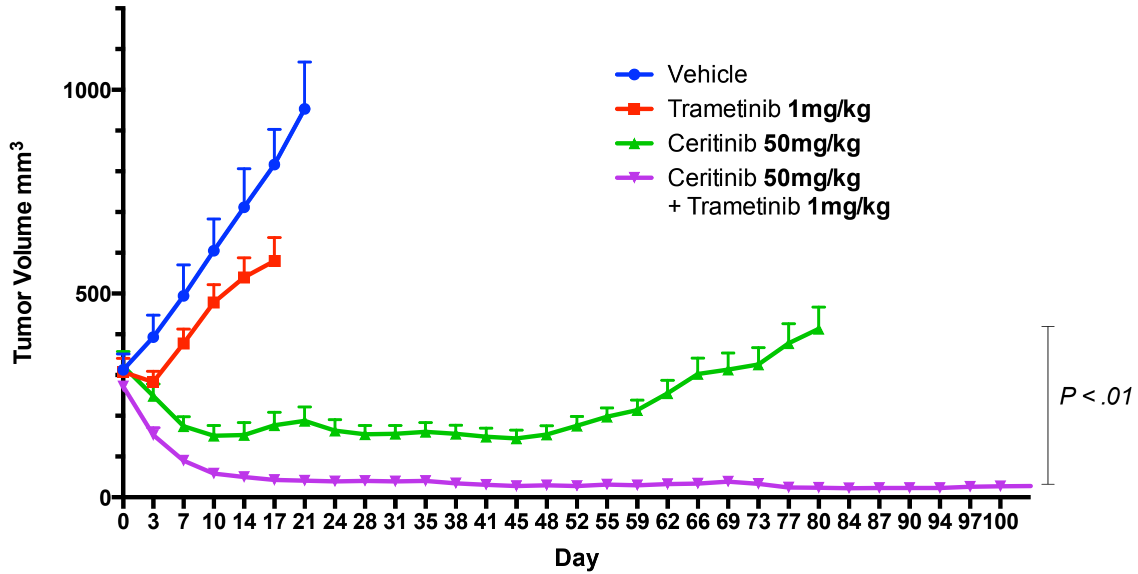

Figure 6. Addition of sub-maximal MEK inhibitor upfront prevents resistance to ALK inhibitor *in vivo*.
Tumor volume (mm^3) of H3122 xenografts during treatment with ceritinib (50mg/kg), trametinib (1mg/kg), or the combination (50mg/kg ceritinib, 1mg/kg trametinib). Values shown are the change in tumor volume from baseline (d=0) $\pm$ SEM. Acquired resistance was defined as tumor regrowth to baseline volumes during continuous (ALK inhibitor) treatment.

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Chapter 5

Conclusion

Conclusion & Discussion

By systematically investigating the molecular basis of EML4-ALK oncogene dependence in lung adenocarcinoma, we establish the RAS-MAPK signaling axis as the primary EML4-ALK effector pathway required for growth and survival in EML4-ALK lung adenocarcinoma cells (**Fig. 1, left panel**). We show for the first time that EML4-ALK activates all three major RAS isoforms, leading to substantial RAS-MAPK signaling output that likely explains the unanticipated specific MAPK signaling dependence we uncovered in EML4-ALK lung adenocarcinoma models. This RAS-MAPK dependence provides a therapeutic opportunity, as sub-maximal MEK inhibitor treatment can enhance the initial anti-tumor effects of ALK inhibitor therapy by dampening RAS-MAPK signaling output (**Fig. 1, middle panels**). This synergistic upfront dual ALK-MEK inhibitor polytherapy can also lead to a more durable response by minimizing the opportunity for tumor cells to survive and then evolve to resistance during initial therapy, thus forestalling the development of acquired resistance (**Fig. 1, middle, right panels**).

The importance of the RAS-MAPK pathway in EML4-ALK tumor survival is highlighted by the resistance mechanisms that emerge upon ALK TKI treatment. Generally speaking, it appears that all confirmed and potential mechanisms of resistance to ALK-inhibition in ALK-fusion positive tumor cells has include rescue of the MAPK pathway. This includes prior work implicating ALK secondary mutations (~30% of cases), upregulation of MAPK-activating bypass tracks such as IGF1R, EGFR, KIT, and HER2, and importantly a case report of an activating MEK mutation[1]. My work has added to the field by implicating genomic amplification (or copy number gain) of KRAS

and/or downregulation of the MAPK phosphatase DUSP6 as clinical mechanisms of resistance to ALK TKI therapy. Importantly, I have uncovered that KRAS genomic amplification occurs in ~20% of crizotinib-resistant patient tumors (second in frequency to the 30% that are explained by secondary ALK mutations). Altogether, my work and others' converges on the RAS-MAPK axis as the central and critical component of EML4-ALK oncogenesis, tumor maintenance, and growth, and thus provides a rational and clinically feasible therapeutic co-target in ALK-positive tumors.

The utility of low-dose (sub-maximal) trametinib is critical for the clinical translation of these findings. In humans, the MAPK-pathway is generally present ubiquitously and is important for various normal physiological processes[2]. Thus, the clinical use of MEK inhibitors has been met with significant toxicity, which ultimately limits their utility both in the monotherapy and combination therapy setting[3]. As of writing, the only approved MEK-inhibitor combinations are with BRAF-V600E inhibitors (such as dabrafenib) in BRAF-mutant melanoma[4]. However, in this indication, the primary target (BRAF-V600E) is only expressed in the tumor cells themselves (dabrafenib does not inhibit wild-type BRAF or RAF1) thus ultimately sparing on-target toxicity in normal cells. Moreover, because MEK lies directly downstream of BRAF-V600E, the amount of MEK inhibition needed to reduce levels to a lethal level is smaller (BRAF-V600E inhibition is already reducing signaling through this pathway). It may be that EML4-ALK positive lung tumors behave in (and can be treated in) much the same way as BRAF-V600E melanomas. EML4-ALK, like BRAF-V600E, is only expressed in the tumor cells, thus an ALK inhibitor would have no systemic on-target toxicity besides a possible role in nervous system development[5]. My studies have shown that MAPK is

downstream of and also the primary effector of EML4-ALK, as it is in BRAF-V600E tumors, thus a small amount of MEK inhibitor would be sufficient for complete abrogation of this pathway in tumor cells specifically (but avoiding complete abrogation of the MAPK pathway in normal cells that do not express EML4-ALK). This is supported by my *in vitro* and *in vivo* data. Low levels of trametinib (1nM) are synergistic with ALK inhibitors *in vitro*, and the combination of sub-maximal 1mg/kg trametinib and ceritinib/crizotinib is well-tolerated in mice (high-dose, 3mg/kg, trametinib is toxic), whilst leading to significantly enhanced tumor responses.

Unfortunately, the clinical development of combination therapies is complicated if the two drugs of interest (drugs A and B) are owned by different pharmaceutical or biotechnology companies. This creates not only pricing issues (very expensive therapy), but also requires both financial and regulatory collaboration between the two companies[6,7]. Thus, as it stands now, it is far easier and more likely to develop a combination therapy when one company owns both drugs A and B. In the case of ALK-MEK, there are fortunately several companies who are able to achieve this. Ceritinib (approved ALK-inhibitor) and trametinib (approved MEK-inhibitor) are both owned by Novartis. Alectinib (ALK inhibitor) and cobimetinib (MEK inhibitor) both belong to Roche. Thus, ceritinib+trametinib or alectinib+cobimetinib combination trials are practically feasible and should be pursued in the ALK-fusion positive front-line setting.

Notably, our data uncover the EML4 component of EML4-ALK (specifically the HELP domain in EML4) as essential for the ability of ALK to engage and signal through RAS to the MAPK pathway, thus establishing MAPK pathway dependence in EML4-ALK lung adenocarcinoma cells. Future studies will more fully explore the role of the

HELP domain in regulating EML4-ALK subcellular localization and signaling. The importance and role of fusion partners in fusion-positive tumor cells is generally poorly characterized. The fusion partner has traditionally been thought to serve mainly as a dimerization domain to facilitate kinase activation. Hence, our studies provide new rationale to explore the function of the fusion partner in the engagement of downstream signaling components by oncogenic fusion proteins more generally (including ROS1, RET, other ALK fusion variants such as KIF5B-ALK).

For example, although ROS1-positive tumor cells are sensitive to ROS1 inhibition, the relative importance of effectors that operate downstream of ROS1 has not been explored[8]. Like ALK fusions, ROS1 fusions have multiple known fusion partners, most notable FIG, SLC34A2, CD74, CCDC6, and others[9-12]. However, unlike ALK fusions, the trans-membrane domain of ROS1 is sometimes included in the fusion product[13]. Thus, it would be interesting to see if the identity of the fusion partner affects the level of “oncogene addiction” or clinical responses.

My studies also provide insight as to why fusion-RTK positive patients (ALK, ROS1, RET, and more recently NRTK1) have had such substantial clinical responses to date. In a phase II trial in NSCLC, crizotinib (which is also a ROS1 inhibitor) showed a 19-month median PFS in ROS1-fusion positive patients[14]. Next-generation ROS1 inhibitors are predicted to have even better responses[15]. Likewise, RET-fusion positive patients have responded to cabozantinib, and thus paved the way for clinical development numerous selective RET-inhibitors[16,17]. It is perhaps the unique biology and potent oncogenic capacity of RTK-fusions that allow them to be such clinically exploitable targets. It is critical to explore if RTK-fusions behave similar to EML4-ALK more

generally.

Kinase-fusions are constitutively active kinases by virtue of ligand-independent dimerization induced by the fusion partner (kinase-fusions are hyper-active)[18]. The expression of these kinases is also controlled by the regulatory elements of the fusion partner, which is typically a highly expressed housekeeping gene (kinase-fusions are highly expressed)[18]. A secondary feature of this "partner-sponsored" regulation is that it leads to high expression of an active kinase which is often not normally expressed in the cell-type of origin (engagement of non-native effector pathways/complexes/cell processes, such is the case with ALK/ROS1/RET/NTRK1 in lung epithelial cells)[18]. Lastly, the fusion partner leads to mis-localization of the kinase, allowing to it engage and activate effectors outside of the local plasma-membrane environment (kinase-fusions are note sequestered to one cellular compartment). All of these features lead to a unique but highly potent oncogenic signaling output that may negate the need to develop further oncogenic events for tumor initiation. This is also supported by the fact that RTK-fusion positive NSCLC's are typically genomically quiet outside of the gene-fusion event (they have low mutation burden and low frequency of tumor suppressor loss)[19]. In this setting, one could imagine a true "dependence" on the RTK-fusions for initiating, maintaining, and driving the oncogenic state. Therefore, wide adoption of appropriate diagnostic measures (RNAseq, exome, FISH, or RT-PCR) are needed in order to identify the presence of RTK-fusions, which include but are not limited too ALK, ROS1, RET, NTRK1-3, FGFR1-4, MET, AXL, PDGFRA-B, SYK, JAK2, ABL, RAF1, BRAF, and EGFR[20,21]. The biology of these fusions (with the exception of EML4-ALK fusions from this thesis) are not studied and thus require further investigation. I would argue, however,

that kinase-fusions represent the most clinically exploitable genetic events in human cancer.

In summary, the functionally essential pathway(s) downstream of oncogenic EML4-ALK has not been defined. Here, we unexpectedly identify the RAS/MEK/MAPK axis as critical in this regard. Filling this mechanistic knowledge gap by uncovering the unanticipated RAS-MAPK dependence in EML4-ALK lung cancer cells allowed us to then test and validate a new therapeutic hypothesis and propose a novel upfront combination therapy approach for immediate testing in EML4-ALK lung cancer patients. Our findings show the utility of deciphering signaling pathway dependencies operating downstream of oncogenic receptor kinases, and have implications for not only understanding and targeting EML4-ALK signaling but also oncogenic receptor kinase signaling in cancer more broadly.

Figure 1

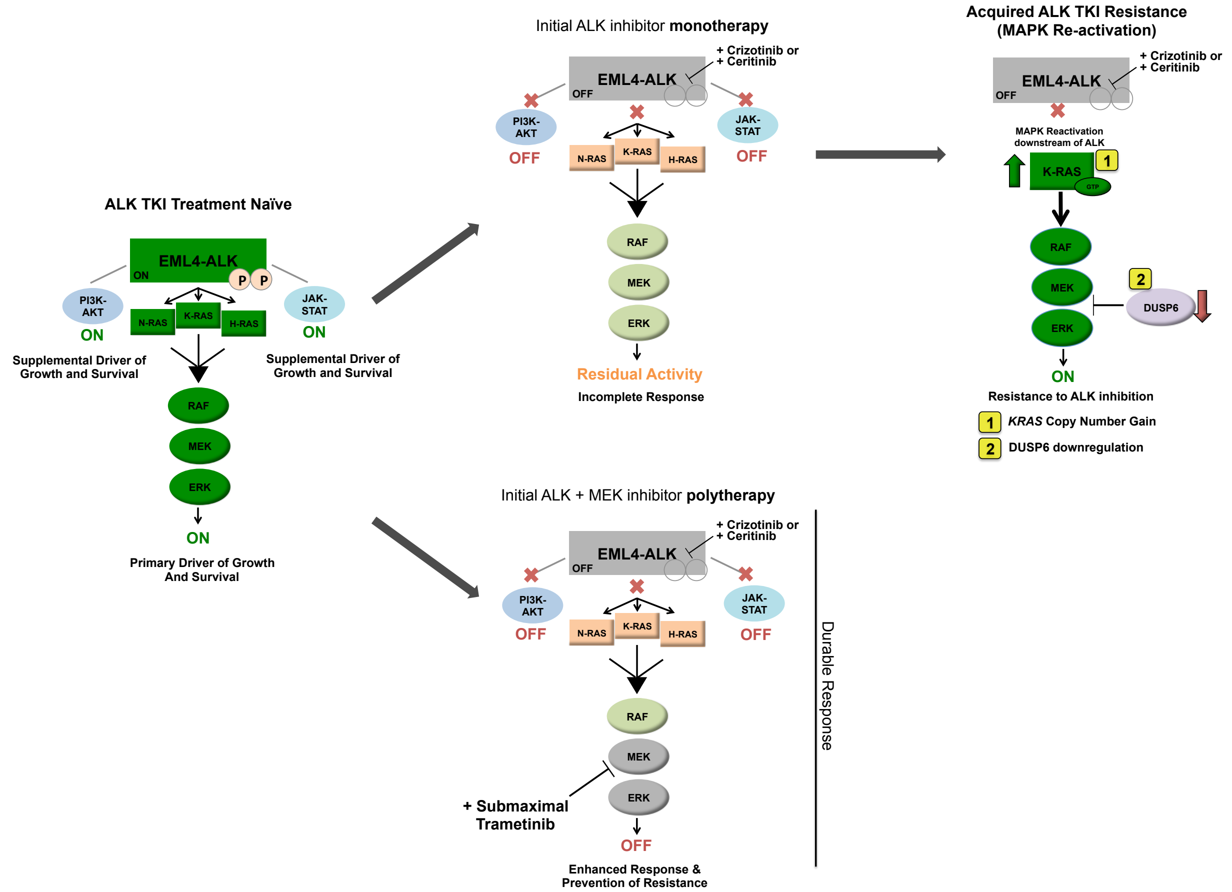

Figure 1. Model depicting the new model for EML4-ALK oncogene dependence. EML4-ALK variant 1 expressing cells are dependent primarily on RAS-MAPK signaling. Shown is the mechanism of enhanced efficacy of combined ALK and (sub-maximal) MEK inhibitor treatment. In the treatment naïve setting, EML4-ALK engages RAS-MAPK signaling as the primary downstream effector pathway as well as other supplemental pathways (including PI3K-AKT and JAK-STAT signaling) to drive tumor cell growth and survival (**left panel**). Upfront ALK monotherapy leads to an incomplete response and tumor cell survival due to residual MAPK activity (**middle top panel**). Eventually, these cells acquire resistance to ALK monotherapy by fully rescuing MAPK downstream of EML4-ALK via (1) $KRAS^{WT}$ copy number gain or (2) downregulation of the MAPK phosphatase DUSP6 (**right top panel**). In contrast, initial ALK + MEK inhibitor polytherapy abrogates this residual MAPK kinase activity to promote greater and more durable upfront responses by minimizing tumor cell survival and MAPK signaling re-activation (**middle bottom panel**).

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Chapter 6

Materials & Methods

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Cell culture. All cell lines were maintained in humidified incubators with 5% CO2 at 37C. The isogenic crizotinib resistance cell lines (CAR1-3, LAR1-3) were maintained in 1uM crizotinib or 200nM ceritinib, respectively. The human lung adenocarcinoma cell lines H3122, STE-1, H2228 were maintained in RPMI-1640 supplemented with 10% FBS and penicillin-streptomycin 100ug ml^{-1}. Derivation of STE-1 was described previously[9]. All cell lines were confirmed to have presence of EML4-ALK via cDNA sequencing and tested negative for mycoplasma contamination.

Compounds. Crizotinib (Selleck Chemicals, Houston, TX, USA), ceritinib (Selleck Chemicals, Houston, TX, USA), trametinib (Selleck Chemicals, Houston, TX, USA), selumetinib (Selleck Chemicals, Houston, TX, USA), ruxoltinib (Selleck Chemicals, Houston, TX, USA), BKM120 (Selleck Chemicals, Houston, TX, USA), BYL719 (Selleck Chemicals, Houston, TX, USA), MK2206 (Selleck Chemicals, Houston, TX, USA), GDC0941 (Selleck Chemicals, Houston, TX, USA) were dissolved in DMSO.

Cell viability and apoptosis assays. For cell titer glo (Promega, Madison, WI, USA) viability experiments, cells were seeded in 96-well plates at 5000 cells/well and exposed to drugs on the following day. At 72h after drug addition, cell titer glo reagent was added and luminescence was measured on a Spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) according the manufacturer instructions. All experimental points were set up as octuplicate replicates. Data are presented as percentage of viable cells compared to control (vehicle treatment) cells. Growth assays were performed via crystal violet staining and quantification. Briefly, cells were seeded in 6-well plates and grown in presence of drug. At 5d-7d, cells were fixed with 4% PFA

and stained with crystal violet. Pictures of stained cells were taken using GE Imagequant. For quantification, stained wells were dissolved with 1% SDS solution, and 470nM absorbance was measured using Spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) and values (denoted as OD) were normalized to control (vehicle treated) cells. All crystal violet images are representative at least three individual experiments. For the lung cancer cell line panel apoptosis assay, 10,000 cells were plated in 96-well plates (triplicates) and exposed to drug the following day. After 24h, caspase-glo reagent (Promega, Madison, WI, USA) was added and luminescence was measured using Spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Values were normalized to vehicle treated cells. Statistical significance was assessed by two-sided unpaired t-tests of means calculated by Graphpad and reported as the P-value for significance.

Generation of TKI-resistant cell lines. To create crizotinib and ceritinib resistant cell lines (Supplementary Fig. 5a), H3122 cells were cultured with increasing concentrations of drug, starting with 250nM crizotinib or 50nM ceritinib, respectively. When cells began proliferating at normal rates, doses of drug were doubled until 1uM crizotinib or 200nM ceritinib was reached. Fresh drug was added every 72-96h. Both sets of resistant lines (CAR, LAR) took approximately 4 months to generate. Exome sequencing comparing parental and resistant lines confirmed generated cell lines were from the same origin. Resistant cell lines (as polyclonal populations) were maintained continuously in TKI.

***In vitro* resistance assay.** Assay was adapted from previous studies[24]. Briefly, cells were plated in 96-well plates at 7500 cells/well (~40% confluency) and drug treatments began the following day. Each treatment group had 40 replicates, and drug was replaced every

72-96h. Resistance was defined as time (in days) for a well to become confluent (90%) in the presence of treatment.

Antibodies and immunoblotting. The following antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA): ALK, pY1604 ALK, STAT3 123H6 (#9319), pY705-STAT3 D3A7 (#9145), AKT (#9272), pS473-AKT D9E (#4060), ERK1/2 (#3493), pT202/Y204-ERK1/2 D13.14.4e (#4370), RAS, DUSP6, cPARP 19F4 (#9546), S6, pS6, $BRAF^{V600E}$, cCaspase 3, horseradish peroxidase (HRP)-conjugated anti-mouse and HRP-conjugated anti-rabbit. The following antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA): GAPDH, Actin, H-RAS, N-RAS, K-RAS.

For immunoblotting, cells were washed in PBS and lysed and scraped with 25mM Tris-HCL (ph 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS supplemented with cOmplete protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and PhosSTOP phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Where indicated, RAS GST-RBD pulldowns were performed on lysates prior to running western blot. Lysates were subjected to SDS-PAGE followed by blotting with indicated antibodies and detection via ECL Prime (Amersham Biosciences, Sunnyvale, CA) or Odyssey Licor (Lincoln, NE, USA) with infrared-conjugated secondary antibodies (IR Dye 800, IR Dye 680).

siRNA experiments. Transfection of siRNA was performed with Dharmafect reagent (Dharmacon ,USA). Smartpool siRNA used in experiments were KRAS, NRAS, HRAS and were purchased from Thermo Scientific and used according to the manufacturer's protocol.

Xenograft studies. H3122 tumor xenografts were generated by injection of $2x10^6$ cells in matrigel in 8-week old NOD/SCID mice. Mice were randomized to treatment groups once tumors reached an average of $150mm^3$ (Fig. 3e, n=8 per treatment group) or $300mm^3$ (Fig. 6c, n=10 per treatment group, Fig. S6c, n=10 per treatment group). Ceritinib was administered at 25mg/kg or 50mg/kg p.o. daily for 5d. Trametinib was administered at 1mg/kg or 3mg/kg p.o. daily for 5d. Ceritinib 25mg/kg or Ceritinib 50mg/kg and Trametinib 1mg/kg were administered together p.o. daily for 5d. STE-1 tumor xenografts were generated by injection of $3x10^6$ cells in matrigel in 8-week old NOD/SCID mice. Mice were randomized into treatment groups once the tumors reached an average of $300mm^3$ (Fig. 3g, n=8 per treatment group). Crizotinib 50mg/kg was administered with Trametinib 1mg/kg or vehicle together p.o. daily. The vehicle for all drugs was .5%methylcelluse/.5% Tween-80.

Viral transduction. Expression vectors for GFP, Myr-AKT, $BRAF^{V600E}$, KRAS-G12C/D/V, MEK-DD were in pBABE retroviral expression plasmids and obtained from Addgene (Cambridge, MA, USA). The KRAS-G12C and –G12D cDNAs contained the HA-tag (Addgene #58901, #58902). EF.STAT3C.Ubc.GFP (CA.STAT3) was obtained from Addgene (Cambridge, MA, USA, plasmid #24983). DUSP6-Myc plasmid was obtained from Addgene (#27975) and inserted into pBABE retroviral expression plasmid. pLenti-KRAS-4B (WT) was a kind gift from Dr. Frank McCormick. The viral titration experiments were performed by exposing H3122 cells to different volumes (concentrations) of viral supernatant (1.0 indicates to 1mL of viral supernatant in 4mL media added). EML4-ALKv1-WT-Myc and EML4-ALK-dHELP-Myc were cloned into the pcDNA plasmid backbone and were kind gifts from Dr. Hiroyuki Mano. 293-GPG

viral packaging cells were transfected with pBABE constructs using Lipofectamine-2000 (Life Technologies, Pleasonton, CA) per manufacturers instructions. Virus containing media was harvested three days post transfection and used to transduce cancer cell lines incubated with 6ug/ml polybrene for 48 hours. Media was changed to standard growth media and puromycin (1ug/ml for H3122, .5ug/ml for STE-1) was added for selection.

shRNA experiments. Lentiviral shRNA (pLKO) plasmids for DUSP6 (1,2), KRAS (1,2) were obtained from Sigma. DUSP6 [(sh#1), (sh#2)] and KRAS[(sh#1), (sh#2)]. Virus was produced by transfection of 20ug pLKO.1-shRNA and 20ug Virapower mixed packaging plasmids. Selection with puromycin was started 48-72h post viral transduction, after which point experiments were performed.

DNA transfections. Transfection of EML4-ALK plasmids into 293T and Beas2B cells was performed using FuGene 6 (Promega, Madison, WI, USA).

RNA analysis. RNA-seq was performed in triplicate for each cell line on Illumina Hi-Seq 2000 using paired-end 100bp reads as described[42]. Reads were aligned and quantified using RSEM. Differential expression analysis between sets of conditions was performed using DESeq and as described[42]. Quantitative PCR (qPCR) was performed on the QuantStudio 12K Flex Real-Time QPCR System using Taqman probes (Applied Biosystems, Life Technologies) specific to the coding regions of the genes assessed.

RAS activation assay. RAS GST-RBD activation kit was obtained from Cytoskeleton (Denver, CO, USA), Cat #BK008. Protocol was per manufacturers instructions. Lysis Buffer for RAS-GTP pulldowns was 50mM Tris (ph 7.5), 10mM $MgCl_2$, .5 M NaCl, and 2% Igepal. Snap-freezing of lysates was performed directly after lysis using liquid nitrogen baths.

Immunofluorescence. Endogenous EML4-ALK staining (in H3122 and STE-1) was performed using an ALK antibody (XP, Cell Signaling Technology). Cells were fixed with 4% PFA and incubated with .1% Triton-X and mounting media used was Prolong Gold + DAPI (Life Technologies). Secondary antibodies (Alexa Fluor 596) were purchased from Thermo Scientific. In Beas2B cells transfected with EML4-ALK, pcDNA-EML4-ALKv1-Myc and pcDNA-EML4-ALK-dHELP-Myc constructs were used. Cells were fixed with 4% PFA and incubated with .1% Triton-X. Primary incubation was done with anti-Myc antibodies obtained from Cell Signaling Technology, and mounting media used was Prolong Gold + DAPI (Life Technologies). Secondary antibodies (Alexa Fluor 596) were purchased from Thermo Scientific.

Immunohistochemistry. 10 micron thick formalin-fixed paraffin embedded (FFPE) tissue sections were stained with the DUSP6 rabbit monoclonal antibody (AbCam ab76310, 1:100 dilution). All stained slides were imaged (at 10x and 20x) and tumor cells were identified and scored (by three independent, blinded, individuals) from 0-4 in order of increasing staining intensity. "0" represents no visible DUSP6 staining in tumor cells.

FISH. Fluorescence *in-situ* hybridization (FISH) assays were performed as previously described[46]. Specimens were classified as KRAS amplification positive if KRAS/CEP12 ratio was > 2.2 or when >20% tumor cells carried clusters of >15 copies per cell. All called amplifications (patient 2, 13, 14) met both criteria.

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