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Localization-based Signaling Pathway Dependence of ROS1 Fusions and a Novel Role for Ras in EML4-ALK-Driven Lung Cancer



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# Localization-based Signaling Pathway Dependence of ROS1 Fusions and a Novel Role for Ras in EML4-ALK-Driven Lung Cancer

by

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DISSERTATION

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This dissertation is dedicated to Drew and my Dad,
the two most wonderful gentlemen

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## Contributions to presented work

### Chapter 2.

I designed and performed all biochemical and functional studies described in this chapter under the supervision of Trever Bivona. Victor Olivas and Golzar Hemmati assisted with the *in vivo* experiments.

### Chapter 3.

I performed the biochemical, functional, and imaging studies described in this chapter in collaboration with Asmin Tulpule and Juan Guan under the supervision of Trever Bivona and Bo Huang. Gorjan Hrustanovic and Asmin Tulpule performed the experiments described in Figure 1 and Figure 6. Juan Guan generated the mNG-A-RAF BEAS-2B lines used in Figure 8.

This chapter contains previously published material (Figures 1 and 6) from: Hrustanovic G., Olivas V., Pazarentzos E., Tulpule A., Asthana S., Blakely C.M., Okimoto R.A., Lin L., Neel D.S., Sabnis A., Flanagan J., Chan E., Varella-Garcia M., Aisner D.L., Vaishnavi A., Ou S.H., Collisson E.A., Ichihara E., Mack P.C., Lovly C.M., Karachaliou N., Rosell R., Riess J.W., Doebele R.C., Bivona T.G.. (2015) RAS-MAPK dependence underlies a rational polytherapy strategy in EML4-ALK-positive lung cancer. *Nature Medicine* 21(9):1038-47

**Chapter 4.**

This chapter contains previously published material from Neel D.S. and Bivona T.G. (2017) Resistance is futile: overcoming resistance to targeted therapies in lung adenocarcinoma. *npj Precision Oncology* 1(3).

## Abstract

### Localization-based Signaling Pathway Dependence of ROS1 Fusions and a Novel Role for Ras in EML4-ALK-Driven Lung Cancer

Dana Neel

Receptor tyrosine kinase (RTK) fusions are recently described drivers in lung adenocarcinoma that arise from chromosomal rearrangements, resulting in the C-terminal kinase domain of an RTK attached to a variety of N-terminal fusion partners. Here, we elucidate the importance of N-terminal partners of ROS1 and ALK fusions in driving oncoprotein localization and downstream signaling pathway activation. ROS1 gene fusions involve rearrangements with several 5' genes, and we have discovered that the resultant N-terminal partner mediates the intracellular localization of the fusion. This localization, in turn, dictates which downstream pathways these fusions are able to engage, which determines signaling pathway dependency in ROS1 fusion-positive cells. We find that some ROS1 fusions are present on endosomes and are able to activate the RAS/MAPK pathway, while the most common fusion, CD74-ROS1, is found on the endoplasmic reticulum (ER), where it is unable to engage this pathway. Mislocalization of CD74-ROS1 from the ER to the endosome results in activation of the MAPK pathway by this fusion, demonstrating that localization is critical in driving downstream signaling pathway activation. These findings have clinical significance, as we reveal that ROS1 fusions that can activate MAPK form more aggressive tumors and are less responsive to the targeted ROS1 inhibitor crizotinib *in vivo*. The most common ALK fusion seen in lung cancer is EML4-ALK variant 1 (v1), and here we demonstrate that EML4-ALKv1

exists in intracellular non-membrane-associated clusters, and that this localization and cluster formation is dependent on oligimerization domains present within EML4. Additionally, we reveal that EML4-ALKv1 is able to engage the RAS/MAPK pathway despite the absence of a membrane, which runs counter to the canonical idea that RAS requires a membrane scaffold for downstream pathway activation. In summary, my findings demonstrate both the importance of N-terminal RTK fusion partners in driving downstream oncogenic signaling pathways, which may inform therapeutic approaches for treatment of ROS1 and ALK fusion-positive patients, and uncovers a novel signaling role for cytoplasmic RAS, representing a shift in how we understand a central node of signaling biology.

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# Chapter 1

Introduction

**Introduction: ROS1 and ALK fusions in lung cancer**

Lung cancer is the leading cause of cancer-related death in the United States.[1] The majority (85-90%) of all lung cancers can be characterized as non-small cell lung cancer (NSCLC), which can be further classified as adenocarcinoma, squamous cell carcinoma, or large cell carcinoma.[2] Until recently, lung cancers were almost solely described and treated based on their histopathologic characteristics. About 30 years ago, mutations in the relatively "undruggable" RAS proteins (primarily K-RAS) were identified in approximately 30% of lung adenocarcinomas, but the driver oncogene in the remaining 70% remained unknown.[3-5] However, advances in genomics over the past decade and a half have revealed several new oncogenic drivers **(Figure 1)**. These drivers include activating mutations in the kinases *EGFR* (~20%), *BRAF* (~3-4%), *PIK3CA* (1-3%), and *ERBB2* (2-4%).[6-13] Identification of these drivers has led to the development of specific, targeted small molecule kinase inhibitors, some of which offer great improvement in survival over traditional chemotherapeutics.[14-19]

Another class of lung cancer drivers not appreciated until recently result from chromosomal rearrangements, fusing a variety of 5' partners onto genes encoding receptor tyrosine kinases (RTKs), producing oncogenic RTK fusions. These include fusions involving the RTKs ROS1, ALK, RET, NRG1, and NTRK1.[20-28] There are several ways a fusion partner may impart oncogenic potential on a kinase **(Figure 2)**.[29] 1) The fusion partner can drive overexpression of the kinase: aberrant expression of a kinase leads to increased activation of downstream signaling pathways, which can lead to higher levels of otherwise normal pro-growth and anti-apoptotic signaling.[30] 2) A fusion partner can promote ligand-free oligimerization of the kinase: typically, an RTK requires

ligand binding to drive receptor dimerization, which enables activating transphosphorylation.[31] In this way, downstream signaling pathways driving proliferation are carefully linked to extracellular signaling. When an RTK fusion partner can promote RTK oligimerization, often through a dimerization domain contained within the N-terminal partner, in the absence of an extracellular signal, the tightly linked regulation of extracellular signals and cell growth and survival is broken.[32-38] 3) Fusions can relieve autoinhibitory structures that suppress kinase activity. Typically, RTKs contain an inhibitory domain in the juxtamembrane region which results in the RTK adopting an autoinhibitory confirmation.[39] In the absence of a ligand, which drives dimerization and relief of this confirmation, normal RTKs will exist in this autoinhibited state. Chromosomal rearrangements and fusion to an N-terminal partner can result in deletion of this domain, allowing the RTK to take an active confirmation in the absence of ligand.[40,41] 4) Fusions can result in mislocalization of the kinase. Often, activation of RTKs at the plasma membrane also leads to ubiquitination, internalization, and degradation of the RTK, thus terminating downstream signaling.[31] Mislocalization of an RTK off of the plasma membrane can disrupt this normal internalization and degradation pathway, leading to prolonged activation of RTK-mediated signaling.[42] Additionally, evaluation of the FIG-ROS1 glioblastoma fusion suggested FIG-mediated mislocalization to the golgi could drive oligimerization of the fusion, promoting downstream signaling.[43] Understanding the mechanism by which a particular N-terminal RTK fusion partner promotes oncogenesis could be critical in designing therapies to block this mechanism that could be using in combination with more classical small molecule tyrosine kinase inhibitors. My thesis project thus focuses on oncogenic fusion

kinases involving ROS1 and ALK, and more specifically on the importance of the N-terminal fusion partner in fusion localization, signaling, and pathway dependency.

ROS1 fusions are found in approximately 1% of all lung adenocarcinoma patients.[20,23,44] These fusions are commonly found in patients who are younger, Asian, and frequently never-smokers.[23,45] ROS1 is one of the few remaining orphan receptor tyrosine kinases; that is, its ligand is unknown. However, the large extracellular domain has high sequence homology to the extracellular matrix (ECM) protein fibronectin, suggesting ECM proteins may serve as ROS1 ligands.[46] The wildtype function of ROS1 is also not well known – ROS-null mice are viable and apparently normal, with the exception of male infertility.[47] ROS1 was originally discovered over half a century ago as the oncogene responsible for tumorigenesis mediated by the avian sarcoma RNA virus UR2.[48,49] Intriguingly, comparison of the proto-oncogene c-ROS1 and its oncogenic counterpart v-ROS1 revealed loss of the extracellular domain in v-ROS, suggesting that loss of this domain may somehow dysregulate ROS1 activity and lead to oncogenesis.[50] This may have implications for the mechanism of oncogenicity of ROS1 fusions seen in human cancers, as all ROS1 fusions in human tumors display loss of virtually all of the extracellular domain of ROS.

ROS1 fusions contain the entire C-terminal kinase domain of ROS, fused to a wide variety of N-terminal fusion partners.[23,44,51] To date, over a dozen N-terminal fusion partners have been identified. These N-terminal partners include CD74, SLC34A2, SDC4, TPM3, EZR, LRIG3, CCDC6, and FIG.[20,22,52-55] The N-terminal fusion partners lack any clear relationship to one another – that is, they don't share similar protein domains or functions. This raised the possibility that not all ROS1 fusions behave the

same. Unfortunately, due to the lack of a known ligand, analysis of pathways activated downstream of wildtype ROS1 have been limited. Most of the signaling information for ROS1 come from studies utilizing chimeric EGFR-ROS1 receptors, where the extracellular ligand-binding domain of EGFR was fused to the C-terminal end of ROS. These studies suggested a wide range of signaling pathways can be activated downstream of ROS, including the RAS/MAPK, PI3K/AKT, VAV3, and STAT3 pathways.[56-60] However, they also revealed that small variations in the protein sequences of these chimeric ROS1 receptors could drastically alter their downstream signaling and transformation potential, again suggesting that a one-size-fits-all approach to understanding ROS1 fusions may be misguided.[57]

One of the most studied ROS1 fusions is FIG-ROS, a fusion discovered in a glioblastoma cell line which is also found, albeit infrequently, in lung adenocarcinoma.[52,61] FIG is a golgi-associated protein, and its recruitment to the golgi is mediated by a coiled-coil domain. FIG-ROS1 fusions contain almost the entirety of the wildtype FIG protein, and as a result, FIG-ROS1 is localized to the golgi apparatus. Deletion of the golgi-associated coiled-coil domain results in loss of FIG-ROS1 from the golgi and loss of its transformation capacity.[43] This suggested that localization of FIG-ROS1 may be critical for its transforming abilities, and hinted that other ROS1 N-terminal fusion partners may be conveying oncogenicity onto ROS1 through altering its localization. However, as previously mentioned, the N-terminal ROS1 fusion partners vary widely in their structure and function, suggesting they may not all localize and act in the same way. Thus, the first part of my thesis project (Chapter 2) involved analyzing which pathways are activated downstream of some of the most common ROS1 fusions

(CD74-ROS1, SDC4-ROS1, and SLC34A2-ROS1) and where the basis for differential pathway activation may lie. Indeed, I found that not all ROS1 fusions activate the same downstream pathways – all three fusions studied activated the JAK/STAT pathway, but while SDC4- and SLC34A2-ROS1 fusions activated the RAS/MAPK pathway, CD74-ROS1 fusions were unable to activate this pathway. Intriguingly, the ROS1 fusions that were able to activate the MAPK pathway grew more quickly and were less sensitive to crizotinib, a targeted small molecular kinase inhibitor with potency against ROS.

Discovering what pathways are activated downstream of an oncogene can be critical for understanding what pathways a cancer cell depends on for survival. While a particular oncogene may be capable of activating multiple downstream pathways, that doesn't mean the cancer cell is equivalently addicted to these pathways. For example, previous work from our lab demonstrated that while EML4-ALK variant 1 (v1) fusions can activate the SHP2/RAS/MAPK, PI3K/AKT, and JAK/STAT pathways, EML4-ALKv1-driven tumors are uniquely dependent on RAS/MAPK signaling for survival.[62] Comprehension of critical downstream signaling pathways enables us to predict targeted therapy resistance mechanisms and potential therapeutic avenues to combat or altogether prevent this resistance by strongly inhibiting a critical survival pathway (discussed in Chapter 4). Identification of RAS/MAPK as a critical pathway in driving some, but not all, ROS1 fusion-driven cells, allowed us to predict that these cells may be sensitivity to RAS/MAPK inhibition. Indeed, we found specific sensitivity of the ROS1 fusions that activate RAS/MAPK pathway, but not CD74-ROS1 fusions, to a SHP2 inhibitor. Altogether, the findings from this part of my thesis project suggest the need to identify and stratify patients with ROS1 fusion-positive tumors by the N-terminal ROS1

fusion partner, because the fusion partner may suggest that a patient would benefit from different or more aggressive treatment.

Another oncogenic fusion recently discovered in lung cancer involves rearrangement of the gene *Anaplastic lymphoma kinase* (*ALK*). *ALK* is a known oncogene, with mutations found in neuroblastoma and rearrangements seen in anaplastic large-cell lymphoma.[63-67] ALK rearrangements are seen in ~2-5% of lung adenocarcinomas.[68-70] Similar to *ROS1* rearrangements, ALK fusions are seen more commonly in patients who are younger, Asian, and never-smokers.[68,71-73] The most common ALK fusions in lung cancer result from rearrangement between ALK and several different exonic breakpoints in *echinoderm microtubule associated protein-like 4* (EML4). There are a variety of EML4-ALK fusions, which differ based on the number of *EML4* exons contained in the fusion, all joined to exon 20 of ALK.[68,74-77] EML4-ALK variant 1 (v1) is the most common EML4-ALK fusion variant.[78] EML4-ALKv1 comprises EML4 exons 1-13, which includes a trimerization domain contained within a basic domain, a hydrophobic HELP domain, and a fraction of a WD repeat domain, all fused to the entirety of the ALK kinase domain.[68] Studies from our lab and others demonstrate that oncogenic ALK fusions are capable of activating a variety of downstream pathways, including those most often associated with oncogenic signaling: RAS/MAPK, PI3K/AKT, JAK/STAT, NfKB, and mTOR, as well as several others.[62,79-83] As previously mentioned, work from our lab demonstrated that while these fusions can activate several downstream pathways, cells harboring EML4-ALKv1 fusions are uniquely dependent on RAS/MAPK signaling.[62] Unlike ROS1 fusions, all identified NSCLC EML4-ALK fusions contain only the intracellular portion of ALK—that is, they lack the ALK transmembrane

domain. Indeed, immunofluorescence revealed that EML4-ALKv1 is localized not on the plasma membrane, like wildtype ALK and other receptor tyrosine kinases, but instead was found in intracellular clusters.[62] This localization raised an interesting question – how can a fusion that is apparently not localized on a membrane activate the RAS/MAPK pathway, which canonically requires a lipid bilayer scaffold for activity? This question served as the basis for my second thesis project (Chapter 3).

RAS proteins are small GTPases that cycle between an active, GTP-bound state and an inactive, GDP-bound state.[84] While RAS proteins have an intrinsic GTPase activity, this molecular on/off switch is greatly enhanced by the presence of GTPase-activated proteins (GAPs) and guanine nuclear exchange factors (GEFs).[85] When RAS proteins bind GTP, they undergo a conformational change in the switch I and switch II regions of the proteins, allowing for effector binding to RAS.[86,87] There are four RAS isoforms in mammalian cells – HRAS, NRAS, and KRAS4a and KRAS4b, which are splice variants of the same gene.[88] All RAS isoforms display high sequence homology, differing mainly in their C-terminal hypervariable regions (HVRs). The sequence differences in these regions lead to distinct patterns of subcellular localization, with KRAS localized on the plasma membrane, and HRAS and NRAS found at the plasma membrane, the endoplasmic reticulum (ER), and more prominently, on the Golgi membrane. Canonically, all RAS proteins require membrane association for their biological activity. In order to reach a lipid bilayer, all RAS proteins undergo intracellular processing. Once translated in the cytoplasm, RAS proteins are farnesylated at the cysteine of their C-terminal CAAX motif.[89,90] Farnesylation of RAS leads to its recruitment to the (ER), where it is further processed by cleavage of the terminal –AAX

residues and carboxymethylation of the farnesylated cysteine.[91,92] At this stage, differences in the HVRs dictate differential fates of the RAS proteins.[93] HRAS, NRAS, and KRAS4a contain cysteines proximal to the C-terminal farnesylated cysteine which are palmitoylated.[94] KRAS4b, on the other hand, has a lysine-rich polybasic region adjacent to the terminal farnesylated cysteine.[95] This polybasic region enables trafficking of KRAS4b directly from the ER to the plasma membrane, while the other RAS isoforms are trafficked through the Golgi to the plasma membrane.[96,97]

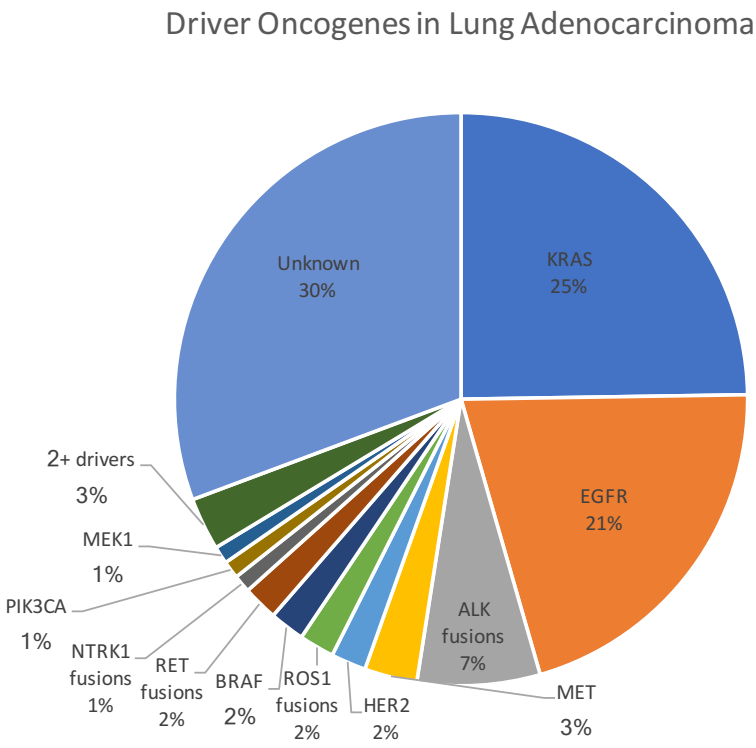
RAS isoforms have been shown to be active both at the plasma membrane and at endomembranes, including the golgi, the ER, and at endosomes.[98,99] A biologic role for non-membrane-associated RAS has not been demonstrated in mammalian cells. Indeed, it is thought that RAS absolutely requires a membrane for activity. Firstly, the major RAS effector proteins, the RAF kinases ARAF, BRAF, and CRAF, are active at membranes, and recruitment of RAF to the membrane by active RAS is thought to be the major step in Ras-mediated RAF activation.[100] In fact, membrane recruitment of Raf kinases alone, in the absence of association with active Ras, has been show to be sufficient to activate these proteins and drive downstream MAPK signaling.[101,102] Secondly, mutation of the CAAX box cysteine to serine, which blocks farnesylation and all subsequent RAS processing, has been shown to inhibit the biologic activity of RAS, again suggesting that RAS requires association with a membrane for its activity.[103] In fact, prevention of this farnesylation step has been evaluated as a therapeutic strategy in treatment of RAS-driven tumors.[104] Farnesyltransferase inhibitors, which showed early efficacy in cell lines, were not as successful as initially hoped, because of rescue of prenylation of the CAAX motif by geranylgeranyltransferases.[105,106]

Geranylgeranylation of the CAAX motif has the same function as farnesylation of this region. For these reasons, the idea that mammalian RAS always requires a membrane for its activity has been widely accepted as fact.

In Chapter 3, I address the question of where EML4-ALKv1 is localized, what dictates it localization pattern, and how it engages its critical downstream pathway, the RAS/MAPK pathway. We demonstrate that these EML4-ALKv1 clusters are not associated with any membrane, and that MAPK pathway adaptors and signaling molecules colocalize with and are active in these clusters, suggesting that RAS itself is active in these non-membrane-associated structures. This is the first demonstration in mammalian cells that non-membrane-associated RAS can not only be functionally active but can also drive oncogenic signaling. There is some precedence in *Drosophila* for cytoplasmic RAS having a functional role. One study suggests signaling via non-membrane-associated RAS is critical for eye development in *Drosophila*, and that in fact only soluble RAS, and not membrane-associated RAS, is able to play this role, but no functional role for mammalian RAS in the absence of a membrane has been described before.[107] Our finding may suggest a broader role for cytoplasmic RAS in both normal and oncogenic signaling. Additionally, it also highlights the significance of RTK fusion localization in oncogenic transformation. Our studies reveal that clustering of EML4-ALKv1 is critical for activation of the RAS/MAPK pathway, which suggests disruption of these clusters may be a feasible way to target EML4-ALKv1-driven tumors.

Understanding how oncogenes drive activation of downstream pathways, and their dependence on specific pathways for survival, is critical for the treatment of tumors. We have demonstrated that not all pathways downstream of an oncogene are

created equal – that is, a particular oncoprotein may be capable of activating several downstream pathways, but may be uniquely dependent on just one for survival. This has specific implications for prediction of resistance to targeted therapies. The discovery of tyrosine kinase-driven tumors has led to the development of specific small molecule tyrosine kinase inhibitors (TKIs), which drastically improve survival compared to conventional chemotherapy.[6,16,108-110] The small molecule TKI crizotinib, which has efficacy against both ALK and ROS1 kinases, improves progression-free survival compared to cytotoxic chemotherapies to 10.9 months and 19.3 months, respectively.[109,111] However, virtually all tumors eventually develop resistance to targeted inhibitors. Resistance can arise through several different potential mechanisms (described in detail in Chapter 4), including on-target "gatekeeper" mutations in the oncogenic kinase, upregulation of other parallel RTKs, or mutational activation of signaling molecules in downstream pathways.[112-121] One way to prevent development of resistance mediated by reactivation of downstream pathways is through dual treatment of tumors in the upfront setting, with both a drug that targets the driver oncogene and one that hits its critical downstream survival pathway.[14,122] Indeed, previous work from our lab suggests that upfront treatment of EML4-ALKv1-driven tumors, which are dependent on MAPK signaling, with both crizotinib and the MEK inhibitor trametinib, can prevent resistance from occurring.[62] Thus, characterization of necessary survival pathways downstream of an oncogene can enable prediction of how a tumor might develop resistance, and hopefully lead to better, more specific treatment, and improved overall survival.




Adapted with permission from "Lung Cancer: Current Therapies and New Targeted Treatments" by Hirsch, Fred R et al. *The Lancet*, Volume 389, Issue 10066, 299 - 311

**Figure 1-1: Driver oncogenes in lung adenocarcinoma**

Prevalence of discovered driver oncogenes in lung adenocarcinoma, many of which are targetable by recently developed small molecule inhibitors. However, up to 30% of lung adenocarcinomas still have no known oncogenic driver.

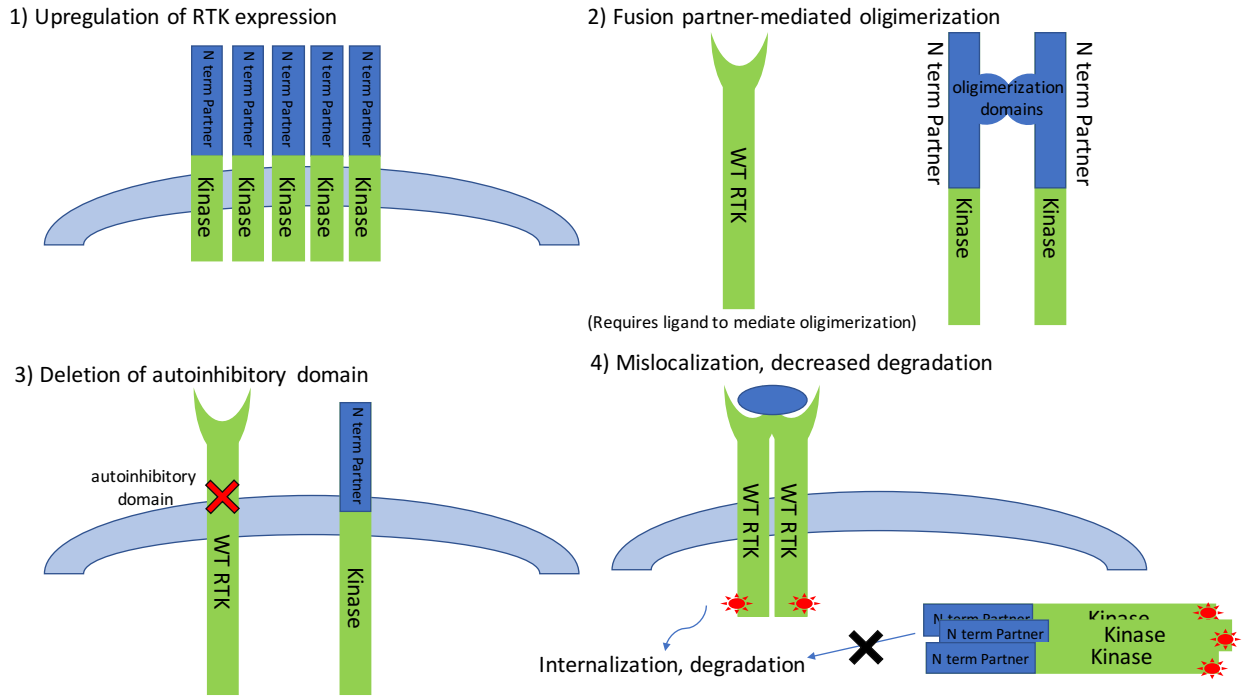



**Figure 1-2: Molecular mechanisms of RTK fusion-mediated oncogenesis**

Oncogenic RTK fusions can lead to cancer through a variety of mechanisms, including upregulation of RTK expression and downstream signaling; fusion-partner mediated oligimerization in the absence of ligand; deletion of juxtamembrane auto-inhibitory domains in the RTK; and mislocalization and decreased degradation of the RTK.

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# Chapter 2

# ROS Fusion Partner Dictates Downstream Pathway Addiction in a Localization-Dependent Manner

## Introduction

Lung cancer is the leading cause of cancer-related death in the United States.[1] Recent genomic advances have allowed the identification of several new oncogenic drivers of lung cancers, which has led to the development of targeted agents to specifically attack these drivers.[2-11] One relatively new class of oncogenes identified in lung cancer arise from chromosomal rearrangements, resulting in the production of novel kinase fusion oncoproteins. This class includes *ALK*, *ROS1*, *RET*, *NTRK1*, and *NRG1* fusions.[6-8,12] All identified lung cancer fusions contain a C-terminal kinase domain attached to a variety of N-terminal fusion partners.[13] There are several potential ways N-terminal fusion partners may contribute to oncogenesis, including driving overexpression of the C-terminal kinase, promoting oligomerization of fusion molecules, releasing auto-inhibitory mechanisms, and mislocalizing the C-terminal kinase.[14] However, the significance of the N-terminal fusion partners in lung cancer oncogenic fusions have not been identified. In fact, lung cancer oncogenic fusions are most often detected by a break-apart fluorescence in situ hybridization (FISH) assay, meaning that only the presence or absence of a specific fusion is described, and usually the N-terminal fusion partner is not identified at all.[15]

Fusions involving the receptor tyrosine kinase ROS are found in 1-2% of all lung adenocarcinomas.[8,16] ROS is one of the last remaining orphan receptor tyrosine kinases, and little is known about the wildtype function of the gene. The wildtype ROS protein contains a large N-terminal extracellular domain, whose structure suggests extracellular matrix proteins may serve as ligands.[17] In ROS fusions, this extracellular domain is lost, leaving the transmembrane and entire kinase domain of ROS fused to a

wide variety of N-terminal fusion partners.[8,18] So far, 10 distinct N-terminal fusion partners have been identified for ROS1 kinase fusions **(Figure 1)**.[19] The most common *ROS1* fusion partner is *CD74* (found in ~50% of *ROS1* fusions).[20] Other commonly observed *ROS1* fusion partners include *SDC4*, *SLC34A2*, *LRIG3*, *EZR*, and *TPM3*.[18,21,22] All of these N-terminal partners lack clearly unifying protein domains or functions, raising the possibility that not all fusions act in the same way. However, whether or not the N-terminal partner in ROS fusions affects signaling, oncogenicity, or response to therapy has not been explored.

In this study, we demonstrate that different ROS fusions engage distinct downstream pathways, affecting both tumor aggressiveness and response to therapy. Additionally, we show that this differential pathway activation is driven by differential subcellular localization of the ROS fusions. These findings have broad implications for both how we diagnose and treat patients with ROS fusion-positive lung cancer, suggesting that identification of the fusion partner is critical.

**Results**

To examine signaling downstream of different ROS fusion proteins, we generated isogenic cell lines expressing some of the most common *ROS1* fusions isolated from patient tumors, including *CD74-ROS1*, *SDC4-ROS1*, and *SLC34A2-ROS1* (**Figure 2**).[19] All of these fusions are topologically predicted to result in a cytoplasmic-facing kinase domain, and expression of all three result in constitutive activation of the kinase. Intriguingly, while all of the ROS fusions activated the JAK/STAT pathway (measured by STAT3 phosphorylation) to an equivalent degree, the ability of ROS fusions to activate

the Ras/MAPK pathway (measured by ERK phosphorylation) varied significantly depending on fusion partner (**Figure 3a**). Both SDC4-ROS and SLC34A2-ROS fusions activate the MAPK pathway. Stunningly, CD74-ROS fusions are basically unable to activate the pathway at all. To confirm whether this differential activation of the MAPK pathway by different ROS fusions was seen in patient-derived models, we used short-term siRNA-mediated knockdown of *ROS1* in *ROS1* fusion-positive patient-derived cell lines that express the same fusions used in our isogenic systems. We observed that knockdown of SDC4-ROS and SLC34A2-ROS fusions, but not CD74-ROS fusions, resulted in suppression of the MAPK pathway (**Figure 3b**). This suggested that the *ROS1* fusion-positive lines that have fusions demonstrated to engage the MAPK pathway may be dependent on MAPK signaling for survival, while the cell line in which the ROS fusion is disconnected from the MAPK pathway would not be addicted to this pathway. Indeed, we found that hyperactivation of the MAPK pathway using a constitutively active MEK was sufficient to rescue cells expressing *SDC4-ROS1* and *SLC34A2-ROS1* fusions (which activate MAPK), but not a *CD74-ROS1* fusion (which does not activate MAPK), from crizotinib sensitivity (**Figure 4a, S1**). In contrast, hyperactivation of the JAK/STAT pathway with a constitutively active STAT3 was not able to rescue cells from crizotinib sensitivity **(Figure S1, S2)**.

Recently, the phosphatase SHP2 has been identified as an important player in signaling downstream of oncogenic receptor tyrosine kinases (RTKs), specifically in activation of the Ras/MAPK pathway.[23] Additionally, previous studies had identified SHP2 as a critical factor in signaling mediated via the FIG-ROS fusion in glioblastoma, albeit via an alternative downstream pathway.[24] This lead us to hypothesize that SHP2

may be mediating MAPK pathway activation downstream of the NSCLC ROS fusions. Indeed, the cell lines which depended on MAPK were sensitive to single-agent treatment with the SHP2 inhibitor RMC-4550. The cell line that is not dependent on MAPK signaling showed resistance to single-agent treatment with this drug (**Figure 4b**). These experiments demonstrated that in cell lines harboring *SDC4-ROS1* and *SLC34A2-ROS1* fusions, but not in those with the more common *CD74-ROS1* fusions, MAPK pathway activation is necessary and sufficient for cell survival.

Next, we wanted to examine what could be mediating differential pathway activation from the different ROS1 fusions. As the differential pathway activation observed downstream of a fusion was the same whether the exonic breakpoint was in *ROS1* exon 32 or 34 (**figure S3**), and the entirety of the ROS kinase domain was retained and identical between the different fusions, this suggests that it must be the N-terminal fusion partner which is driving the differential signal pathway activation. Previous studies of the FIG-ROS fusion, found primarily in glioblastoma, suggested that subcellular localization to the golgi, mediated by the coiled-coil domain contained within the FIG fusion partner, was important for the transforming ability of that particular fusion.[25] By extension, we hypothesized that differential subcellular localization of other ROS fusions may be driving the differential pathway activation we observed. Using immunofluorescence and confocal microscopy, we examined subcellular localization of SDC4-ROS, SLC34A2-ROS, and CD74-ROS fusions, both in isogenic BEAS2-B lung epithelial lines we engineered to express these fusions (ROS B2Bs) and in patient-derived cell lines **(Figures 5 and 6)**. Surprisingly, we found that there was non-identical subcellular distribution of the different ROS fusions. SDC4-ROS and SLC34A2-ROS,

which are able to activate the MAPK pathway, were found in punctate structures that colocalized with the endosomal marker EEA-1. CD74-ROS, which does not activate RAS/MAPK, was found in a more diffuse pattern that displayed perinuclear enhancement and colocalized with calnexin, a marker of the endoplasmic reticulum (ER).

As differential localization correlated with differential pathway activation, we wanted to test whether subcellular localization was required for pathway activation. Wildtype CD74 is the invariant chain, a type II transmembrane receptor which is involved in trafficking of MHC molecules through the ER to the endolysosome. CD74 contains a 15 amino acid N-terminal cytoplasmic extension, which anchors it into the ER.[26,27] We created a FYVE zinc finger domain-tagged CD74-ROS construct to target the fusion to endosomes.[28] Immunofluorescence of ROS B2Bs expressing this construct reveals relocalization of CD74-ROS from the ER to punctate structures, similar to those seen with SDC4-ROS and SLC34A2-ROS (**Figure 7a**). Strikingly, expression of FYVE-CD74-ROS leads to increased activation of the MAPK pathway, suggesting that localization of ROS fusions is critical in mediating RAS/MAPK pathway activation (**Figure 7b**). Thus, the differential activation that is observed between ROS fusions is due to different subcellular localization of the fusions, which is conferred by the N-terminal fusion partner.

Finally, we wanted to determine whether there was any functional significance to the differential ability of these ROS fusion oncoproteins to activate the RAS/MAPK pathway. Unfortunately, none of the *ROS1* fusion-positive patient-derived lines have been successfully grown as tumor xenografts. Thus, to examine tumor growth *in vivo*,

we generated NIH-3T3 cells expressing the *SDC4-ROS1* and *CD74-ROS1* fusions, and looked at their ability to form tumors in mice. As expected, and as described previously, NIH-3T3 cells expressing both fusions were able to form tumors in mice, while control 3T3 cells expressing an empty vector was not (**Figure 8a-b)**. Stunningly, SDC4-ROS fusions formed much more aggressive tumors, which grew substantially faster than CD74-ROS1 fusion-driven tumors (**Figure 8c**). Additionally, while both tumor types responded to the ROS inhibitor crizotinib, CD74-ROS-positive tumors displayed stable disease upon crizotinib treatment, while SDC4-ROS-positive tumors still continued to grow, only showing a decreased growth rate (**Figure 8d**). This is striking, because it suggests that expression of a *ROS1* fusion that is able to activate the MAPK pathway results in tumors that are both more aggressive and less responsive to the targeted inhibitor than tumors expressing *ROS1* fusions that do not activate MAPK.

**Discussion**

Genomic advances have led to improved classification of lung tumors, from characterization solely on histopathologic features to identification of a tumor's specific driver oncogene. Discovery of novel oncogenes has led to the development of specific targeted agents to precisely attack tumor cells. Some of these newly discovered oncoproteins are kinase fusions, and small molecule inhibitors of oncogenic kinases have led to greatly improved patient survival. One example of this is the 19 month progression-free survival observed in patients harboring ROS1 fusion-positive tumors treated with the tyrosine kinase inhibitor crizotinib.[9]

Currently, diagnosis of ROS1 fusions is primarily via break-apart FISH, and the N-terminal fusion partner is not identified. Our study demonstrates that while all ROS fusions examined activate the JAK/STAT pathway to a similar degree, they vary greatly in their ability to activate the MAPK pathway. SDC4-ROS and SLC34A2-ROS fusions are able to activate the MAPK pathway while CD74-ROS is not. We found that this differential MAPK pathway activation is due to different subcellular localization of ROS fusions, which is dictated by the N-terminal fusion partner. What mediates this differential ability for pathway activation from the ER and the endosomes remains to be elucidated. One possibility is that there is differential accessibility to a positive or a negative regulator of the MAPK pathway. Alternatively, the degree to which the ROS1 fusion is able to dimerize or associate with other kinase-activating factors may be mediated by subcellular localization. Understanding how localization regulates the ability of a fusion to activate specific signaling pathways could be critical for discerning other important cofactors driving oncogenic signaling.

The patient-derived *CD74-ROS1* cDNA utilized in our studies contains an ER-targeting motif, which anchors the ROS fusion to the ER, preventing it from activating MAPK. Interestingly, the shorter isoform of wildtype CD74 lacks this N-terminal ER-targeting motif, leaving open the possibility that some CD74-ROS tumors may express this shorter isoform, and may be able to engage MAPK.[27] The ability of individual fusions to activate the MAPK pathway is directly correlated with rate of tumor growth and sensitivity to crizotinib, suggesting that current diagnostics identifying on the presence or absence of a fusion may be insufficient, and precise identification of the fusion partner may be critical in stratifying patients for treatment. Finally, while many

*ROS1* fusion-positive tumors initially respond to crizotinib, virtually all tumors become resistant to therapy. We found that MAPK pathway activation is necessary and sufficient for survival of cells expressing SDC4-ROS and SLC34A2-ROS, suggesting MAPK pathway reactivation may be a mechanism of resistance to crizotinib monotherapy. In fact, there are isolated reports in the literature of RAS mutations or upregulation driving resistance to crizotinib in the setting of these ROS fusions.[29,30] Identification of the downstream pathways activated by an oncogene can be critical to predicting what pathway a tumor may reactivate in resistance, allowing for upfront combination therapy, (such as crizotinib plus a MAPK pathway inhibitor) to delay or prevent resistance from arising.

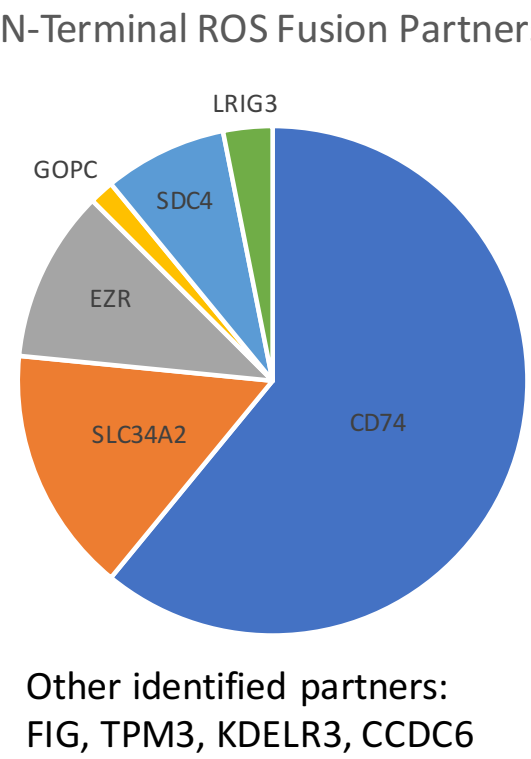
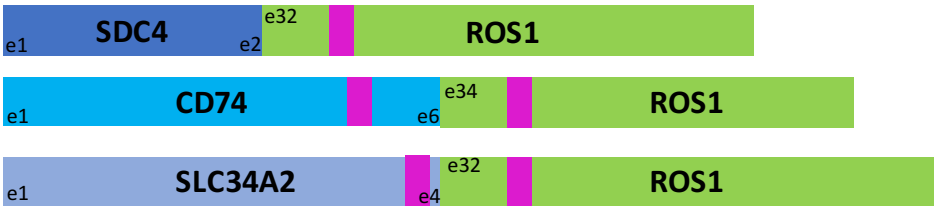



**Figure 2-1: Identified N-Terminal ROS fusion partners**

Prevalence of ROS fusion partners present in COSMIC data set (in pie chart), and other ROS fusion partners identified in case reports.

**a**




**b**

| N-terminal fusion partner | ROS1 exonic breakpoint | Number | Proportion |
|---|---|---|---|
| SDC4 (e1-2) | e32 | 3 | 75% |
| SDC4 (e1-2) | e34 | 1 | 25% |
| CD74 (e1-6) | e32 | 3 | 8% |
| CD74 (e1-6) | e34 | 33 | 92% |
| SLC34A2 (e1-4) | e32 | 8 | 100% |
| SLC34A2 (e1-4) | e34 | 0 | 0% |

**Figure 2-2: ROS1 fusions used in this study and relative prevalence of exonic breakpoints**

**a)** Diagram of the commonly occurring ROS1 fusions, which were utilized in this study. Pink denotes transmembrane domain. **b)** COSMIC analysis of ROS fusions demonstrate bias within fusions for specific exonic breakpoints.

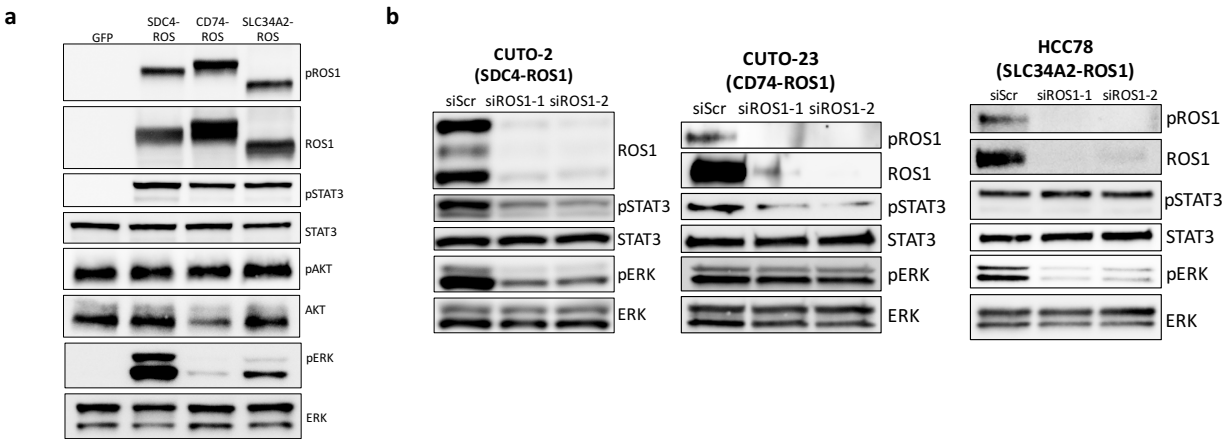



**Figure 2-3: ROS fusion partners dictate differential activation of downstream pathways**

**a)** Immunoblot of 293Ts transfected with GFP, SDC4-ROS, CD74-ROS, or SLC34A2-ROS. **b)** Immunoblot of ROS fusion-positive patient-derived cell lines with 55 hour siRNA-mediated knockdown of ROS.

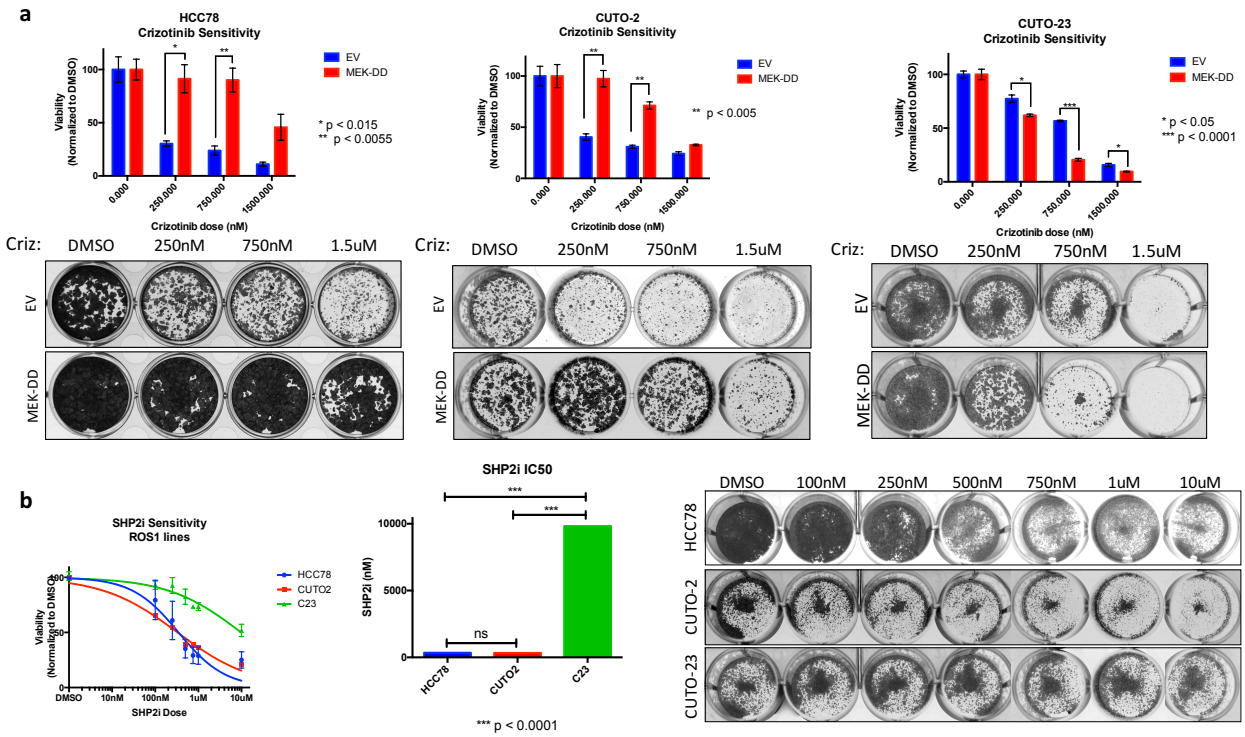



**Figure 2-4: MAPK pathway signaling is necessary and sufficient for survival of SDC4-ROS1-positive and SLC34A2-ROS1-positive lines, but not a CD74-ROS1 positive line**

**a)** Crystal violet staining and quantification of the ROS fusion-positive patient-derived cell lines HCC78, CUTO-2, and CUTO-23, expressing empty vector or constitutively active MEK-DD, treated with DMSO or increasing concentrations of the ROS inhibitor crizotinib. **b)** Crystal violet staining and quantification of HCC78, CUTO-2, and CUTO-23 cell lines treated with DMSO or increasing concentrations of the SHP2 inhibitor RMC-4550.

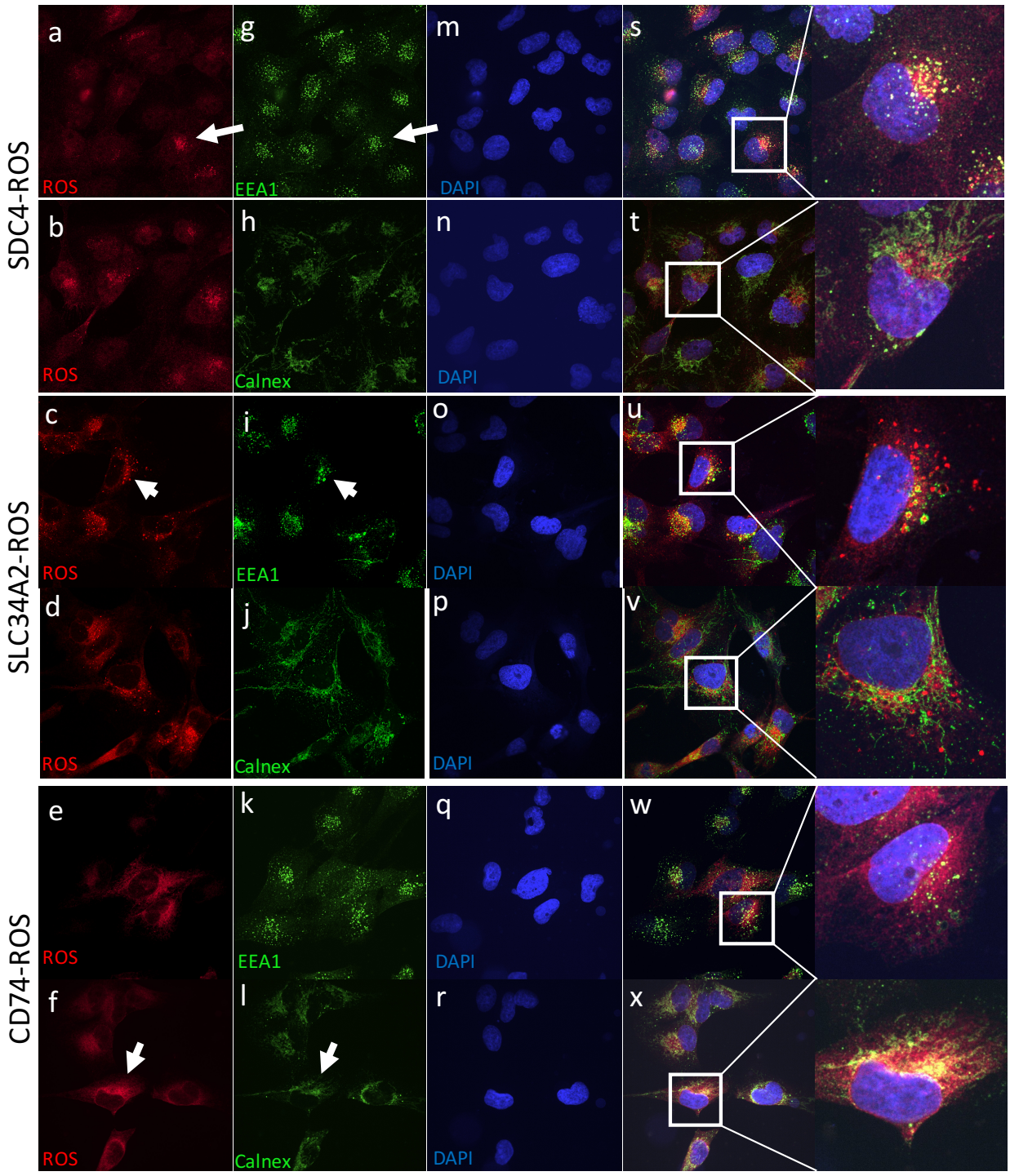



**Figure 2-5: Localization of ROS in isogenic BEAS-2B system reveals different localization of fusions.** Immunofluorescence and confocal microscopy of BEAS-2B cells expressing SDC4-ROS, SLC34A2-ROS, and CD74-ROS. **Rows 1,2** = SDC4-ROS; **Rows 3,4 =** SLC34A2-ROS; **Rows 5,6 =** CD74-ROS. Antibodies used were specific for**: a-f** = ROS1; **g,i,k** = EEA1; **h,j,l** = Calnexin; and **m-r** = DAPI. **s-x** = composite of left 3 lanes, increased magnification of single cell.

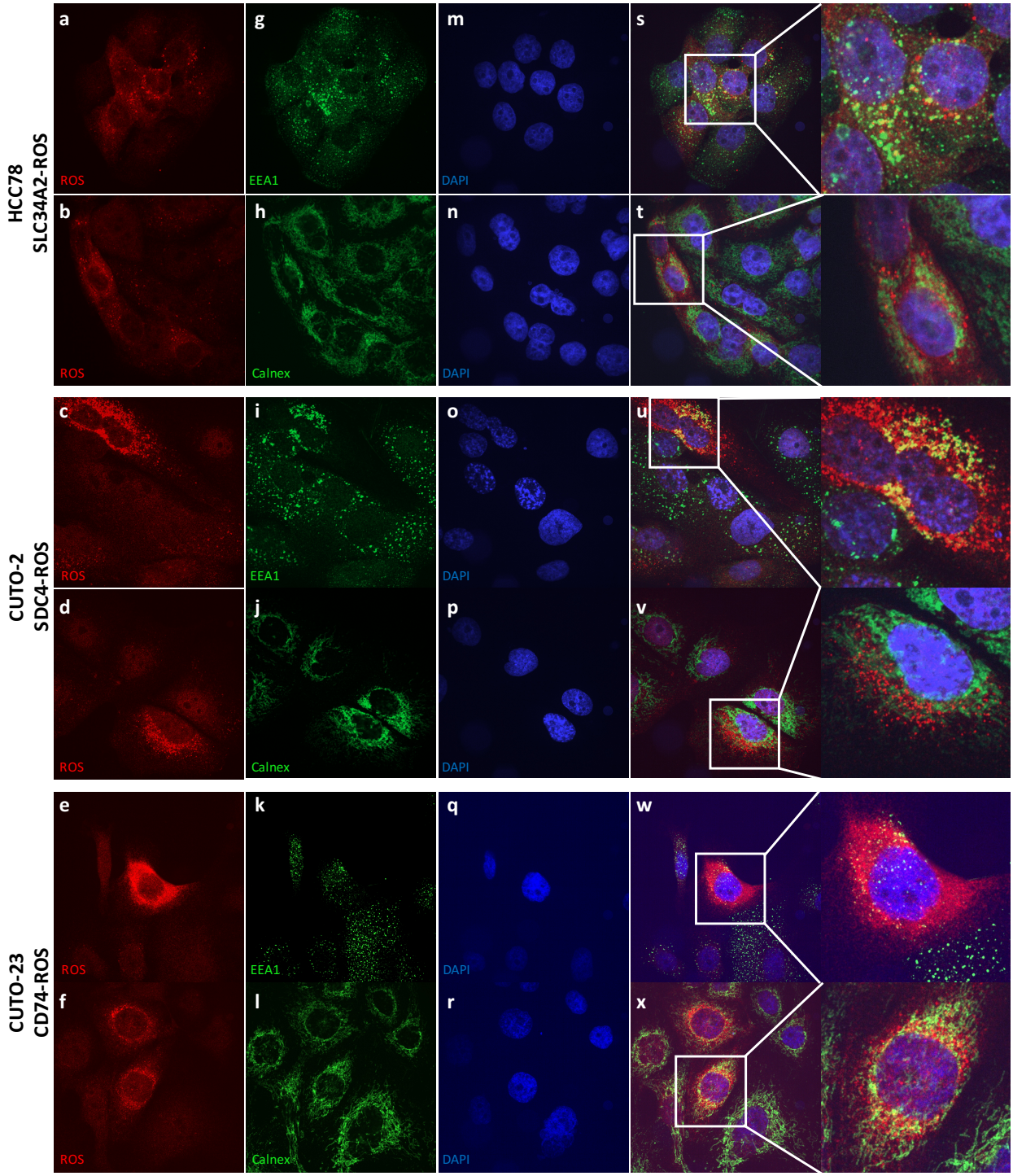



**Figure 2-6: Localization of ROS in patient-derived cell lines reveals different localization of fusions**. Immunofluorescence and confocal microscopy of patient-derived cell lines expressing SDC4-ROS, SLC34A2-ROS, and CD74-ROS. **Rows 1,2** = CUTO-2; **Rows 3,4** = HCC78; **Rows 5,6** = CUTO-23. Antibodies used were specific for: **a-f** = ROS1; **g,i,k** = EEA1; **h,j,l** = Calnexin; and **m-r** = DAPI. **s-x** = composite of left 3 lanes, increased magnification of single cell.

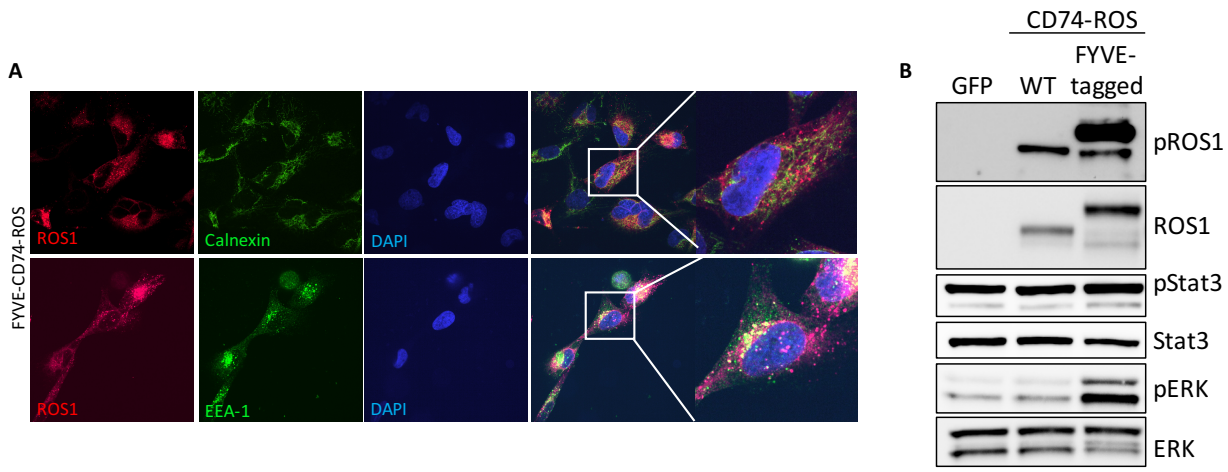



**Figure 2-7: Localization of ROS1 dictates engagement of downstream signaling pathways.**

**a)** Immunofluorescence and confocal microscopy of BEAS2-B cells stably expressing an endosome-targeted FYVE-tagged CD74-ROS construct and stained with the indicated antibodies. Far right panel = increased magnification of single cell.

**b)** Immunoblot of BEAS2-B cells transfected with GFP, WT CD74-ROS, or FYVE-CD74-ROS.

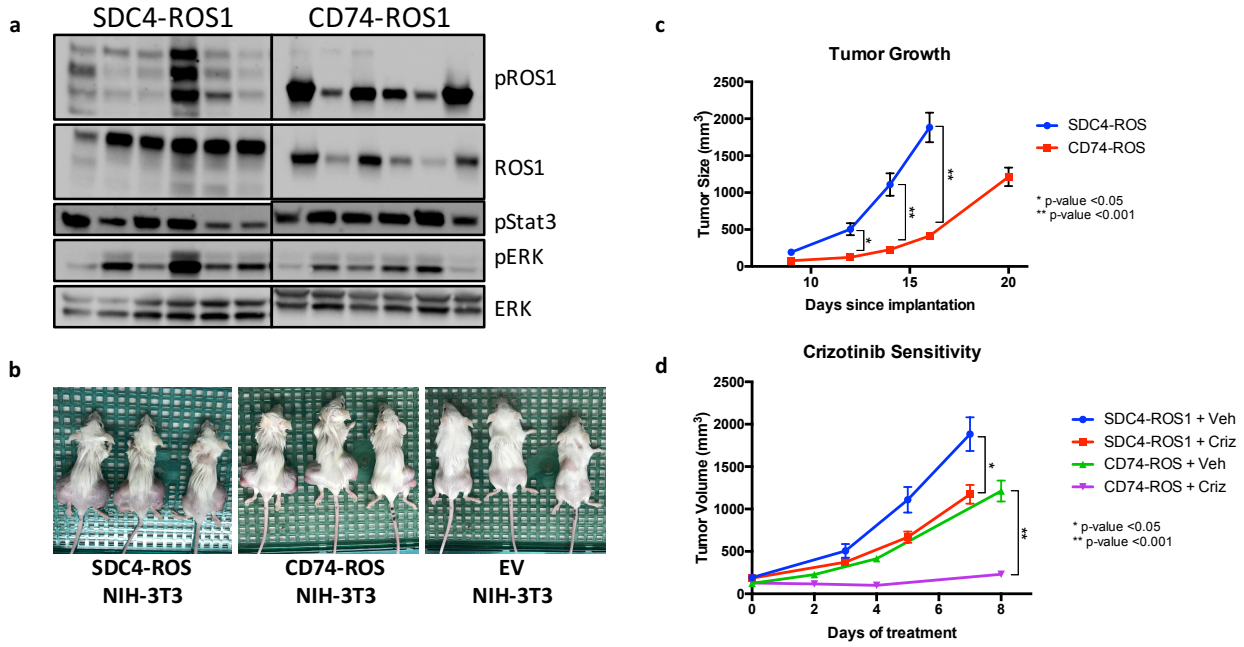
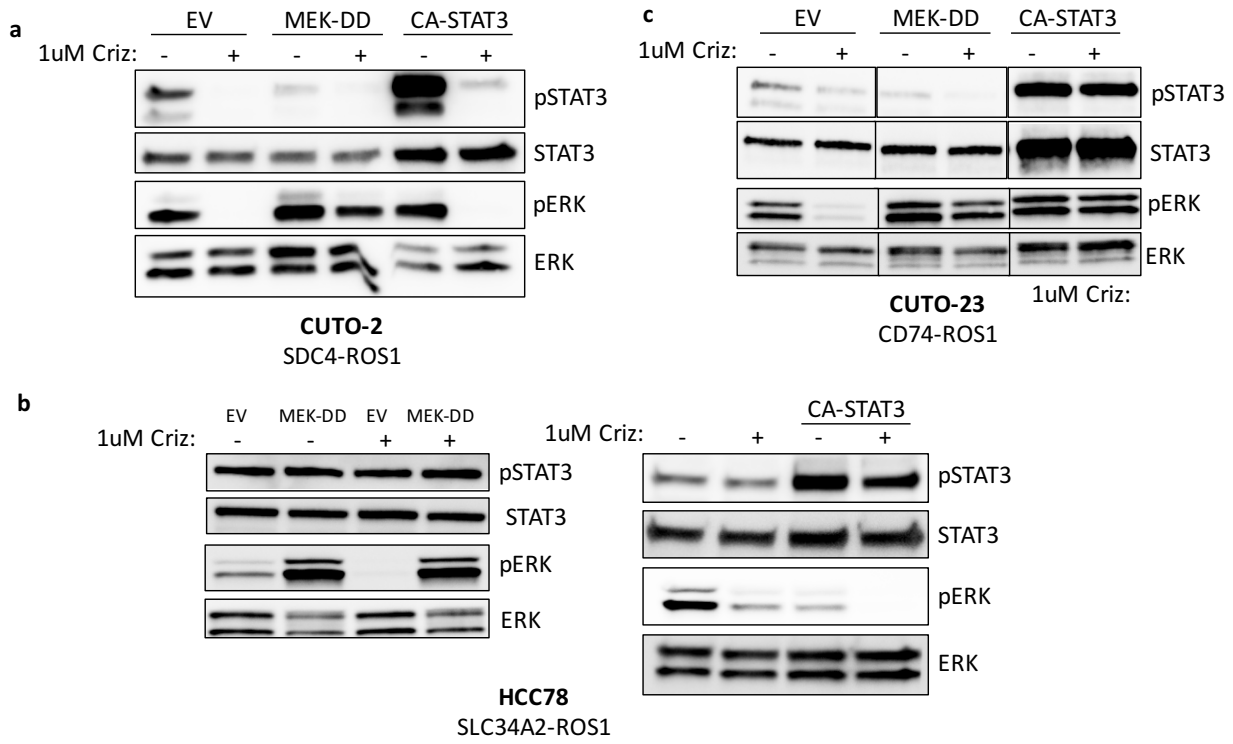


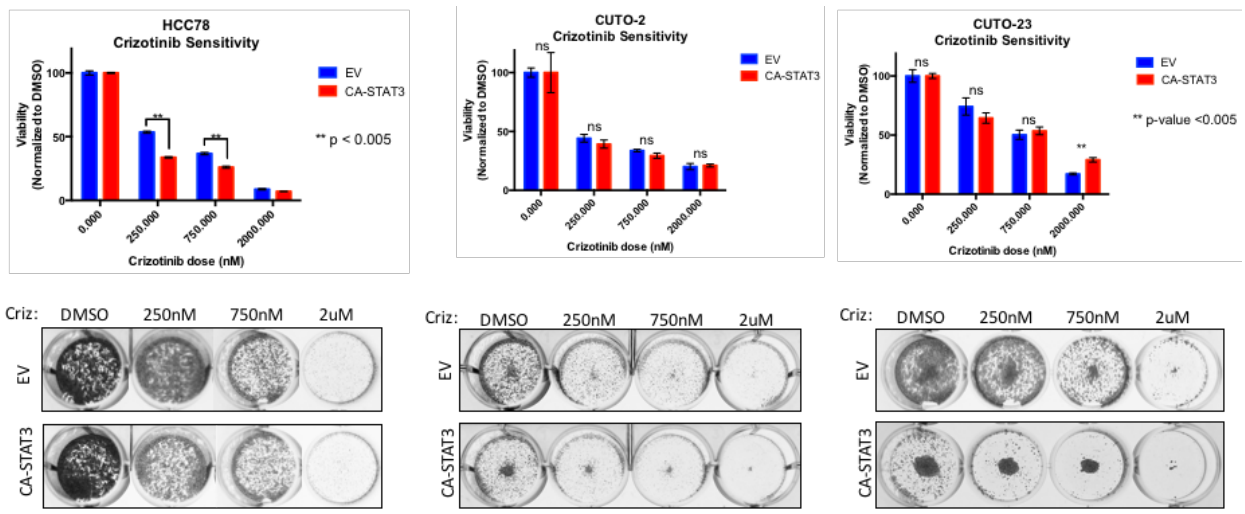

**Figure 2-8: MAPK pathway activation is associated with increased tumorigenicity and decreased sensitivity to crizotinib *in vivo*.**

**a)** Immunoblot of ROS1 fusion expression and signaling in NIH-3T3 tumor xenografts. **b)** Tumors arising from indicated cell lines implanted in flanks of SCID mice **c)** Tumor growth rates in flank xenografts of NIH-3T3 cells stably expressing SDC4-ROS and CD74-ROS. **d)** Growth rates of SDC4-ROS and CD74-ROS NIH-3T3 tumor xenografts treated with vehicle or crizotinib.



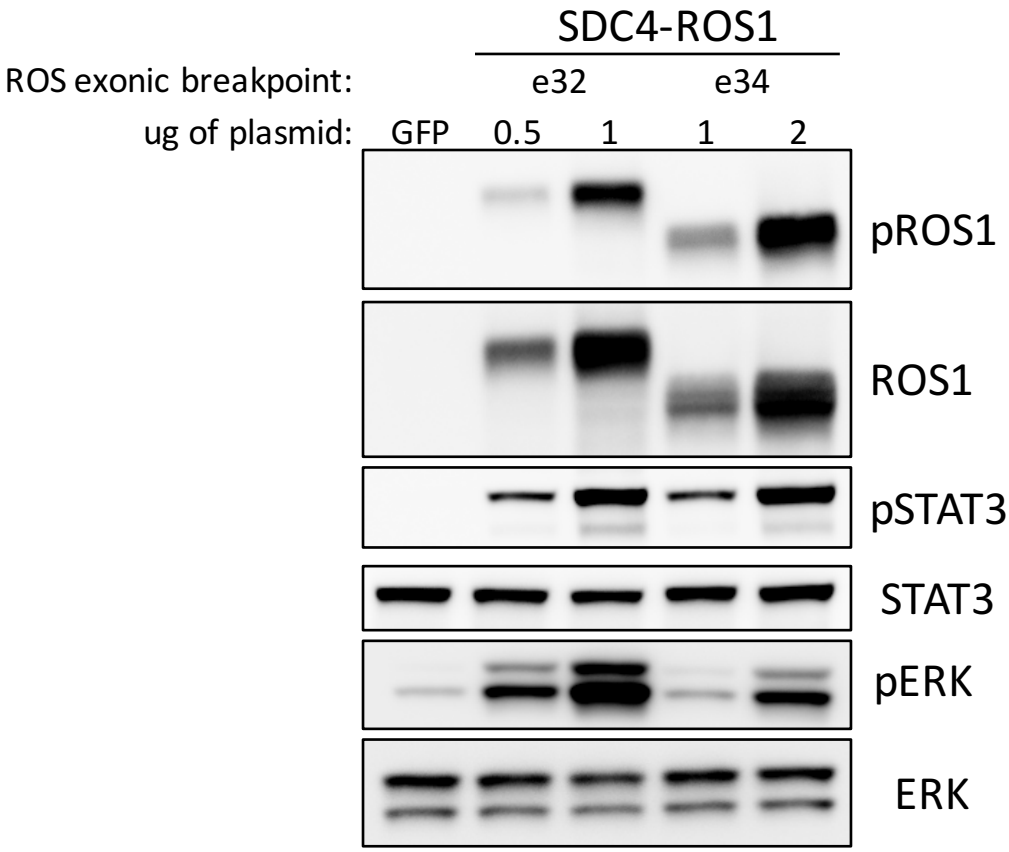

**Supplementary Figure 1: Expression of constitutively active MEK-DD and STAT3 in patient-derived ROS1-positive cell lines**

**a)** Immunoblot of CUTO-2 cells harboring the SDC4-ROS fusion expressing EV, MEK-DD, or CA-STAT3 treated with DMSO or 1uM Crizotinib. **b)** Immunoblot of HCC78 cells harboring the SLC34A2-ROS fusion expressing EV, MEK-DD, or CA-STAT3 treated with DMSO or 1uM Crizotinib. **c)** Immunoblot of CUTO-2 cells harboring the CD74-ROS fusion expressing EV, MEK-DD, or CA-STAT3 treated with DMSO or 1uM Crizotinib.




**Supplementary Figure 2: JAK/STAT pathway activation is not able to rescue ROS fusion-positive patient-derived cells from crizotinib sensitivity**

Crystal violet staining and quantification of the ROS fusion-positive patient-derived cell lines HCC78, CUTO-2, and CUTO-23, expressing empty vector or constitutively active STAT3, treated with DMSO or increasing concentrations of the ROS inhibitor crizotinib.




**Supplementary Figure 3: ROS1 exonic breakpoint does not determine fusion's ability to engage MAPK pathway**

Immunoblot of 293T cells expressing SDC4-ROS fusions harboring a *ROS1* breakpoint in either exon 32 or exon 34. Both are able to activate the MAPK pathway when equivalently expressed.

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adenocarcinoma. *Genome Res.* **22,** 2109–2119 (2012).
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# Chapter 3
# EML4-ALK variant 1 drives RAS activation through a novel, membrane-independent mechanism

## Introduction

The most common oncogenic fusions found in lung adenocarcinoma involve the receptor tyrosine kinase (RTK) ALK, most often resulting from rearrangements between *EML4* and *ALK*.[1-4] Several EML4-ALK fusions have been described, with varying breakpoints in *EML4*; all resulting fusion proteins contain some portion of the N-terminus of EML4 fused to the entire intracellular domain (exons 20-27) of ALK.[1,5-7] The most common EML4-ALK variant, EML4-ALK variant 1 (v1), contains exons 1-13 of EML4, which contributes a trimerization domain, a HELP domain, and a portion of a WD repeat domain, to the fusion protein.[1,8] Intriguingly, however, this fusion, unlike almost all other RTK fusions in cancer, lacks a transmembrane domain, as do all ALK fusions identified in lung cancer.[1]

EML4-ALKv1 activates several downstream pathways, including the PI3K/AKT, RAS/MAPK, and JAK/STAT3 pathway.[9-11] Previous work from our lab demonstrated, however, that survival of EML4-ALK-driven cells is uniquely dependent on signaling through the RAS/MAPK pathway.[11] Patient-derived EML4-ALK-positive cell lines were sensitive to single-agent treatment with MEK inhibitors, but not to treatment with JAK inhibitors or PI3K/AKT inhibitors. Additionally, constitutive activation of the RAS/MAPK pathway in EML4-ALK-driven cells via mutant $KRAS^{G12V}$ or MEK-DD was sufficient to rescue the cells from sensitivity to the ALK inhibitor crizotinib. Finally, EML4-ALK promotes GTP-loading onto RAS, which led us to ask how EML4-ALK, without a TM domain to anchor it in a membrane, or any obvious way to associate with a membrane, was able to engage RAS, given that RAS canonically requires a membrane to signal.[12-15]

**Results**

To understand how EML4-ALK is activating RAS, we first had to identify where in the cell EML4-ALK is localized. Using immunofluorescence and confocal microscopy, we localized endogenous EML4-ALK in patient-derived cell lines, and observed that EML4-ALK was present in intracellular punctate clusters (**Figure 1a**). Exogenous expression of EML4-ALK in nontransformed lung epithelial BEAS2-B cells revealed the same intracellular distribution (**Figure 1b**). As EML4-ALK engages RAS, we sought to identify whether or not these clusters were in fact intracellular membranous structures. To this end, we performed biochemical fractionation of the EML4-ALK-positive patient-derived cells in the presence and absence of a mild detergent. In this assay, membrane-bound structures will sediment in the absence of detergent, but will be released into the supernatant in the presence of the detergent. Calnexin, EGFR, and EEA-1, which are all associated with different membrane compartments in the cell and were used as controls for this assay, all moved from the pelleted fraction to the supernatant fraction in the presence of detergent. To our surprise, EML4-ALK was not removed from the pelleted fraction upon detergent treatment, suggesting that EML4-ALK is not associated with any endomembrane **(Figure 2a-b)**.

Given that EML4-ALK does not appear to be on a membrane, we next asked if RAS had to be membrane-associated in order for it to be activated and signal to the MAPK pathway in the context of EML4-ALK. To address this, we used the C185S mutant of KRAS, which is unable to be prenylated and thus stays cytoplasmic and is not trafficked to any membranes.[16] Intriguingly, we found that prenylation-defective,

cytoplasmic RAS is activated by EML4-ALK, but not by the membrane-associated oncoprotein EGFR L858R **(Figure 3a,b)**. Additionally, RAS C185S can productively signal in EML4-ALK-positive cell lines (**Figure 3c)**. This suggests that in the context of EML4-ALK, RAS does not require a membrane for activation or productive downstream signaling.

Next, we wanted to identify what components of EML4-ALK were mediating the observed clustering. Previous groups had identified the trimerization domain as required for wildtype EML4 self-association, so we hypothesized it may also be necessary for EML4-ALK clustering.[17] Additionally, the HELP domain has been shown to be required for full EML4-ALK-mediated transformation, and it has been suggested that the HELP domain may mediate interactions of EML4 with other subcellular structures.[1,18] Thus, we generated deletion mutants of EML4-ALK lacking the trimerization domain (dTD) or the HELP domain (dHELP), as well as a kinase-dead (KD) version of EML4-ALK (K589M) (**Figure 4a,b)**. Both immunofluorescence of flag-tagged versions of these mutants and live-cell imaging of YFP-tagged mutants revealed that both the trimerization domain and the HELP domain were required for EML4-ALK clustering – both of these mutants were cytoplasmic and displayed no clusters **(Figure 5c-f)**. Surprisingly, the kinase-dead version of EML4-ALK also did not form clusters (**Figure 5g-h)**. This suggests a stochastic mechanism of EML4-ALK clustering, where an initial phosphorylation event is required to form the "seed" of the cluster, and then interactions through both the trimerization and HELP domains are required for subsequent oligimerization steps resulting in final cluster formation.

We next wanted to see if clustering was required for RAS/MAPK activation. Strikingly, the clustering-deficient EML4-ALK dHELP mutant was unable to activate the RAS/MAPK pathway, as shown both by the absence of downstream phospho-ERK induction and the lack of EML4-ALK dHELP-mediated RAS-GTP loading **(Figure 6a,b)**. This strongly suggested a role for clustering in RAS/MAPK pathway engagement. Additionally, we demonstrated by immunoprecipitation that only wildtype EML4-ALK, and not the cluster-deficient mutants, were able to associate with the MAPK adaptor protein Grb2 **(Figure 7a)**. Grb2 serves as a bridge between an activated RTK and RAS activation by binding to phosphorylated tyrosines in the RTK cytoplasmic tail and recruiting the RAS-GEF SOS1, which activates RAS by accelerating RAS-GTP loading.[19] Live-cell imaging of a cell expressing GFP-tagged Grb2 revealed relocalization to clusters upon cotransfection with BFP-EML4-ALK **(Figure 7b)**.

Finally, we wanted to directly link these intracellular EML4-ALK clusters to RAS activity. We already determined that EML4-ALK activated the RAS/MAPK pathway through increasing RAS-GTP loading, we demonstrated that EML4-ALK was found in non-membrane-associated intracellular clusters where it interacted with MAPK adaptors, and we showed that non-clustering mutants of EML4-ALK were unable to load GTP onto RAS or associate with MAPK pathway adaptors. However, the direct functional significance of these clusters remained uncharacterized. To address this, we used CRISPR/Cas9 to knock mNeonGreen2 exon 11 into BEAS2-B cells in-frame with *A-RAF*, allowing us to visualize localization of endogenous A-RAF.[20] Strikingly, transfection of these cells with fluorescently-tagged EML4-ALK leads to redistribution of A-RAF, which is normally diffusely cytoplasmic, into the EML4-ALK clusters **(Figure 8)**.

A-RAF binds to active RAS via its Ras-binding domain (RBD). Thus, the enrichment of A-RAF in EML4-ALK clusters is consistent with the presence of active RAS in these clusters, demonstrating that EML4-ALK clusters are functional, and drive RAS/MAPK pathway activation in the absence of a membrane through recruitment of MAPK adaptor proteins and increasing RAS-GTP loading in the clusters.

**Discussion**

We have demonstrated for the first time in mammalian cells that RAS is able to productively signal in the absence of a membrane, which represents a paradigm shift in how we should think about RAS biology. Whether this finding suggests a broader role for cytoplasmic/non-membrane-associated RAS in other cellular contexts, perhaps even in a wildtype setting, or is unique to EML4-ALK positive cells remains to be seen. However, EML4-ALK fusions are not the only non-membrane-associated proteins known to be linked to RAS – the classic cancer fusion oncoprotein, BCR-ABL, also lacks a transmembrane domain, and is known to activate RAS, suggesting cytoplasmic RAS may be functioning in this context as well.[21-24]

The discovery that cytoplasmic RAS is functional may have broad implications for therapeutic strategies in RAS/MAPK-dependent contexts. Farnesyltransferase inhibitors (FTIs) have been in clinical development as a way to treat some RAS-driven tumors by preventing the farnesylation of the C-terminal CAAX box cysteine in RAS.[25] Blocking farnesylation prevents the initial trafficking of RAS from the cytoplasm to the endoplasmic reticulum. Despite successes in cell lines, FTIs have in general been a failure in treated cancer in the clinic.[26] This is in part due to compensatory

geranylgeranylation of RAS, but perhaps cytoplasmic RAS is able to play a role in FTI resistance as well.[27] Additionally, our findings suggest that FTIs would never be effective in the context of EML4-ALKv1, because trapping RAS in the cytoplasm would not prevent EML4-ALK-mediated RAS/MAPK pathway activation.

Finally, we have shown for the first time the functional significance of the EML4-ALK clusters, and have demonstrated that preventing EML4-ALK from forming clusters blocks downstream RAS/MAPK pathway activation. This suggests that dissolution of these clusters by disruption the EML4-mediated oligimerization may represent a new avenue for targeting EML4-ALK-positive tumor cells.

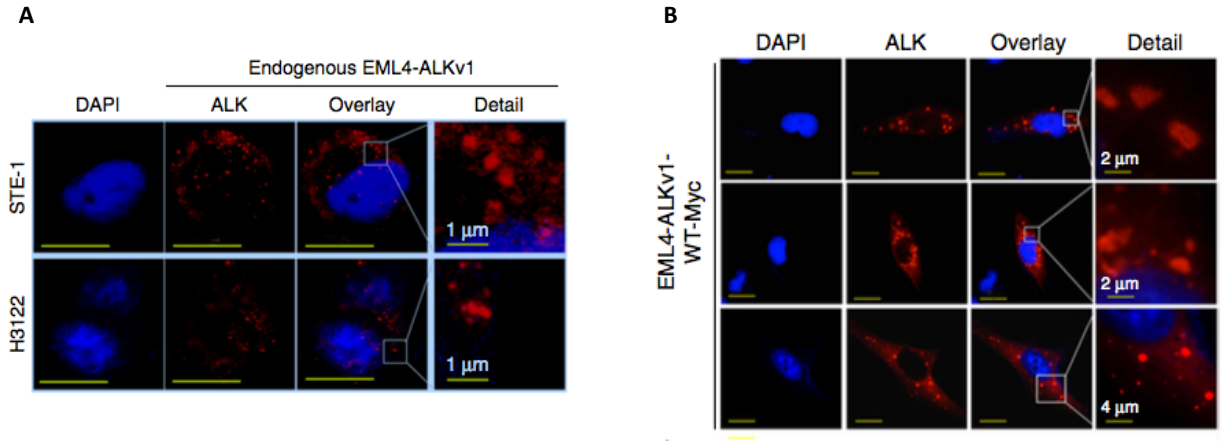


**Figure 3-1 Localization of EML4-ALK variant 1**[11]

**a)** Anti-ALK immunofluorescence in the patient-derived EML4-ALK-positive cell lines STE-1 and H3122. **b)** Anti-Myc immunofluorescence in BEAS2-B cells transfected with a Myc-tagged EML4-ALK variant 1.

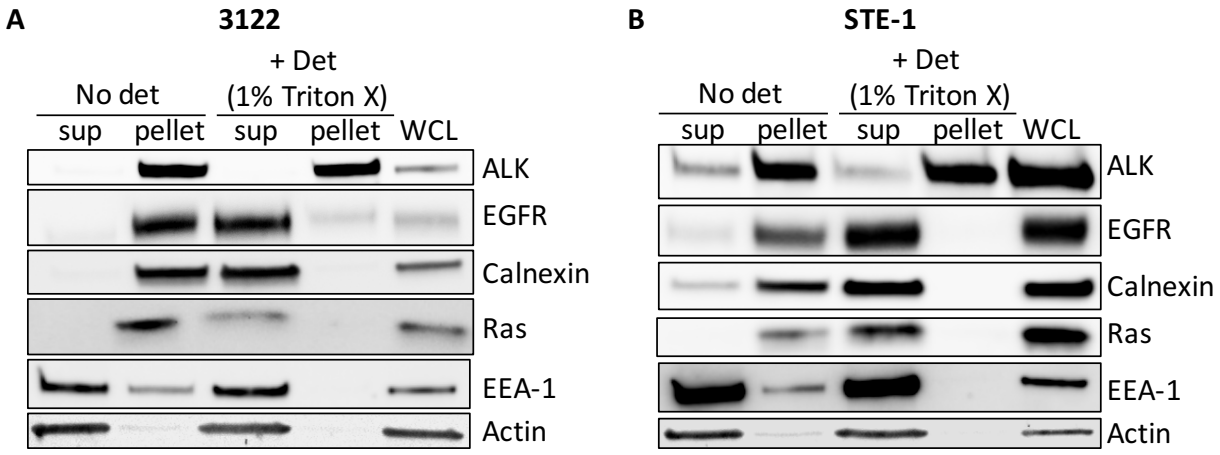


**Figure 3-2: Ultracentrifugation of EML4-ALK-positive cell lines**

Ultracentrifugation of the patient-derived EML4-ALK-positive cell lines, 3122 **(a)** and STE-1 **(b)** in the absence or presence of a mild membrane-disrupting detergent.

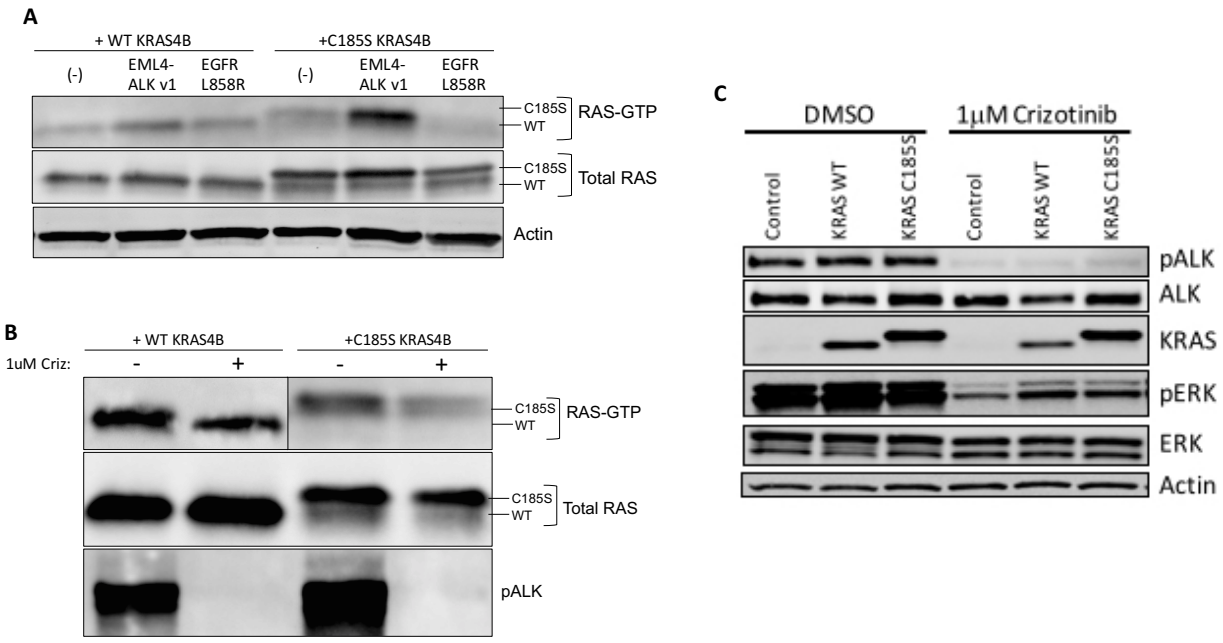


**Figure 3-3: RAS C185S can be loaded by EML4-ALKv1 and signal productively downstream to the MAPK pathway**

**a)** Expression of WT or C185S KRAS4B in BEAS2-B cells, along with no vector, EML4-ALKv1, or EGFRL858R. **b)** Expression of WT or C185S KRAS4B in the 3122 cell line. **c)** Expression of EV, WT KRAS4B, or C185S KRAS4B in 3122 cells in the presence and absence of 1uM crizotinib

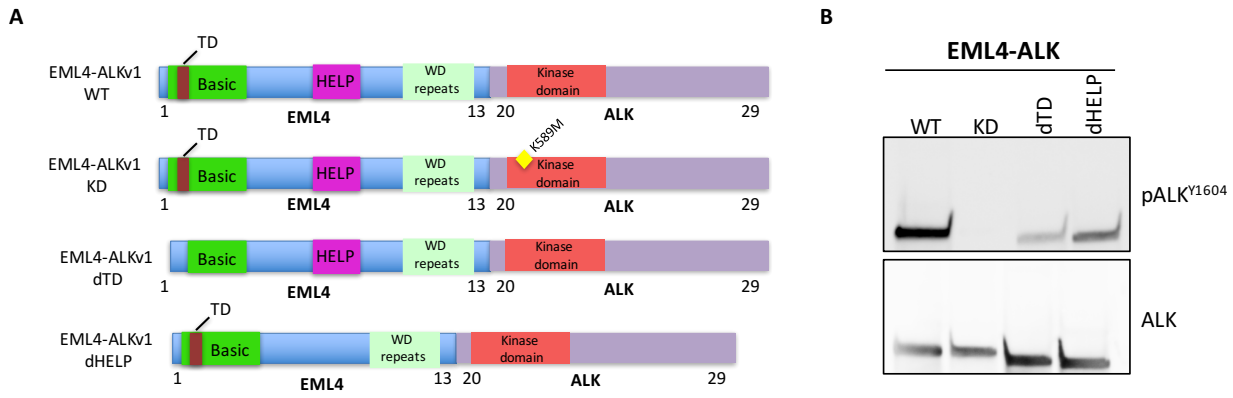


**Figure 3-4: Generation of EML4-ALK mutants**

**a)** Schematic of EML4-ALK deletion mutants. **b)** Western blot of EML4-ALK deletion mutants stably expressed in BEAS2-B cells.

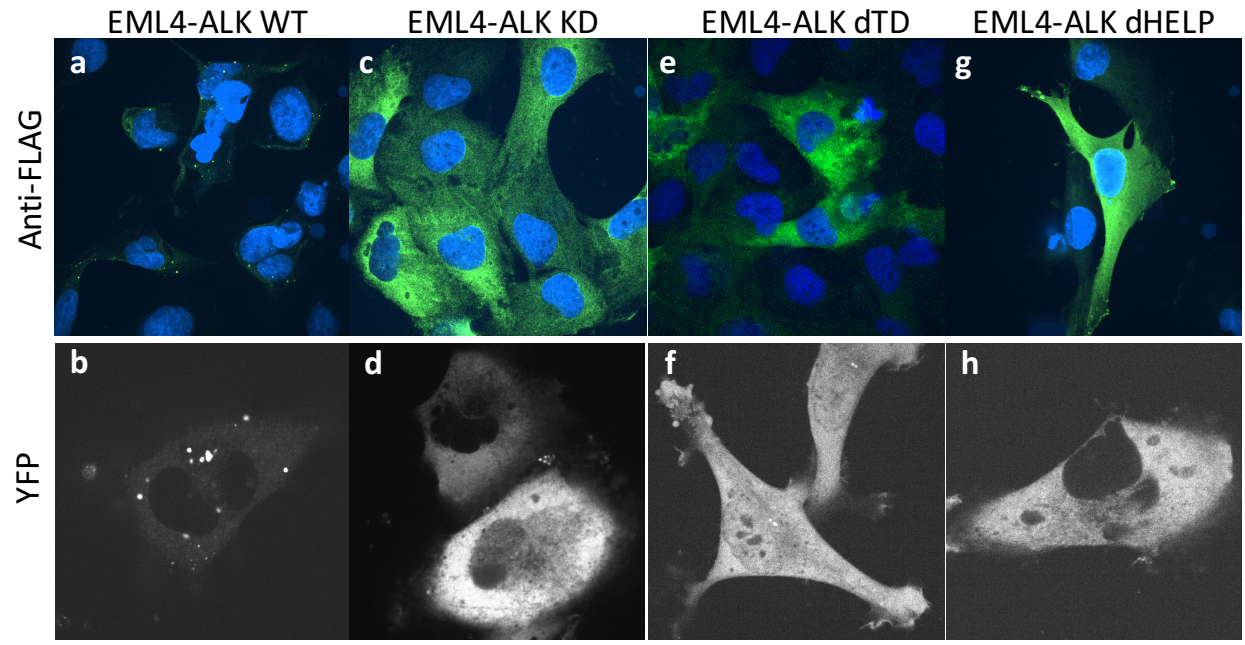


**Figure 3-5: Localization of EML4-ALK mutants**

**a,c,e,g)** Anti-FLAG immunofluorescence staining of indicated 3XFLAG-EML4-ALK constructs expressed in BEAS2-B cells.

**b,d,f,h)** Live cell imaging of YFP-tagged EML4-ALK constructs expressed in BEAS2-B cells

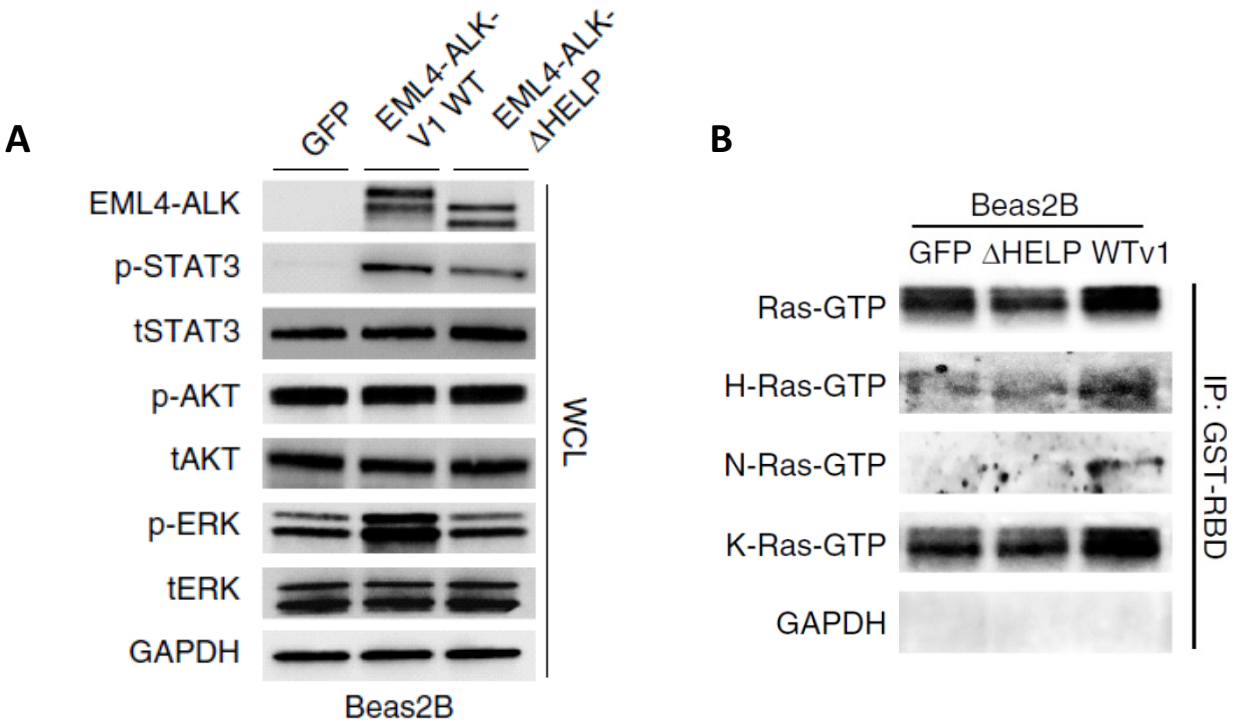


**Figure 3-6 EML4-ALK dHELP mutant is unable to activate the RAS/MAPK pathway**[11]

**a)** Western blot of BEAS2-B cells transfected with GFP, EML4-ALK WT, or EML4-ALK dHELP. **b)** Ras-GTP pulldown in BEAS2-B cells transfected with the indicated constructs

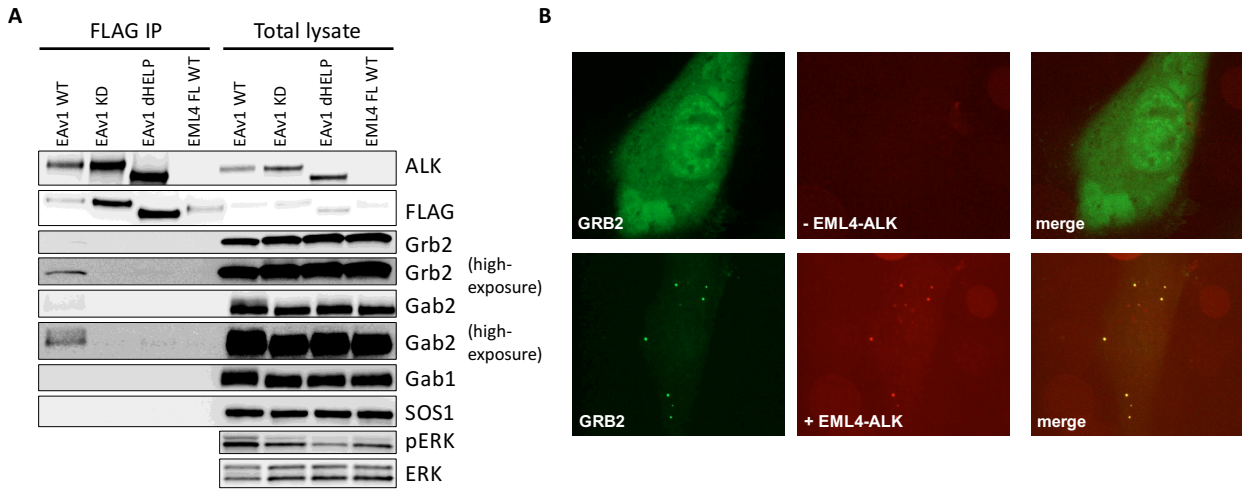


**Figure 3-7 EML4-ALK WT associates with the MAPK pathway adaptor Grb2**

**a)** FLAG immunoprecipitation in BEAS2-B cells stably expressing 3XFLAG-tagged EML4-ALK WT and mutants. **b)** Live-cell imaging of BEAS2-B cells transiently transfected with GFP-Grb2 in either the absence (top) or presence (bottom) of WT EML4-ALK.

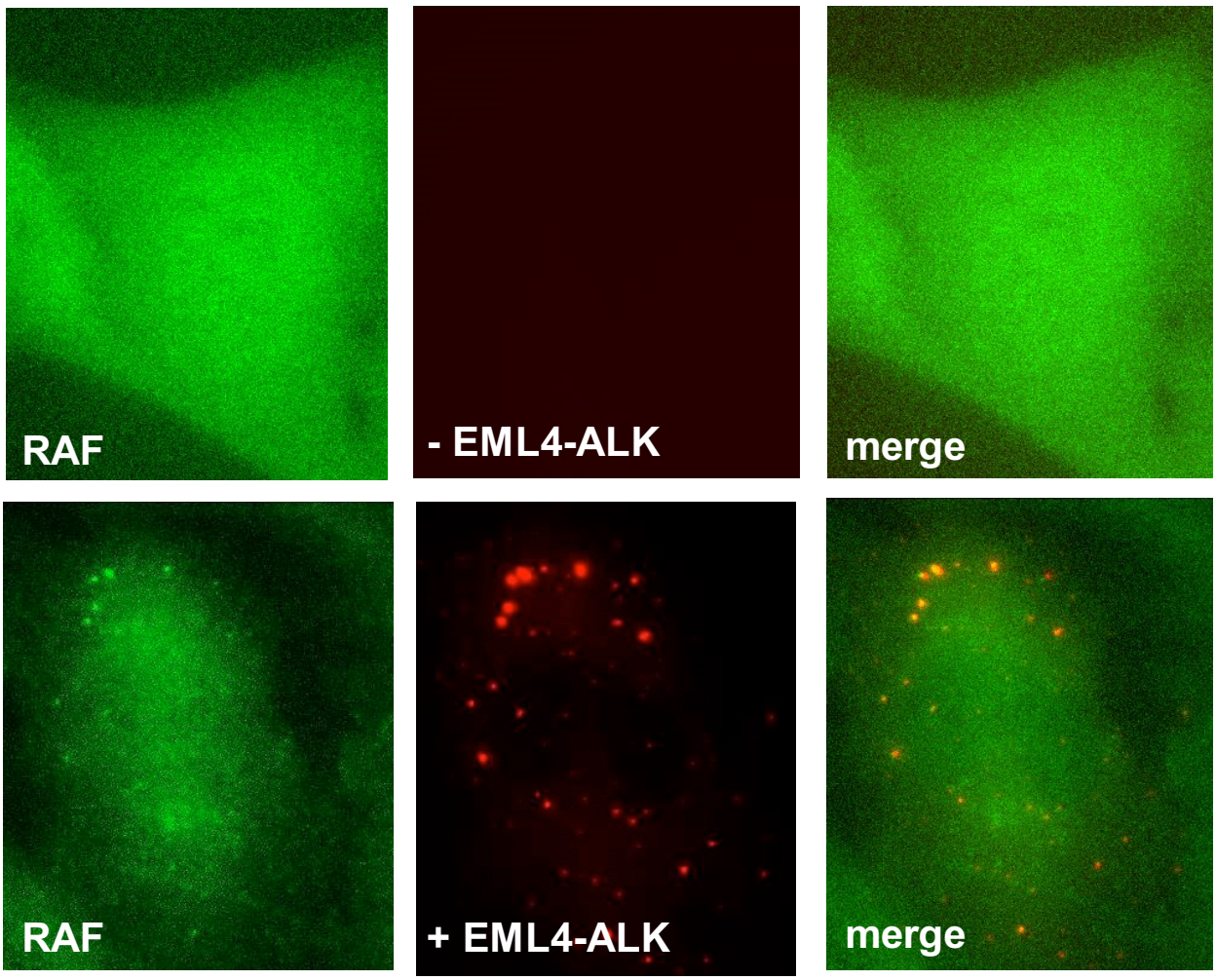


**Figure 3-8: A-RAF is enriched in WT EML4-ALK clusters**

Live-cell imaging of mNG2-A-RAF BEAS2-B cells in the absence (top) or presence (bottom) of WT EML4-ALK expression

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# Chapter 4
# Mechanisms of Resistance to Targeted Therapies in Lung Adenocarcinoma and Strategies to Overcome Resistance

## Introduction

The advent of genomics has led to the identification of specific "driver" mutations in oncogenic kinases and the development of targeted small molecule inhibitors to block their tumor-driving functions. These specific inhibitors have been a clinical success, and often significantly prolong the lives of individuals with cancer. Inevitably, however, the treated tumors recur as resistance to these targeted therapies develops.[1] Here, we review the major mechanisms by which a cancer cell can evade targeted therapy, focusing on mechanisms of resistance to kinase inhibitors in lung cancer. We discuss the promising concept of rational upfront polytherapy in lung cancer, which involves concurrently targeting multiple proteins in critical signaling pathways in a cancer cell to prevent or delay resistance.

## Targeting Driver Oncogenes in Lung Adenocarcinoma.

A significant fraction of lung adenocarcinomas harbor activating mutations in targetable oncogenes. These include mutations in *EGFR* (~11%) and *BRAF* (~7%), and activating gene rearrangements involving *ALK* and ROS1 (1-2%), all of which encode protein kinases and result in hyperactivation of downstream signaling pathways that drive cell growth, proliferation, and survival.

The identification of these driver kinases has led to the clinical use of small molecule kinase inhibitors that suppress these oncoproteins — erlotinib, gefinitib, afatinib, osimertinib for mutant EGFR, vemurafenib and dabrafenib for mutant BRAF, and crizotinib, ceritinib, alectinib for ALK and/or ROS1 gene rearrangements.[2-10] These targeted drugs function as ATP competitive inhibitors. Additionally, inhibitors of kinases

that are activated downstream of these oncoproteins have been developed for use as either monotherapy or in combination with inhibitors of the upstream oncoprotein. The MEK1/2 inhibitor trametinib is one such drug – it inhibits MAPK pathway activation by binding to and blocking MEK in an allosteric fashion. All of these inhibitors have shown efficacy over conventional chemotherapies in patients harboring the cognate genetic driver kinase.

**Mechanisms of Resistance to Targeted Therapies**.

Unfortunately, the initial clinical response to targeted kinase inhibitors is almost always temporary, as acquired resistance to these drugs invariably develops. Many mechanisms of resistance to each targeted therapy have been identified, but can be generally categorized into three predominant classes (**Figure 1**): (1) those that alter the driver oncogene, (2) those that activate a critical signaling pathway(s) in a parallel or downstream fashion, and (3) those that drive pro-survival signaling through a different signaling pathway. A fourth class of resistance encompasses histological transformation from one cell lineage such as epithelial to another such as neuroendocrine or mesenchymal. This last class is generally poorly understood.

## Alteration of the Driver Oncogene

### Gatekeeper Mutations and Other "On-Target" Mechanisms of Resistance.

Small molecule kinase inhibitors bind to their target through non-covalent bonds within the ATP-binding pocket. Cancer cells can develop resistance to specific small molecule kinase inhibitors by mutating a so-called "gatekeeper" residue within the

pocket. This residue is often small in the native oncoprotein with the secondary resistance-associated mutation resulting in a bulky amino acid substitution. How gatekeeper mutations cause resistance to small molecule inhibitors remains incompletely understood. Initial studies showed that the gatekeeper mutation both creates a residue that cannot hydrogen bond with the inhibitor and sterically hinders inhibitor binding in the pocket, while leaving the pocket's ATP-binding affinity unchanged.[11] The effect of the gatekeeper amino acid substitution is to prevent kinase-inhibitor binding while allowing retention of the ability of the kinase to bind ATP. More recently, data demonstrating that gatekeeper mutants can retain sensitivity to structurally similar but irreversible inhibitors suggest that steric hindrance may not explain the gatekeeper mechanism of resistance in all cases, and instead that the function of a gatekeeper mutation could be to bind ATP more strongly to decrease the ability of the ATP competitive kinase inhibitor to bind in the pocket.[12] Which of these mechanisms is responsible for gatekeeper mutation-mediated inhibitor resistance may depend on the kinase in question.

The gatekeeper T790M mutation in EGFR is found in ~50% of EGFR-mutant patients who develop resistance to EGFR inhibition.[13,14] Gatekeeper mutations have similarly been identified in cancers that become resistant to ALK inhibitors (L1196M). Additionally, other "on-target" mutations in EGFR and ALK have been described which are found at other residues within the ATP-binding domain and cause targeted inhibitor resistance.[15-17] While the gatekeeper threonine mutation has been identified as an *in vitro* cause of BRAF inhibitor resistance, this mutation has not been seen in patients.[18] This may be because many BRAF inhibitors induce paradoxical activation of wild-type

RAF.[19] Thus, upregulation of wild-type RAF or RAS signaling can activate the same pathways that are downstream of mutant BRAF. These signaling events may obviate the selective pressure to acquire resistance to so called “paradox activating” RAF inhibitors by mutation of the driver oncogene itself.

The prevalence of on-target mutations as a mechanism of resistance has led to the creation of new inhibitors that can inhibit both the original oncoprotein and its resistance-associated mutated form. While this strategy does improve patient survival in the short term, mechanisms of resistance emerge to these inhibitors as well.[4,20]

Other alterations in the driver oncogene can lead to resistance as well. Studying $BRAF^{V600E}$ mutant lung adenocarcinoma, our group identified that a switch from expression of a full-length $BRAF^{V600E}$ to a shorter splice variant was capable of mediating resistance to the BRAF inhibitor vemurafenib.[21] This splice variant is able to dimerize and activate downstream MAPK pathway signaling despite presence of the RAF inhibitor. This mechanism of resistance was observed initially in BRAF-mutant melanomas, indicating conservation across different tumor histologies.[22]

Finally, changes in the level of the targeted driver oncogene can also cause inhibitor resistance. By increasing the levels of the driver oncogene, an inhibitor appears to be less potent, because there will be fewer molecules of inhibitor per molecule of oncoprotein. Upregulation of $BRAF^{V600E}$ was identified as a driver of resistance in inhibitor-insensitive melanomas.[23] Intriguingly, loss of the initial driver oncogene and replacement with a new driver, also known as “oncogene swap”, has recently been described as a cause of targeted inhibitor resistance.[24] In this instance, a cancer cell loses the targeted driver oncogene and upregulates a new oncogene, such that

oncogenic signaling driving cell survival is mediated by the new oncogene and the inhibitor of the initial oncogene no longer has a substantial impact on cancer cell survival.

## Reactivation of Critical Signaling Pathways

### Parallel Activation of Signaling Pathways.

One way that cancer cells can evolve resistance to a targeted therapy without altering the target oncogene is by upregulating expression and/or activation of a protein that signals through the same signaling pathway. In this way, despite continued suppression of the initial driver oncoprotein by the small molecule inhibitor, critical parallel signaling persists under the control of the newly upregulated protein activity.

Amplification of wildtype *MET* in the setting of EGFR mutant lung cancer was one of the earliest examples of this type of mechanism of resistance.[25-27] Mutant EGFR heterodimerizes with ErbB-3 to activate the PI3K/Akt signaling pathway. Upon inhibition of EGFR with a targeted inhibitor, this pathway is suppressed. *MET* amplification leads to the reactivation of this pathway by forming a MET-ErbB-3 heterodimer.[28] Thus, despite continuous suppression of EGFR by the inhibitor, a critical downstream signaling pathway is reactivated, and resistance to the inhibitor emerges. Upregulated expression of the MET ligand HGF, leading to hyperactivation of MET, has been found to drive resistance to EGFR inhibition through a similar mechanism as *MET* amplification.[29,30] Clinical trials are underway to test the effect of dual inhibition of MET and EGFR to overcome this mode of resistance.[31] Additionally, ErbB-3 blocking antibodies are currently under clinical development.[32] Amplification of the ERBB family

member *HER2* has also been identified as a possible mechanism of resistance to EGFR inhibitors, by similarly activating ErbB3 and downstream PI3K signaling.[33] Inhibition of this key signaling pathway downstream of the activation node is another way to overcome resistance.[34]

Upregulation or activation of non-ERBB family member receptor tyrosine kinases to reactivate downstream signaling pathways and cause resistance has also been identified. IGF-1R pathway activation can mediate resistance in both *EGFR*-mutant and *EML4-ALK* positive lung cancer.[35-37] Overexpression of the tyrosine kinase AXL, which is able to engage multiple downstream signaling nodes also activated by EGFR, was identified by our group and others as a driver of EGFR inhibitor resistance.[38] Similar findings were observed in ALK gene rearrangement positive lung adenocarcinoma.[39]

Similarly, resistance to Raf inhibition can result from upregulated levels of EGF, which activates EGFR and drives downstream pathway activation in $BRAF^{V600E}$ mutant lung adenocarcinoma.[21] Combinatorial inhibition of multiple kinases could be a therapeutic possibility in cases where activation of alternative receptor tyrosine kinases is responsible for driving resistance.

**Downstream Activation of Signaling Pathways.**

Cancer cells can become resistant to targeted inhibitors by amplifying or acquiring activating mutations in pathway genes downstream of the driver oncogene. Thus, despite continued silencing of an oncoprotein's activity by its inhibitor, there is persistent pathway activation downstream of the reactivated pathway node.

Activating mutations in or amplification of genes involved in MAP kinase pathway signaling have been identified as a mechanism of resistance to targeted inhibitors of several driver oncogenes. BRAF mutations drive resistance to targeted inhibition of EGFR, while KRAS amplification leads to ALK kinase inhibitor resistance in EML4-ALK-drive lung cancer.[40,41] Similarly, downregulation of genes that negatively regulate the MAP kinase pathway have also been implicated in resistance. Decreased expression of the phosphatase *DUSP6* leading to rescue of phospho-ERK and reactivation of MAPK signaling was observed in ALK inhibitor-resistant cell lines and patient samples.[40] Similarly, downregulation of *NF1*, leading to reduced levels of the Ras-GTPase activating protein neurofibromin and thus increased Ras activity, has been identified as a driver of resistance to EGFR inhibition in lung cancer.[42]

Along with signaling through MAPK, the PI3K pathway is another major signaling pathway that is commonly hyperactivated downstream of driver oncogenes. Several lines of evidence suggest that downstream reactivation of this pathway may be a mechanism of acquired resistance to targeted inhibitors. First, concurrent *EGFR* and *PIK3CA* mutations are seen in patients with acquired resistance to EGFR inhibition and are correlated with a poor response to EGFR-targeted therapy; furthermore, PIK3CA mutants can drive resistance to EGFR inhibitor *in vitro*.[43,44] These mutations lead to increased phosphorylation of PIP2, generating the second messenger PIP3 and activating AKT. Similarly, homozygous loss of *PTEN*, which normally acts to dephosphorylate PIP3 and suppress this signaling pathway, has also been identified as a mechanism of EGFR inhibitor resistance *in vitro* in lung and other cancers.[45,46] However, the clinical significance of these mutations with regard to inhibitor sensitivity

remains controversial. While concurrent mutations in the PI3K pathway with EGFR and KRAS mutations are associated with a poorer prognosis overall, PI3K mutations do not alter sensitivity or clinical responses to targeted inhibitors in EGFR-mutant lung cancer, suggesting reactivation of this pathway may not actually be a cause of targeted inhibitor resistance in patients.[47]

## Alternative Pathway Activation

### Activation of Pro-Survival Signaling Pathways.

Besides reactivation of critical pro-growth pathways downstream of a driver oncogene, activation of pro-survival signaling networks has also been implicated in targeted inhibitor resistance. Our group discovered that, upon inhibition of EGFR, an NFκB-containing complex is rapidly recruited and activates a downstream pro-survival signaling pathway that limits EGFR inhibitor-mediated cell death.[48,49] Co-treatment of cells with EGFR and NFκB inhibitors prevents the emergence of this resistance in pre-clinical models. [49]

Activation of pro-survival genes also limits response to MAPK pathway inhibitors in BRAF and RAS-driven tumors. Our group and others identified the YAP pathway as a key mediator of response to MAPK pathway inhibition in these tumors.[50-52] In some tumors, YAP levels are elevated and drive transcription of downstream targets, including *BCL2L1*, which encodes the anti-apoptotic BCL-xL protein. These tumors are more resistant to MAPK pathway inhibitors because, even without pro-survival MAPK pathway signaling, these tumors maintain activation of alternative pro-survival signaling pathways through YAP activation.

These mechanisms of resistance are distinct from those discussed earlier, as they involve engagement of pathways separate from those activated by the driver oncogene prior to inhibitor treatment. In both cases described here, combined inhibition of the driving oncogenic pathway and the resistance-mediating pro-survival pathway is an effective way to overcome targeted inhibitor resistance[49,50].

## Additional Mechanisms of Resistance

### Epigenetically Regulated Drug Tolerance.

Global epigenetic changes have been observed in response to small molecule inhibitor treatment. Sharma and colleagues found that the H3K4 histone demethylase KDM5A was upregulated in inhibitor-resistant EGFR-mutant cells, and this overexpression was required for drug resistance.[53] Intriguingly, IGF-1R signaling was found to be required for establishment of this drug-resistant cell population, and *KDM5A* upregulation was linked to IGF-1R signaling. This finding suggests that upregulation of IGF-1R could contribute to inhibitor resistance through multiple mechanisms, both by reactivating critical signaling pathways (as discussed earlier) and by altering a cell's epigenetic landscape.

### Germline Disruption of Apoptosis

Germline polymorphisms in pro-survival signaling pathways have also been implicated in targeted inhibitor resistance. Ng and colleagues identified a common polymorphism in *BIM*, a pro-apoptotic protein, as a mediator of intrinsic targeted inhibitor resistance in EGFR-driven lung cancer and chronic myeloid leukemia.[54]

Normally, MAP kinase pathway signaling activated by the driver oncogene suppresses BIM and it's pro-apoptotic function. Upon treatment with a targeted inhibitor, this suppression is released, restoring the pro-death function of BIM. However, some patients harbor a polymorphism in *BIM* that lacks the critical pro-apoptotic BH3 domain, thus rendering BIM ineffective in driving apoptosis upon oncogene inhibition and causing intrinsic resistance to the targeted inhibitor. In these cases, Ng *et al* suggest BH3 mimetics in combination with targeted oncogene inhibition may be efficacious in combating intrinsic resistance.

**Upregulation of Drug Transporters.**

One non-specific mechanism of small molecule inhibitor resistance is upregulation of drug efflux pumps. Expression of these pumps has long been associated with resistance to a variety of cytotoxic cancer therapies, as they reduce intracellular drug concentration. Some small molecule tyrosine kinase inhibitors are targets of efflux pumps, and in some cases, expression of specific drug transporters have been reported to induce drug resistance to targeted inhibitors of EGFR and ALK.[55,56]

**Epithelial-to-Mesenchymal Transition.**

The epithelial-to-mesenchymal transition (EMT) has been observed in association with acquired resistance to targeted inhibitors in a variety of oncogene-driven cancers, including EML4-ALK and EGFR mutant lung adenocarcinoma.[38] EMT is observed as a morphologic change in cells, decreased epithelial markers (like E-

cadherin) and upregulated mesenchymal markers (like vimentin), as well as increased migration and invasion properties of cells. However, whether EMT is directly responsible for acquired resistance or is an associated but not causal change remains controversial. For example, induction of EMT by TGFB signaling is associated with ALK inhibitor resistance in an EML4-ALK positive cell line, and knockdown of the mesenchymal marker vimentin restores sensitivity in an EML4-ALK acquired resistance model that displays EMT.[57] However, other groups have found that reversal of the EMT phenotype in ALK inhibitor-resistant EML4-ALK lines can be induced despite continued resistance to the targeted inhibitor.[58] Thus, whether or not EMT is a driver of resistance or just a consequence associated with the functional resistance mechanism remains to be elucidated.

**Transformation to Small Cell Carcinoma.**

Finally, histologic transformation of non-small cell lung adenocarcinoma to small cell carcinoma is occasionally observed as a mechanism of resistance to multiple driver oncogene-targeted therapies, including inhibitors of EGFR and EML4-ALK.[59-61] Interestingly, these tumors retain expression of the original mutant oncogene, and do not appear to have acquired any of the resistance mechanisms discussed above. Analysis of these small-cell-transformed tumors revealed loss of *RB1*, a hallmark of small cell lung cancer.[62,63] Loss of *RB1* may be the alteration that induces this non-small-cell to small-cell transformation, resulting in a molecular switch from dependence on the original driver oncogene to a different survival program regulated by *RB1* loss.[64]

## Emergence of Resistance

Most patients will have an incomplete response to a given targeted therapy — that is, their tumors will not completely regress and they will have some degree of residual disease from which acquired resistance subsequently emerges.[10] While much research over the past decade has focused on identifying mechanisms mediating acquired resistance to targeted inhibitors, there is still much to be learned about what other factors might be involved in preventing an initial complete response and supporting residual disease. This population of tumor cells that never completely responds to therapy likely functions as a transition state culminating eventually in a drug-resistant tumor (acquired resistance). Therefore, understanding the mechanisms involved in the survival of this subpopulation of residual tumor cells is critical for progress.

How resistance emerges in a tumor remains unclear. One possibility is that certain resistance mechanisms exist in a subset of tumor cells prior to treatment with a targeted agent. These pre-existing resistant cells are then selected for upon introduction of the targeted inhibitor. Many tumors show a great amount of intratumoral heterogeneity prior to therapy, and there is evidence of the existence of tumor cells harboring resistance mutations prior to treatment with targeted inhibitors.[65-68] Another possibility is that the treatment of tumor cells with a targeted inhibitor induces a drug-tolerant population of cells from which resistance can then emerge based on the stochastic presence of genetic variation due to the underlying mutation rate in replicating cells.[53,68] A third hypothesis is that targeted inhibition of an oncogene itself somehow induces epigenetic or genetic changes, leading to resistance. Likely, any or

all modes of resistance emergence may occur in an individual patient, depending on the intratumoral heterogeneity present prior to treatment and the characteristics of the cancer type and inhibitor used. However, if critical signaling pathways can be predicted and targeted in a patient in an upfront manner, the emergence of resistance through either mechanism described here may be delayed or prevented entirely, offering a promising approach to combat the heterogeneity and adaptiveness of most cancers.

**Development of Rational Upfront Polytherapy Strategies.**

Despite initial frustrations over the inevitable emergence of resistance to targeted kinase inhibitors, new insights into understanding these mechanisms of resistance provide hope for the development of more durably effective treatment strategies. The emergence of resistance can suggest which pathway(s) are critical for the survival of cells driven by specific oncoproteins, suggesting the potential of upfront combinatorial inhibition of the driver oncoprotein and the crucial pathway further downstream. Preclinical studies from our group have demonstrated that upfront treatment of EML4-ALK positive lung tumors with both an ALK inhibitor and a MAPK pathway inhibitor can significantly delay or even prevent onset of resistance.[40] The development of clinical trials to test this upfront therapy in patients is underway. Similar findings were observed in EGFR mutant lung adenocarcinoma preclinical models and individuals with BRAF V600E lung adenocarcinoma.[69,70]

So far in this review, we have covered the myriad ways a cancer cell can develop resistance to targeted therapies. But how can the knowledge of how cancer cells evade inhibitors translate into real benefits for patients? Now that many pathways of resistance

have been identified, these specific resistance mechanisms can be monitored for in the patient once they start inhibitor treatment. In many cases, additional drugs are available that can be combined with the initial inhibitor to overcome emerging resistance. The fact that tumor cells shed their DNA, which can be detected in the blood of patients, has recently begun to be harnessed to effectively obtain serial biopsies of a patient's disease over the course of treatment. In this way, physicians can easily monitor for the emergence of genetic alterations associated with resistance to inhibitors, and potentially deploy combination therapies to short-circuit the emergence of resistance before it is fully established in the patient.[71,72] Early pre-clinical and clinical data suggest it may be critical to combat resistance by treating it before it truly emerges – once a tumor has recurred on a macroscopic level, it appears to be less sensitive to inhibitors targeting its resistance mechanism as when a tumor is initially co-treated with inhibitors targeting both its driver oncogene and a common resistance mechanism.[73,74] This clinical experience exemplifies the importance of early low-level detection of residual disease and tumor recurrence using liquid biopsies (and where feasible on-treatment tumor biopsies) so resistance can be addressed prior to the appearance of macroscopic disease, and of understanding the signaling pathways that are most critical to tumor survival to allow for upfront polytherapy to prevent resistance from ever appearing.[10]

While this review mostly focuses on resistance to targeted therapies in lung cancer, the mechanisms can be broadly applicable to other tumor types as well. First, many other tumors harbor the same driver oncogenes discussed here, are targetable with the same small molecule inhibitors, and show similar patterns of resistance mechanisms. Second, identification of resistance mechanisms that may emerge and

then upfront treatment of patients with combinations of inhibitors to delay or prevent this resistance from occurring is a concept that is generalizable to virtually any tumor type. Better understanding how tumor cells escape from the detrimental effects of targeted therapies holds promise for our ability to prioritize and deploy synergistic drug combinations that chronically control and potentially eliminate the ability of the tumor to evolve full resistance. While drug combinations may show clinical toxicity, prioritizing combinations of agents with synergistic anti-tumor effects could offer a therapeutic window. Alternatively, sequential or alternating drug schedules could be tested, where appropriate, to mitigate clinical toxicity and maintain anti-tumor efficacy.

Altogether, the path to chronic cancer control is clearer and brighter due to the increasing understanding of the biological basis of resistance and the arrival and adoption of emerging technologies that allow us to chart the molecular course of cancer evolution in individual patients during treatment.

**Figure 1.**

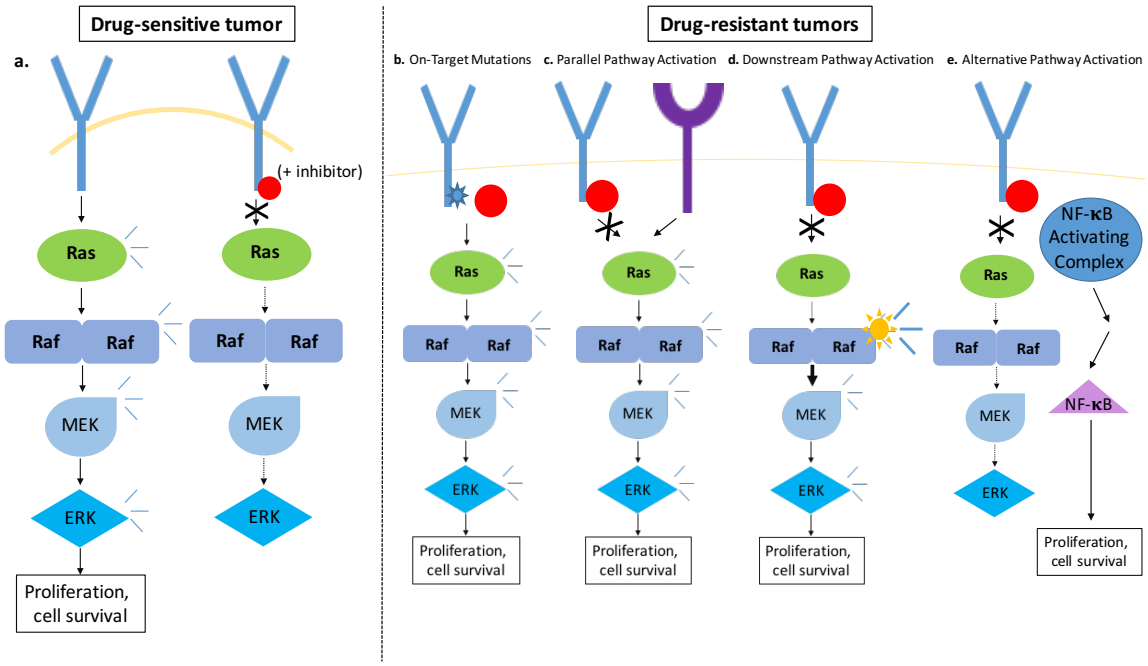


**Figure 4-1: Mechanisms of Resistance to Targeted Therapies**

**a)** Example of a drug-sensitive tumor. Downstream signaling is decreased upon addition of a targeted inhibitor. **b-e)** Examples of mechanisms promoting drug-resistant tumors. **b.** On-target mutations block the ability of the drug to bind to and inhibit the target oncoprotein, allowing continued signaling to promote tumor survival. **c.** Upregulation of a distinct receptor tyrosine kinase sustains signaling through a critical signaling pathway despite continued inhibition of the primary oncoprotein with the targeted drug. **d.** Mutational activation of a protein involved in a critical downstream signaling pathway reactivates the pathway below the level of inhibitor blockade. **e.** Activation of pro-survival signaling networks can prevent inhibitor-mediated apoptosis.

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# Chapter 5
Conclusion

Our work has highlighted the importance of 5' fusion partners in mediating oncogenic transformation and signal pathway dependency in receptor tyrosine kinase (RTK) fusion-driven lung cancers, a previously underappreciated role. We demonstrate for the first time that different ROS fusions signal differentially to downstream signaling pathways, that these signaling differences arise from differential localization of the fusions, and that localization is mediated by the 5' fusion partner. We also show that ROS fusions that activate the MAPK pathways are dependent on the MAPK pathway for survival and specifically sensitive to MAPK pathway inhibition. Additionally, tumors driven by ROS fusions that are capable of activating the MAPK pathway are more aggressive and less responsive to targeted therapy. Current diagnostics for ROS fusions employ a break-apart fluorescence in situ hybridization (FISH) assay, which describes the presence or absence of a ROS fusion in a tumor sample, but does not identify the fusion partner.[1] Our findings demonstrate the importance of identification of the 5' fusion partner and suggests a need for stratification of patients based on fusion partner, as certain subsets of patients may benefit from addition of a MAPK pathway inhibitor or more aggressive treatment. Additionally, understanding which pathways a tumor depends on for survival may be critical in predicting and preventing resistance from arising, and knowing the 5' fusion partner could allow us to predict this pathway dependency. For example, our data demonstrate that SLC34A2-ROS1 and SDC4-ROS1 fusions are uniquely dependent on the RAS/MAPK pathway for survival, suggesting reactivation of this pathway may occur in resistance; indeed, two separate groups have described RAS upregulation or mutation in the contexts of these fusions.[2,3]

There are many different 5' fusion partners associated with ROS1, with case reports identifying new fusion partners published often. We were only able to interrogate the signaling and pathway dependency of the most common ROS1 fusions, but it will be important to translate this work into a more broadly applicable model for therapeutic purposes. Our data demonstrate the importance of ROS1 fusion subcellular localization in downstream pathway activation. One potential way to predict pathway dependency for an uninterrogated ROS1 fusion may be to develop a clinically-usable fluorescence-based assay that can be used to precisely identify the subcellular localization of a ROS1 fusion in patient formalin-fixed paraffin-embedded (FFPE) samples.[4] Alternatively, the importance of MAPK pathway activation in driving tumor growth and stunting response to crizotinib in our isogenic ROS1 fusion NIH-3T3 system suggests that an immunohistochemistry (IHC)-based approach to patient stratification for therapy may be beneficial.

While we have demonstrated a clear dependence on ROS1 localization for specific downstream pathway activation, determining what dictates this differential activating ability remains to be elucidated. One possibility is that there is differential accessibility to critical signaling pathway adaptors. That is, there may be a positive regulator that is only accessible to ROS1 when it is localized at endosomes, or there could be a negative regulator that exists in higher concentrations at the endoplasmic reticulum (ER). Alternatively, the ability of individual ROS1 fusion molecules to dimerize may be hindered at the ER, or the endosome may be a more permissive environment for dimerization. In fact, the precise mechanism by which ROS1 fusion kinases are activated remains controversial. All other receptor tyrosine kinases (RTKs) act as

dimers or oligomers—most exist at the plasma membrane as monomers, and are induced to dimerize by ligand binding the extracellular domain, although ligand is not always required for dimerization.[5,6] Some oncogenic RTKs harbor mutations that promote ligand-free dimerization and activation, like the common EGFR L858R mutant.[7] Other oncogenic RTK fusions contain 5' partners harboring dimerization or oligimerization domains, promoting constitutive dimerization, like EML4-ALK.[8-10] How ROS1 fusions dimerize, however, or whether they even require dimerization for activity at all, remains unclear. First, the fact that wildtype ROS1 has an unknown ligand makes studying the wildtype receptor difficult. Additionally, no group has been able to express the wildtype full-length ROS1 kinase *in vivo* in mammalian cells.[11] Finally, some groups have suggested that ROS1 fusions do not dimerize, and instead may function as active monomers that require a certain subcellular environment for auto-activation.[12,13] If ROS1 fusions do function as monomers requiring specific cofactors for self-activation, differential localization to the ER or endosomes may affect the degree of activation and thus which downstream signaling pathways are able to be engaged.

Finally, what dictates ultimate subcellular localization of the different ROS1 fusions remains to be elucidated. Both SDC4 and SLC34A2 encode proteins (Syndecan-4 and Solute Carrier Family 34 Member 2, respectively) that are transmembrane proteins themselves, whose wildtype forms can reside on the plasma membrane.[14,15] What leads their resultant ROS1 fusion proteins to exist and signal from intracellular membrane compartments, with no identifiable presence on the plasma membrane, is unknown. It is possible that these fusions reside briefly at the plasma membrane and are rapidly internalized, or perhaps they are directly targeted to

intracellular endosomal structures. Wildtype SDC4, a heparan sulfate receptor, exists predominantly in intracellular membranes, and there is some suggestion it may signal from these compartments.[14] Thus, SDC4-ROS1 fusions may copy normal SDC4 trafficking and signaling patterns. The long isoform of CD74, on the other hand, is known to be anchored in the ER; thus, that CD74-ROS1 exists on the ER seems related to the wildtype localization of its 5' fusion partner. Intriguingly, CD74 localization is phosphorylation-dependent; that is, exit of wildtype CD74 from the ER is regulated by phosphorylation of its N-terminal cytoplasmic tail.[16,17] The presence of CD74-ROS1 overwhelmingly on the ER suggests this regulation is somehow lost in the fusion protein, but raises the possibility that some cells could regain this function, relocalizing CD74-ROS1 to endosomes and enabling CD74-ROS1 to activate the MAPK pathway as well. Whether this happens in patients remains to be seen.

We and others have shown localization of ROS1 fusions to be critical for signaling; in fact, Charest *et al* demonstrated that FIG-ROS1 fusions that are mislocalized from the Golgi apparatus still have kinase activity but lack transforming ability, again exemplifying the importance of localization for productive signaling.[12] Thus, understanding what factors regulate subcellular localization could open up new avenues for development of therapeutics that disrupt critical localization of ROS1 fusions.

Our work on ALK fusions demonstrates not only the importance of RTK fusion localization on downstream pathway activation, but also uncovers a novel mechanism of RAS activation in the absence of a membrane. We found that EML4-ALK fusions form intracellular, non-membrane-associated clusters in the cytoplasm, and that this clustering requires the trimerization and HELP domains of EML4, as well as the kinase

activity of ALK. These clusters recruit and activate RAS and the downstream MAPK pathway. We also demonstrate for the first time that unmodified cytoplasmic RAS is able to signal productively.

Our finding that EML4-ALK is able to activate cytoplasmic RAS in a membrane-independent manner raises the possibility that cytoplasmic RAS may also be functional in other cellular contexts. For example, it is possible that other ALK fusions might form similar, signaling-competent clusters. EML4-ALK cluster formation is mediated, at least in part, by oligimerization domains contained within EML4. ALK now has many identified fusion partners, and several of them harbor similar protein-protein interaction domains.[9,18] For example, the anaplastic large cell lymphoma (ALCL) ALK fusion partner nucleophosmin (NPM) mediates oligimerization of NPM-ALK molecules, and the oligimerization domains are required for NPM-ALK-mediated transformation.[19] Intriguingly, NPM-ALK has been characterized as colocalizing with "cytoplasmic granules."[20] The N-terminal fusion partner Kinesin-1 heavy chain in the recently-characterized ALK fusion KIF5B-ALK, also donates an oligimerization domain to the fusion.[21-23] It is possible that the oligimerization domain contributed by the N-terminal fusion partner requires some sort of sequence specificity – for example, both EML4 and KIF5B contain N-terminal microtubule-association domains.[8,24] Whether loss of the transmembrane domain and gain of any nonspecific oligimerization domain is sufficient for ALK cluster formation remains to be seen.

Beyond ALK fusions, cytoplasmic RAS may signal in conjunction with other oncogenic fusions. BCR-ABL, arguably one of the most well-studied oncoproteins, is known to interact with MAPK pathway adaptors, activate RAS, and depend on

RAS/MAPK signaling for survival and oncogenesis.[25-28] Additionally, similar to EML4-ALK, BCR-ABL-driven transformation is dependent on tetramerization mediated by a coiled-coil domain in BCR.[29] BCR-ABL is well-characterized as a cytoplasmic, non-membrane-associated fusion protein, and a review of the literature reveals that cytoplasmic BCR-ABL appears to be localized in intracellular clusters. [30,31] All of these findings suggest that BCR-ABL clusters may be signaling to the MAPK pathway through cytoplasmic RAS, which would represent a major shift in our understanding of the molecular requirements for RAS activation.

Finally, the identity of the EML4-ALK clusters we have discovered remains uncharacterized. The clusters are not associated with any membrane, but form clearly functional intracellular signaling aggregates. Liquid droplets are recently characterized intracellular structures that are not membrane-bound, but instead are phase-separated concentrations of proteins.[32,33] Several functional intracellular structures, including nucleoli, P-bodies, Cajal bodies, stress granules, and HP1-associated heterochromatin, have been identified as liquid droplets.[34-37] Preliminary studies suggest EML4-ALK clusters share features with these well-characterized liquid droplets: that is, they have delayed fluorescence recovery after photobleaching (FRAP) and they sediment like the P-body protein DCP1B (a component of a known liquid droplet) in our ultracentrifugation experiment. Additionally, considering EML4-ALK clusters as functional liquid droplets may help explain how EML4-ALK is able to engage the RAS/MAPK pathway in the absence of a membrane. The membrane serves as a local concentrator of signaling factors involved in the MAPK pathway, bringing together multiple signaling components to a distinct site where they can associate and interact with one another.[38,39] Liquid

droplets can act in a similar manner as spatiotemporal concentrators of signaling molecules.[32] Thus, EML4-ALK clusters may be liquid droplets that facilitate recruitment and concentration of MAPK pathway signaling proteins, including RAS, thereby bypassing the requirement for a membrane. While more conclusive studies are required to confirm that EML4-ALK clusters are liquid droplets, this would be the first example of a phase-separated liquid compartment driving an oncogenic signaling pathway.

In summary, we have discovered that localization of two different RTK fusions found in lung adenocarcinoma is critical in activation of downstream signaling pathways. Our work on ROS1 fusions has revealed the importance of the 5' fusion partner in dictating intracellular distribution, which drives specific downstream pathway activation and addiction. This finding suggests a shift in the way we diagnose and treat patients with different ROS1 fusions. Our research on ALK fusions has revealed that EML4-ALK exists in an intracellular non-membrane-bound compartment, and that EML4-ALK clustering is mediated by domains in the 5' fusion partner. We have also demonstrated for the first time that RAS is able to signal to the MAPK pathway in the absence of a membrane, through recruitment of MAPK signaling pathway components to EML4-ALK clusters. These findings have broad-reaching implications for both our understanding of the cell biologic mechanisms driving EML4-ALK-positive cancers and, more generally, of basic RAS signaling biology.

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# Chapter 6
## Materials & Methods

**Materials & Methods**

**Cell Culture.** All cell lines were maintained in a humidified incubator at 37°C, 5% $CO_2$. The patient-derived ROS1-positive lung adenocarcinoma lines HCC78, CUTO-2, and CUTO-23, the patient-derived ALK-positive lung adenocarcinoma lines H3122 and STE-1, and the normal lung epithelial line BEAS2-B, were all maintained in RPMI-1640 supplemented with 10% FBS and 100ug/mL of penicillin/streptomycin. HEK-293T cells and NIH-3T3 cells were maintained in DMEM-High Glucose supplemented with 10% FBS and 100ug/mL of penicillin/streptomycin. CUTO-2 and CUTO-23 cells were a generous gift from Dr. Robert Doebele (University of Colorado, Denver, CO, USA). STE-1 cells were a kind gift from Dr. Christine Lovly (Vanderbilt University, Nashville, TN, USA).

**Compounds**. Crizotinib (Selleck Chemicals, Houston, TX, USA) and the SHP2 inhibitor RMC-4550 (Revolution Medicines, Redwood City, CA, USA) were dissolved in DMSO for *in vitro* experiments. Crizotinib was dissolved in 0.5% methylcellulose prep with 0.5% Tween 80 for *in vivo* experiments.

**Antibodies**. The following Cell Signaling Technology (Danvers, MA, USA) antibodies were used: phospho-ROS1 (Y2274, #3078), ROS1 (#3287), phospho-ALK (Y1604, #3341), ALK (#3633), phospho-STAT3 (Y705, #9145), STAT3 (#9139), phospho-AKT (S473, #5012), AKT (#2920), phospho-ERK (Y202/204, #4370), ERK (#4694), Grb2 (#3972), Gab1 (#3232), Gab2 (#3239), SOS1 (#5890), EEA1 (#3288), Calnexin (#2679), Anti-rabbit IgG, HRP-linked Antibody (#7074), Anti-mouse IgG, HRP-linked Antibody (#7076). The following Sigma-Aldrich (St Louis, MO, USA) antibodies were used: FLAG M2 (#F1804), Beta-Actin (#A2228). The following EMD-Millipore (Billerica,

MA, USA) antibodies were used: Total RAS (05-516). The following Santa Cruz Biotechnology (Santa Cruz, CA, USA) antibodies were used: EEA1 (sc-6415), H-RAS (sc-53959), N-RAS (sc-31), K-RAS (sc-30), GAPDH (sc-365062). The following Abcam (Cambridge, UK) antibodies were used: Calnexin-Alexa Fluor 488 (ab202574). The following Life Technologies Thermo Fisher Scientific (Waltham, MA, USA) antibodies were used: Alexa Fluor 488 Donkey Anti-Mouse (#21202), Alexa Fluor 499 Donkey Anti-Goat (#11055), Alexa Fluor 594 Donkey Anti-Rabbit (#21207).

**DNA transfections.** 293T cells were transiently transfected using TransIt-LT1 transfection reagent (Mirus Bio LLC, Madison, WI, USA).

**Immunoblotting.** For immunoblotting, cells were washed with ice-cold PBS and scraped in ice-cold RIPA buffer [25 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, supplemented with 1X HALT protease inhibitor cocktail and 1X HALT phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA)]. Lysates were clarified with sonication and centrifiugation. Lysates were subject to SDS/PAGE followed by blotting with the indicated antibodies. Signal was detected using Amersham ECL Prime reagent (GE Healthcare Life Sciences) and chemiluminescnce on an ImageQuant LAS 4000 (GE Healthcare Life Science, Chicago, IL, USA). 293T cells were serum starved (0%S) for 5 hours and ROS1 BEAS2-B cells were serum starved (0%S) for 24 hours prior to lysate collection.

**siRNA knockdown.** Cells were seeded in 6-well plates. The following day, siRNA were resuspended to a final concentration of 5uM in serum-free medium with DharmaFECT transfection reagent (Thermo Fisher Scientific), then pipetted onto cells. Lysates were harvested 55 hours later. The following ROS1 siRNAs from Sigma-Aldrich were used:

Hs01_00183685 (siROS1 #1) and Hs01_00183690 (siROS1 #2). Non-targeting control siRNA was purchased from Dharmacon (GE Life Sciences).

**Constructs.** Lentiviral expression constructs for SDC4-ROS and CD74-ROS were generous gifts from Dr. Christine Lovly (Vanderbilt University, Nashville, TN, USA). Lentiviral expression construct for SLC34A2-ROS was a generous gift from Dr. Monika Davare (OHSU, Portland, OR, USA). The lentiviral expression construct for KRAS4B WT was a kind gift from Dr. Frank McCormick (UCSF). The C185S mutation in this construct was generated using QuikChange Lightning site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). The retroviral expression constructs for MEK-DD (#15268) and CA-STAT3 (#24983) were purchased on Addgene. The YFP-EML4-ALKv1 WT construct was a kind gift from Dr. Richard Bayliss (University of Leeds, Leeds, UK). The EML4-ALK kinase dead and deletion mutant constructs were generated using QuickChange Lightning (Agilent). The EML4-ALK-WT-Myc construct was a kind gift from Dr. Hiroyuki Mano (University of Tokyo, Tokyo, Japan). The N-terminal 3X Flag-tag vector was a gift from Dr. Nevan Krogan (UCSF). The GFP-tagged Grb2 expression construct was purchased on Addgene (#86873).

**Viral transduction.** 293T viral packaging cells were plated in 10cm dishes the day prior to transfection. They were transfected with lentiviral or retroviral expression constructs and the appropriate packaging plasmids using TransIt-LT1 transfection reagent (Mirus Bio LLC, Madison, WI, USA). Viral supernatants were collected 48-72 hours post-transfection and used to transduce cell lines in the presence of 1X Polybrene for 24 hours. 72 hours post-infection, media was changed to standard growth media plus the appropriate selectable marker (1ug/mL puromycin for all lines expect NIH-3T3, which

were selected with 2ug/mL puromycin). CA-STAT3-infected cells were sorted on a BD FACSAria II (BD Biosciences, San Jose, CA) for GFP-positivity.

**Crystal Violet Assays.** Cells were seeded in 12-well plates at 10% confluency and treated with drug the following day. They were grown for 6-8 days, then fixed with 4% paraformaldehyde and stained with crystal violet. Pictures of stained cells were taking using transillumination on an ImageQuant LAS 4000 (GE Healthcare, Chicago, IL, USA). Crystal violet was dissolved in 500ul 1% SDS and quantified based on 470nM absorbance using a SpectraMax spectrophotomer (Molecular Devices, Sunnyvale, CA, USA). Relative cell viability was determined by normalizing to DMSO-treated control. All crystal violet images are representative and quantification values arise from $\geq$ n = 3 experiments. Statistical significance was determined by multiple t-test analysis using Prism 6 (Graphpad Software, La Jolla, CA, USA).

**Immunofluorescence.** Cells were seeded in 4-well Lab Tek II Chamber Slides (Thermo Fisher Scientific). The following day, cells were fixed for 15 minutes with 4% paraformaldehyde, washed, and incubated in blocking buffer for 1 hour (1X PBS with 1% BSA and 0.3% Triton X-100). Blocking buffer was aspirated and cells were incubated with primary antibody overnight in the dark at 4°C. The following day, cells were washed, incubated with fluorophore-conjugated secondary antibody for 1 hour at room temperature in the dark, washed, then mounted using ProLong Gold Antifade reagent with DAPI (Cell Signaling Technology, Danvers, MA). Slides were analyzed using a Nikon Ti microscope with a CSU-W1 spinning disk confocal (Nikon Imaging Center, UCSF).

**Ultracentrifugation.** Cells were seeded in 10cm dishes and harvested the following day by scraping into buffer A [10mM Tris-HCl (pH7.4), 1mM EDTA, 250mM Sucrose, supplemented with 1X HALT protease inhibitors (Thermo Fisher Scientific, Waltham, MA)]. Lysates were gently sonicated on minimum intensity and cleared by centrifugation. Lysate was then split equivalently to two ultracentrifugation tubes, and one tube was supplemented with 1% Triton X-100. Lysates were then ultracentrifuged at 100,000xg for 1 hour at 4°C in an Optima MAX Ultracentrifuge (Beckman Coulter, Brea, CA). Supernatant and pelleted fractions were separated, resuspended with Laemmli sample buffer, boiled, and analyzed by SDS-PAGE.

**Immunoprecipitation.** Cells were seeded in 10cm dishes and harvested the following day by scraping into IP buffer [0.5% NP40, 50mM Tris (pH 8.0), 150nM NaCl, supplemented with 1X HALT protease inhibitors and 1X HALT phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA)]. Lysates were gently sonicated on minimum intensity and cleared by centrifugation. 1mg protein was incubated with 50ul anti-FLAG M2 magnetic beads (#M8823 – Sigma-Aldrich, St Louis, MO, USA) for 3 hours on a nutator at 4°C. Beads were washed in 1XTBS, then protein was eluted off beads with 250ng/ul FLAG peptide (#F3290 – Sigma-Aldrich, St Louis, MO, USA). Samples were boiled in Laemmli sample buffer and analyzed by SDS-PAGE.

**Ras-GTP pulldown.** RAS activity was assessed using the Ras Pull-down Activation Assay (#BK008- Cytoskeleton Inc, Denver, CO, USA). Cell lysates were collected and snapfrozen as recommended by protocol. Assay performed according to manufacturer instructions.

**Xenografts.** NIH-3T3 xenografts were generated by injecting $1x10^6$ cells in matrigel in flanks of 8-week old NOD/SCID mice. Mice were randomized to treatment groups once tumors reached a size of 150 $mm^3$ (n = 6 tumors per treatment group). Crizotinib was administered at 50mg/kg daily by oral gavage.

**Live-cell imaging.** Fluorescent protein-expressing cells were cultured in 35mm glass bottom culture dishes (MatTek Corporation, Ashland, MA, USA) and analyzed by confocal microscopy on a Nikon Ti microscope with a CSU-W1 spinning disk confocal (Nikon Imaging Center, UCSF).

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