

TARGETING CANCERS DRIVEN BY THE RECEPTOR TYROSINE KINASE ROS1

By

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A DISSERTATION

Presented to the Department of Cell and Developmental
Biology and
the Oregon Health & Science University
School of Medicine
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

May 2022

School of
Medicine
Oregon Health & Science University

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Acknowledgements

“The most beautiful experience we can have is the mysterious – the fundamental emotion which stands at the cradle of true art and true science.”

Albert Einstein

There are many people that I would like to thank for their support throughout my educational career and doctoral work. I first would like to start with thanking my parents, Usha and Rajan, for instilling in me an insatiable curiosity and interest in science and medicine. Both my parents have achieved advanced degrees in STEM fields and from a young age I was exposed to the math and science required to ultimately pursue a career as a physician-scientist. More importantly, they have been a constant source of positive emotional support during the good and the bad times and taught me how to accomplish my goals and objectives in an ethical and honest manner.

Next, I would like to thank my doctoral advisor, Dr. Monika Davare. Dr. Davare is an extremely accomplished scientist whom I have been fortunate to learn from as her first graduate student and one of the first members of her lab. Dr. Davare always made herself available to advise and assist with science whenever I needed it despite her busy schedule. I did not have a background in wet-lab research or cancer biology prior to coming to OHSU and Dr. Davare gave me the opportunity to learn and improve in this field. Over the last five years, I have seen the lab transform from a small lab of 3 to having three times the amount of personnel funded by multiple federal and society grants. I have had the unique opportunity to participate in the building of a new lab and hope that I have contributed positively to the lab's present and future success. I thank the members of the Davare lab for helping me grow as an individual during my time in graduate school.

In addition to Dr. Davare, I would like to thank my co-mentor and dissertation advisory committee member, Dr. Dan Marks. Dr. Marks, as the head of the Papé Family Pediatric Research Institute, has provided crucial departmental support to my lab in pursuit of our scientific goals. I would also like to thank the rest of my dissertation advisory committee, including Dr. Melissa Wong and the chair of the DAC, Dr. Jeffrey Tyner, for devoting their valuable time to support me in my pursuit of a Ph.D. They have provided apt recommendations and insight at important stages of my research and have always shown a willingness to work with me throughout my training. I also thank Dr. Tyner and Dr. Steve Kurtz for providing numerous small-molecule compounds that were crucial for my research. I also would like to thank Dr. Jacoby and Alexis Young from the M.D./Ph.D. program and Dr. Philip Copenhaver and Lola Bichler from the Cell and Developmental Biology program for their help in navigating my Ph.D.

I would like to thank my mentors prior to coming to OHSU: Dr. Yashar Kalani and Dr. Neil Crawford. I had the opportunity to work with both at Barrow Neurological Institute and it was their support and encouragement that allowed me to pursue a dual degree track. They continue to provide mentorship to this day.

I would also like to thank my past and present fellow students at OHSU, in particular Dr. Katherine Michaelis, Dr. Kevin Burfeind, Dr. Kelsey Priest, Dr. Derek Zachman, Dr. James

Goodman, Dr. Samuel Huang, Dr. Jevgenia Zilberman-Rudenko, Dr. Cheryl Claunch, Dr. Katie Lebold, Dr. John Butler, and Dr. Mollie Marr, for providing advice, support, and feedback throughout my time in the M.D./Ph.D. program. These students taught me how to carry out my responsibilities throughout my time at OHSU and I am grateful for their investment of time in helping me to reach my goals.

Finally, I would like to thank my two steadfast friends throughout my time at OHSU, Dr. Sunil Joshi (also a fellow classmate in the M.D./Ph.D. program) and Dr. John Michaels. These are friends that went above and beyond to aid me throughout my Ph.D. and time at OHSU, who encouraged and motivated me regardless of if the science was going well or not. They provided resources and reagents free of charge with nothing asked for in return, they took time out of their schedules to edit my writing and watch my practice presentations. I would not have succeeded in my Ph.D. without their help, and I am forever grateful to them for this.

Abstract

Genetic aberrations that cause constitutive activation of the receptor tyrosine kinase (RTK), ROS1, can drive cancer formation. Treatment of these cancers with tyrosine kinase inhibitors (TKIs) can lead to cancer reduction in patients. While ROS1-TKIs have achieved notable success in the clinic, especially in the treatment of non-small-cell lung cancer (NSCLC), patients eventually stop responding to treatment, as seen with other forms of targeted therapy. Therapeutic resistance represents a formidable challenge to overcome as secondary alterations either in ROS1 itself or in alternative proto-oncogenes and tumor-suppressors can reduce TKI efficacy. Furthermore, we still have a rudimentary understanding of the spectrum of ROS1-independent alterations that can cause therapeutic resistance. In addition to combating resistance, an outstanding question exists as to if single point mutations in ROS1 can promote constitutive activation of the receptor like what is observed with other RTKs. If so, a need would also exist to test the efficacy of ROS1-TKIs against these alterations. Research presented in this dissertation attempts to address these unmet clinical and research needs. In chapter 2, I present data that may increase durability of ROS1-TKI treatment in the future by elucidating a novel mechanism of therapeutic resistance to ROS1-TKI: overexpression of the proto-oncogenic transcription factor, MYC. In doing so, I also propose several classes of therapeutic compounds that could combine with ROS1-TKI to overcome this mechanism of resistance. In chapter 3, I present data that potentially expands the scope of ROS1-TKI treatment by identifying novel gain-of-function missense mutations in the kinase domain of ROS1 that promote oncogenesis and demonstrate that ROS1-TKI can effectively attenuate tumors driven by such alterations. Such findings both

increase the durability and expand the scope of TKIs that can effectively treat ROS1-driven cancers.

Chapter 1: Introduction

Developing precision weapons against cancer – a primer

Cancer has befuddled humanity for thousands of years, so much so that it continues to inhabit an enigmatic sphere in today's public discourse. The challenge that this disease poses has meant that governments continue to devote millions, if not billions, of dollars to better understand and confront it. In fact, in the recent State of the Union from March 1, 2022, in the midst of numerous crises ranging from a pandemic to a large-scale European war, the president of the United States devoted a significant portion of his speech to address the “number two cause of death in America”: he stated his goal to reduce cancer mortality by 50 % over the next 25 years and announced a new agency, Advanced Research Project Agency-Health (ARPA-H) to specifically focus on devoting significant resources to solving the issues that cancer present (1). Such attention and action towards fighting cancer demonstrate the burden that such an ancient disease continues to inflict on the modern, high-tech world.

In many respects, cancer represents the perfect evolution of human cells in their ability to proliferate regardless of roadblocks either internal or external to the cell. Over the years, researchers attempted to define the key characteristics that distinguish cancer cells from normal cells. This culminated into a general framework where cancer is thought to be represented by six hallmarks: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, and resisting cell death (2).

Researchers have made much progress towards better understanding each of the six hallmarks of cancer, but the question inevitably turns to how to utilize, or “weaponize”, this information to defeat cancer. While blunt instruments like chemotherapy and radiation, which eliminate both cancerous and normal cells, continue to be important mainstays in cancer treatment, there has been a greater push in the last couple of decades to increase the precision by

which we can attack cancer cells. Much of these efforts centered around directly shutting down gain-of-function oncogenes, which are transcribed and translated into proteins that promote the conversion of normal cells to cancer cells. With the advent of next-generation sequencing (NGS) technologies, scientists were able to characterize an increasing number of these oncogenes in subsets of a number of cancer types, which expanded the potential number of therapeutic targets that may be exploited for greater precision in cancer treatment. As oncogenic signaling in cells is largely facilitated by cascades of enzymes that transfer organic functional groups, like phosphates, from one substrate to another, scientists developed small-molecule compounds that can bind competitively or non-competitively to the active sites of aberrant enzymes to attenuate their impact on altering cell signaling networks.

This dissertation will focus on a class of oncogenes that relate to the receptor tyrosine kinase, ROS1. As I will explain in the remaining sections of this chapter, receptor tyrosine kinases (RTKs) are found to exhibit aberrant over-activation that promotes cancer formation. I will discuss how RTKs normally operate, then I will discuss in the context of the ROS1 receptor how these proteins can be altered to promote oncogenic transformation. Finally, I will discuss the use of the small-molecule compounds, tyrosine kinase inhibitors (TKIs), and their ability to shut off overactive ROS1 signaling and cancer cell proliferation.

Normal Structure and Function of Receptor Tyrosine Kinases

In the human genome, researchers have discovered 58 different receptor tyrosine kinase (RTK) genes that can be further subdivided into 20 families (3-5). RTK genes code for proteins that localize the plasma membrane and link extracellular signals to intracellular cell signaling changes. RTKs have multiple domains that allow them to fulfill this function: an extracellular domain (ECD), single-pass transmembrane helix, and then an intracellular region that consists of a juxtamembrane domain (JM), tyrosine kinase domain (TKD), and C-terminal domain (CTD) (6).

While the intracellular domain tends to be conserved across many RTKs, the structure of the ECD can vary greatly (7). The primary way that the ECD varies across RTKs is in the number and type of protein motifs present; such motifs include fibronectin type III-like domains, cadherin domains, leucine and/or cysteine-rich repeats, and immunoglobulin-like loop motifs (7). Ultimately, the diversity of the ECD structures found across RTK families means that RTKs are likely to bind distinct classes of ligands (8).

On a general level, RTKs are activated by ligand binding to the ECD, which leads to a conformation shift in the receptor that allows for the RTK to catalyze the transfer of a phosphate group from ATP to a variety of adaptor signaling proteins (6). These adaptor proteins then activate a number of signaling pathways like RAS-RAF-MEK-ERK, PI3K/AKT, and JAK/STAT (3). These pathways can then regulate a multitude of cellular processes that include but are not limited to cell survival, proliferation, and migration (9). Because of their powerful ability to change cell phenotypes, RTKs are tightly regulated in normal physiological conditions by a

number of intrinsic and extrinsic mechanisms (10,11). Over the next few paragraphs, I will summarize what we know about these inhibitory regulatory mechanisms and then discuss the series of structural steps that lead to RTK activation from ligand binding.

RTKs initially reside in monomeric or oligomeric states prior to a ligand binding the ECD (12,13). A notable example of this is seen with both the EGFR and ErbB2 receptors, where the unstimulated EGFR receptor can reside in monomeric form in one cell type while forming oligomers in other cell types; in contrast, unstimulated ErbB2 primarily assembles into large oligomers that can have as many as 10 copies of the receptor bound in a complex (13). In contrast, members of the insulin receptor family tend to form dimers that are held together by disulfide bonds prior to activation (14).

Nonetheless, the inactive state across all RTKs is characterized by an inactive TKD, due to the presence of both intrinsic structural regulation and extrinsic protein tyrosine phosphatases (15). The intrinsic mechanisms of autoinhibition can be subdivided by the region of the RTK. In the ECD, certain RTKs have domains in the ECD that suppress ligand binding. FGFR represents one receptor where researchers provide evidence that the acid box domain and immunoglobulin-like domain 1 seem to suppress FGF and heparan sulfate binding to the FGFR1 ECD, and that when these domains are eliminated, affinity increases between these ligands and FGFR1 (15-17). Unpublished data from my laboratory also suggest that certain regions of the ECD in the RTK ROS1 also suppresses receptor activation, as deletions of certain motifs in the ECD of ROS1 lead to autophosphorylation of the receptor. As a result, the ECD can play a role in autoinhibition.

The most studied region with regards to autoinhibition is the intracellular juxtamembrane domain (JM), which consists of a few dozen residues in between the transmembrane helix and the TKD (18). The JM both promotes and inhibits RTK activity (18). Several mechanisms exist by which the JM is thought to autoinhibit an RTK. Analyses of the crystal structures of several

RTKs, including FMS-like tyrosine kinase 3 (FLT3) (19) and KIT (20), showed that residues in the JM interact with the activation loop in the TKD to keep an RTK inactive. Importantly, mutations that disrupt the autoinhibitory function of the JM lead to constitutive RTK activation (21). Publications also show that the positively charged amino acids present in the N-terminal region of the JM can interact with negatively charged phosphatidylinositol-4,5-bisphosphate (PIP2) domains found on the lipid bilayer; this interaction anchors the JM in such a way that it removes inhibitory interactions of the JM on the TKD, thus promoting activation (22-25).

The TKD and CTD also have roles in the autoinhibition of RTKs. With regards to the TKD, multiple studies demonstrate that the activation loop (A-loop) itself serves as an autoinhibitory motif, whereby in the absence of ligand binding, the A-loop lodges inside the active site and prevents ATP binding (18,26,27). Once ligand binding occurs, the loop swings out from the active site, and the RTK can then bind ATP.

The CTD can also prevent RTK activation in a similar manner as the A-loop of the TKD, whereby residues from the CTD also interact with the active site and repress ATP binding in certain RTKs like Tie2 (28).

In addition to intrinsic regulatory mechanisms that maintain RTK inactivation, extrinsic protein tyrosine phosphatases also help to maintain RTKs in a low-activity state. Studies in mice demonstrated that reducing the expression of protein-tyrosine phosphatase 1B (PTP-1B), a protein that normally dephosphorylates the insulin receptor (INSR), increases insulin receptor activation and reduces obesity, thus suggesting that PTP-1B is essential to keep INSR inactive (29,30). Studies on other RTKs supported these findings: for example, knocking out and overexpressing protein-tyrosine phosphatases were used to demonstrate that protein-tyrosine phosphatase activity is needed alongside the intrinsic autoinhibitory mechanisms to keep RTKs inactive (10).

Given the foregoing discussion of how RTK autoinhibition is regulated, the question becomes how RTKs are activated. To understand this issue, one needs to understand the structure of the TKD. The TKD consists primarily of two lobes, one small N-terminal lobe with 5 beta-strands that is connected by several residues to a larger alpha-helical C-lobe (26). Key features in the N-terminal lobe include the alpha-C helix and the P-loop, a motif rich in glycine (6,31). The active site of the TKD enzyme is a space that forms between N-terminal and C-terminal lobes; ATP can then bind in this region when ligand binding induces a structural reversal of the intrinsic autoinhibitory mechanisms discussed above (26).

One of the initiating events prior to ATP binding consists of structural changes to the activation loop (A-loop). The A-loop is flanked by two conserved motifs, DFG and APE, and in an inactive state blocks ATP from binding the active site (32). When the ligand binds to the ECD of an RTK, a dimer forms and trans-phosphorylation of tyrosines in the A-loop allows for ATP to bind the active site. Interestingly, it was only found recently how this transphosphorylation occurs. Chen et al. (12) found that although the intracellular kinase dimer complex that forms to facilitate A-loop transphosphorylation is energetically unfavorable (due to one A-loop electrostatically repelling the A-loop on the other molecule), ligand-induced dimerization provides the energy required to overcome this electrostatic repulsion and push the two A-loops together. The asymmetric complex that results from this event features one of the A-loops acting as an enzyme kinase to promotes phosphorylation of tyrosines on the other A-loop. Once the A-loop tyrosines are phosphorylated, the A-loop swings out of the active site, where the initial segment of the A-loop, the DFG (aspartate-phenylalanine-glycine) faces into the active site (DFG-in). In the active (DFG-in) state of a TKD, the aspartate coordinates ATP inside the active site points into the active site to coordinate the ATP. In the inactive state of the TKD (DFG-out), the phenylalanine instead of the aspartate points into the active site (32).

There are several structural interactions that then stabilize ATP in the active site to promote catalysis. One of the important interactions involves a salt bridge with the lysine residue found at the N-terminal lobe surface and the glutamic acid residue of the alpha-C helix in the N-terminal lobe, thereby helping to anchor the ATP in place (33). Another interaction consists of the P-loop associating with the beta-phosphate of ATP, so that the gamma-phosphate can be transferred successfully (26,31,34). A third interaction then consists of the need for both the A-loop and catalytic loop (located in the C-terminal lobe) to interact with the alpha-C helix; an aspartate residue in the catalytic loop can then function as a base and remove a proton from a tyrosine to facilitate transfer of a phosphate group from ATP to the de-protonated tyrosine (26,33).

TKD activation, which is characterized by A-loop transphosphorylation, then triggers phosphorylation of tyrosines in other areas of the RTK, including the CTD. Tyrosines in other areas like the CTD can then serve as docking sites for adaptor proteins (PLC-gamma, SHP2) that are modified to carry signals to downstream signaling pathways mentioned previously (3). The key feature of these adaptor proteins is that they contain phosphotyrosine-recognition domains, such as the Src homology 2 (SH2) domain or the phosphotyrosine-binding (PTB) domain (7,9). Thus, it is this final step of adaptor protein binding that facilitates transfer of extracellular signals via ligand-binding to intracellular signaling pathways to influence cellular outcomes.

The receptor tyrosine kinase ROS1

Adapted from: Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. ROS1-dependent cancers — biology, diagnostics and therapeutics. Nature Reviews Clinical Oncology 2021;18(1):35-55 doi 10.1038/s41571-020-0408-9 PMID - 32760015.

Normal structure and function of the receptor tyrosine kinase, ROS1

The receptor tyrosine kinase (RTK), ROS1, is the largest RTK in the human genome. The *ROS1* gene is located on the reverse strand of chromosome 6 and consists of 44 exons (**Fig. 1.1A**). The large size of the receptor is mediated by the significant N-terminal extracellular domain encoded by the first 34 exons of the *ROS1* gene. This extracellular domain contains nine fibronectin type III repeats and three beta-propeller domains (**Fig. 1.1B**) (35). Sequence analysis of ROS1 reveals that like other RTKs, ROS1 consists of an extracellular domain, single-pass transmembrane domain, and intracellular tyrosine kinase and C-terminal domains. Furthermore, such sequence analysis infers the following: one, that homology exists between ROS1, anaplastic lymphoma kinase (ALK), and other members of the insulin receptor RTK family, and two, that *ROS1* is a conserved RTK related to the protein sevenless from *Drosophila melanogaster* and roller-3 from *Caenorhabditis elegans* (36-38). It is important to note that no crystal or cryo-EM structure of the full-length ROS1 receptor currently exists.

Much of what we assume about the role of ROS1 in normal physiological function comes from the discovery of the ROS1 sequence. Initially, researchers in the 1980s found that the tyrosine kinase function that ROS1 exhibits was linked to the UR2 avian sarcoma retrovirus; this protein was initially named v-ros (39,40). Interestingly, the v-ros protein in the UR2 retrovirus was found to promote oncogenic transformation of chicken embryo fibroblasts and neuroretinal cells (39-43). Researchers then cloned the human form of the *ROS1* gene by generating

complementary DNA to the *v-ros* gene in the UR2 retrovirus and then combining this with placental genomic DNA from humans to clone and determine the sequence of human *ROS1* (44). This work is complemented by similar efforts to clone *ROS1* homologues and orthologues in other animals (45-47).

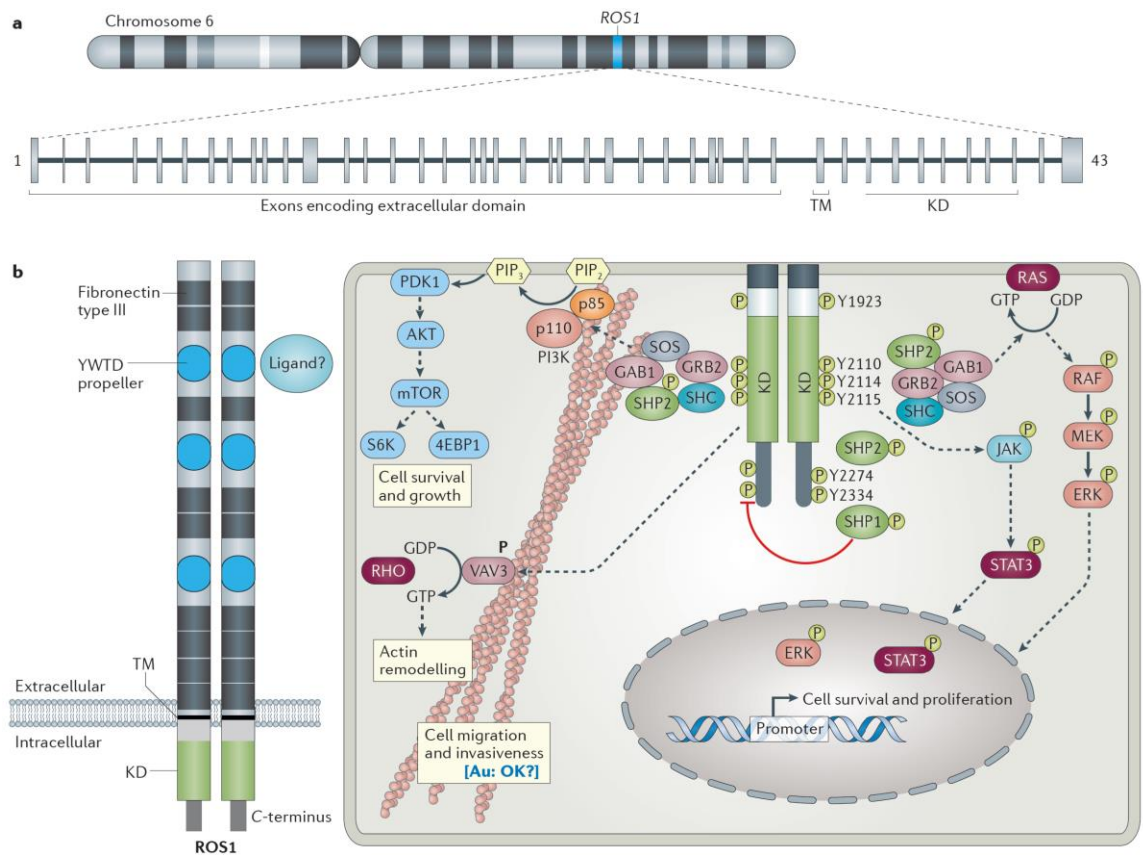


Figure 1.1: *ROS1* gene, structure, and signaling. A. Chromosome and gene map of *ROS1*. **B.** *ROS1* domain structure, predicted from sequence analysis compared to other receptor tyrosine kinases. **C.** Cell signaling pathways that are activated downstream of *ROS1* activity (canonical signaling pathways found with other receptor tyrosine kinases). *Reproduced with permission from: Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. ROS1-dependent cancers — biology, diagnostics and therapeutics. Nature Reviews Clinical Oncology 2021;18(1):35-55 doi 10.1038/s41571-020-0408-9 PMID - 32760015.*

Ultimately, little is known about the tangible role that the ROS1 RTK satisfies in normal cell physiology, as there is no ligand that we confidently know binds to the ROS1 extracellular domain. A significant portion of what we know about the effect of ROS1 on cells comes from the effects on downstream signaling that were elucidated in multiple studies using v-ros and chimeric receptor constructs that combine the extracellular domain of a non-orphan RTK (like EGFR) with the intracellular ROS1 domain that can then be stimulated with the known ligand (i.e. EGF for EGFR-ROS1) (48-54). Evidence from these studies indicates that ROS1 activation adheres to what is known about canonical RTK activation: ligand binding triggers autophosphorylation of specific tyrosine residues in the intracellular domain that can recruit adaptor proteins. The recruitment of SH2 domain-containing and other known RTK-associated adaptor proteins stimulates signaling via the RAS–RAF–MEK–ERK, PI3K/AKT and JAK–STAT pathways, which promote various phenotypes like cell survival, growth, and proliferation (38,49,52-54) (**Fig. 1.1C**). Non-receptor tyrosine phosphatases including SHP1 and SHP2 likely also interact with the intracellular ROS1 domain. SHP2 likely promotes ROS1 signaling, while SHP1 acts as a negative regulator by dephosphorylating ROS1 and reducing downstream signaling (48,55).

While we have not definitively identified ligands that interact with the extracellular domain of ROS1, several studies suggested new avenues to elucidate the role of ROS1 in various cellular phenotypes. For example, multiple studies found that germline knockout of ROS1 in mice leads to male infertility (56-63). Features observed that led to infertility in these ROS1-knockout mice involved the lack of spermatozoa maturation, bent tails on spermatozoa preventing their ability to enter the oviduct, loss of the initial segment of the epididymis, and a loss of osmolyte balance in the early part of the epididymis (56,59-63). Ultimately, a key study in 2014 by Jun et al. found that generating transgenic mice with kinase dead ROS1 leads to failed sperm

maturation and infertility, which is due to the loss of ROS1 kinase activity that normally turns on the RAS-RAF-MEK-ERK pathway that promotes differentiation of the initial segment of the epididymis (58). Alongside this finding, another group found in 2014 that ROS1 can play a role in pathological vascular remodeling post stent deployment via activation of ROS1 due to oxidative and reductive stress that promotes smooth muscle cell proliferation that contributes to atherosclerosis; this study also found that inhibition of ROS1 with the TKI, crizotinib, can reverse the pathologic smooth muscle cell proliferation (64). Finally, two studies in 2020 proposed potential ligands that activate ROS1. The first observed that the neural epidermal growth factor-like like 2 (NELL2) lumicrine factor, secreted by testicular germ cells, associates with the extracellular domain of ROS1 to promote differentiation of the initial segment of the epididymis in mice and that knockout of NELL2 leads to similar infertility observed in ROS1-knockout mice (65). The second study found that ribonuclease 7 (RNase7), an immunomodulatory factor, can bind to ROS1 and promote increased tumorigenesis in hepatocellular carcinoma and that this is reversed with ROS1-TKI treatment (66). Although both studies provide interesting preliminary data to suggest links between these potential ligands and ROS1, it should be noted that my lab has not been able to reproduce these findings in our ROS1 model systems. Hence, more work needs to be done to better establish that NELL2 and RNase7 specifically form protein-protein interactions with the extracellular domain of ROS1, rather than promoting indirect activation of ROS1.

The role of ROS1 in cancer

While several studies found a role for ROS1 in different physiological settings, the greatest impact of ROS1 biology and inspiration for the development of ROS1 inhibitors is in the realm of cancer. In general, ROS1 promotes oncogenesis due to aberrant overactivation of the

receptor that leads to unrestricted downstream signaling through RAS-RAF-MEK-ERK, PI3K/AKT, and JAK/STAT pathways. This can occur in various ways, but the most common mode of ROS1 over-activation involves the participation of *ROS1* in oncogenic gene fusions, while overexpression of the ROS1 protein (such as via gene amplification), transcription of alternative constitutively active splice variants, and gain-of-function activating mutations in the *ROS1* gene represent potential alternative modes of ROS1 over-activation that have been speculated about but not definitely been proven to promote oncogenesis.

ROS1 fusion oncogenes

The majority of what is known about ROS1-driven oncogenesis prior to my doctoral work has involved *ROS1* fusion oncogenes that in all cases consist of a 5' segment of a partner gene fusing to the intracellular regions of the *ROS1* gene (**Fig. 1.2C**). Studies identified 55 different 5' partner genes that associate with the intracellular portions of the *ROS1* gene that code for the tyrosine kinase and C-terminal domains (**Fig. 1.2A**). Cancers where *ROS1* fusion oncogenes are detected include non-small-cell lung cancer (NSCLC), adult and pediatric gliomas, Spitzoid neoplasms and inflammatory myofibroblastic tumors (IMTs). Furthermore, not all *ROS1* fusion 5' partner genes are found in every cancer type. For example, in NSCLC, *CD74-ROS1* is the most common *ROS1* fusion (~44%), followed by *EZR-ROS1* (16%), *SDC-ROS1* (14%) and *SLC34A2-ROS1* (10%) (**Fig. 1.2B**). By contrast, *GOPC-ROS1* is the most common (~75%) *ROS1* fusion in adult glioma and only a couple of other fusion partners have been identified in this cancer type. Also, *GOPC* is currently the only *ROS1* fusion partner identified in cholangiocarcinomas (67,68).

ROS1 fusions are formed via different types of chromosomal aberrations that can vary between cancer types. For example, in adult gliomas, *ROS1* fusions arise largely through small deletions in the q arm of chromosome 6 (69), while the majority of recurrent *ROS1* fusions in NSCLCs, apart from *EZR-ROS1*, result from chromosomal translocations (70-78).

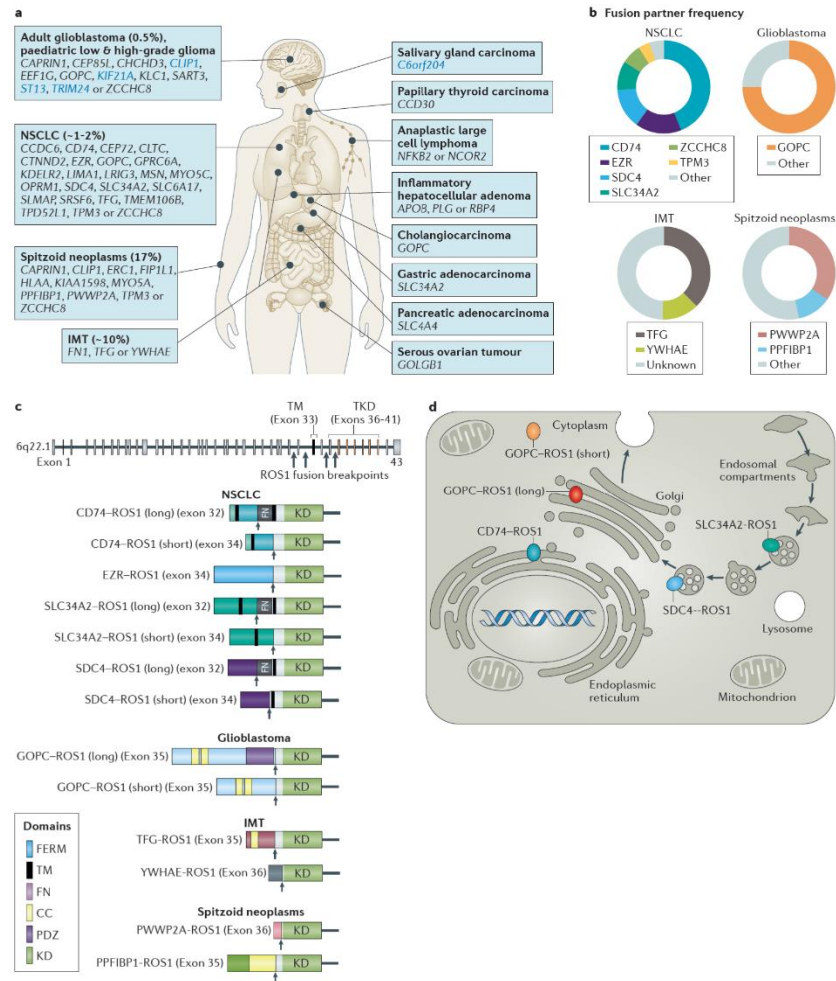


Figure 1.2: ROS1 fusion-driven cancer epidemiology and structure. A. Types of cancer with *ROS1* fusion oncogenes with prevalence data for certain cancers. **B.** Frequency and distribution of *ROS1* fusion oncogenes in non-small-cell lung cancer, glioblastoma, inflammatory myofibroblastic tumor (IMT). **C.** Structure of various *ROS1* fusion oncoproteins with *ROS1* gene map showing chromosomal breakpoints. **D.** Subcellular localization of certain *ROS1* fusion oncoproteins. Reproduced with permission from: Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. *ROS1*-dependent cancers — biology, diagnostics and therapeutics. *Nature Reviews Clinical Oncology* 2021;18(1):35-55 doi 10.1038/s41571-020-0408-9 PMID - 32760015.

How do ROS1 fusions promote oncogenesis?

ROS1 fusion oncogenes are transcribed and translated into chimeric fusion proteins that feature a constitutively active ROS1 TKD that activates canonical signaling pathways as mentioned previously (79). One interesting quirk about the various ROS1 fusion proteins is that downstream signaling seems to be influenced by the subcellular localization conferred by the N-terminal portion of the protein contributed by the 5' partner gene (**Fig. 1.2D**). A recent study by Neel et al. in fact found that ROS1 fusion proteins that localize to endosomes (SDC4–ROS1 and SLC34A2–ROS1) have a greater capacity to activate the RAS-RAF-MEK-ERK pathway than CD74-ROS1, a fusion that localizes to the endoplasmic reticulum; interestingly, this differential activation does not extend to other signaling pathways, such as JAK/STAT, suggesting that the ability of certain downstream adaptors unique to the ERK pathways are restricted in compartments they can access (80). What is consistent is that multiple studies demonstrate that ROS1 fusion proteins, regardless of subcellular localization, can promote oncogenic transformation of a variety of cell types *in vitro* and *in vivo* (53,54,67,81-83).

ROS1 fusion oncogenes can be found with other potential driver alterations in tumors (74,84); however, *ROS1* fusions tend to not be found with other driver alterations in a single tumor (85). ROS1 fusions alone are sufficient to cause tumor formation in a number of models, although cooperation with other oncogenic alterations can lead to more aggressive disease. In a genetically engineered mouse model of glioma, for example, the combination of the most common ROS1 fusion protein in this cancer type, GOPC-ROS1, with *CDKN2A* deletion results in highly aggressive tumor formation with reduced latency and increased penetrance compared with expression of GOPC–ROS1 alone (54). In addition to this observation, it has been found that alterations that activate the PI3K/AKT pathway occur concurrently in most *ROS1*-rearranged

gliomas (54,79) and that concurrent expression of Kras^{G12D} and GOPC–ROS1 increases lung metastasis in a mouse model of cholangiocarcinoma relative to that observed with either oncogene alone (86). Thus, *ROS1* fusions are extremely potent oncogenes that can cause tumor formation in multiple organ systems with or without other oncogenes or loss-of-function alterations in tumor suppressors.

Clinical and histological features of *ROS1*-fusion-positive cancers

ROS1 fusions occur at variable frequencies in a range of cancers. These include subsets of NSCLC (1–2.5%), gastrointestinal cancers (including 1–9% of cholangiocarcinomas) (67,68), in addition rare cancers like Spitzoid neoplasms (17%), IMTs (10%), gliomas (0.5-1%), and anaplastic large cell lymphomas (87) (**Fig. 1.2A**).

NSCLC represents the cancer type in which *ROS1* fusions are most common. This is likely due to the high incidence of NSCLC relative to other types of cancer (88). Patients that have *ROS1*-fusion-positive NSCLC have a median age of 50 years at time of diagnosis and intriguingly, 80% tend to be non-smokers, which is peculiar for a disease where smoking represents a major risk factor (89-91). Another correlative risk factor with *ROS1*-fusion-positive NSCLC is a higher rate of venous thromboembolism (92). Unlike risk factors or demographic trends, *ROS1*-fusion-positive cancers do not have tendencies with regards to pathological classification. For example, *ROS1* fusion-positive gliomas have been found to be low grade or high grade (93-95).

How are *ROS1* fusion oncogenes detected clinically?

There are several ways to detect *ROS1* fusions in clinical tumor samples, with each method having pros and cons (96,97). The traditional method involves the use of

immunohistochemistry (IHC) to detect ROS1 fusions at the protein level (98). It has been observed that diffuse ROS1 staining of moderate to strong intensity is closely associated with the presence of a *ROS1* fusion (99). The issue is that pattern and intensity of staining vary depending on the IHC antibody and fusion protein detected (100). Luckily, in the case of ROS1 fusion protein detection, the two antibodies used (SP384 and D4D6) have similar sensitivity and specificity values >90% (101). A major disadvantage of this approach is that it only detects ROS1 protein expression that may or may not be specific to fusion protein expression. Any tissues that have elevated expression of ROS1^{WT}, like proliferating pulmonary epithelial cells or giant osteoclast cells can be found to have ROS1 positivity via IHC (98,100). As a result, the two approaches listed below (fluorescent “break apart” probes and next-generation sequencing) are likelier to be used as more clinical centers modernize their fusion detection approaches.

A slightly more modern approach involves the use of fluorescent “break apart” probes that can bind to the 5’ and 3’ ends of the unmodified *ROS1* gene, where an intact gene would show a yellow color (merge of green and red fluorescent signals) while a *ROS1*-fusion-positive tumor cell would show separate green and red segments that demonstrate presence of chromosomal aberration splitting the *ROS1* gene apart; a positive test is a scenario where greater than or equal to 15% of cells contain separate green and red signals (89). A major disadvantage with this approach is that while fusion oncogenes formed from large chromosomal aberrations like translocations can be easily detected with this approach, subtler changes in chromosomal structure that lead to fusion oncogenes (like intra-chromosomal deletion leading to *GOPC-ROS1*) cannot be detected, thus leading to false negative results; as a result, fluorescent “break apart” probes are often insufficient and other diagnostic assays are required to confirm the presence of *ROS1* fusion oncogenes (102).

Finally, the increasingly common method of detecting *ROS1* fusions consists of next-generation sequencing (NGS) of tumor cells; this has been employed partly to get past the issues cited before with fluorescent “break apart” probes (103). Tumor DNA can be sequenced from biopsy samples (both tissue-derived and cell free DNA) in two ways: through amplicon-based or hybrid capture-based approaches (targeted genomic sequencing) (104). Of these methods, hybrid capture represents the better approach to detect fusion oncogenes of many types as instead of probing for a specific region that may or may not have been affected by chromosomal breaks, one can enrich larger sections of chromosomal DNA that can then be cut up and sequenced (105). While the traditional approach to perform NGS involves probing genomic DNA, recent advancements in processing of NGS data (such as newer algorithms) now allow for the ability to detect fusion oncogenes with mRNA data (106). The detection of fusion oncogenes at the mRNA level prevents potential issues that may come from sequencing introns, which can have a lot of repeated elements. RNA can be sequenced either with a multiplex amplicon sequencing format (ArcherDX; FDA Breakthrough Device designation (107,108)) or with traditional RNA-seq. In NSCLC, a study showed that RNA-based amplicon sequencing using the ArcherDX assay identified driver alterations in 14% of cancers and *ROS1* fusions in 4% of cancers that had previously undergone targeted or whole genome sequencing (107). Thus, as NGS-based approaches become more common, RNA-seq of cancer biopsies or cell free DNA samples should represent the optimum way to detect *ROS1* fusion oncogenes.

Overexpression, alternative splice variants, and mutations in *ROS1*

The only proven oncogenic drivers that involve *ROS1* overactivation are fusion oncogenes. With that said, other patterns have been observed that point to other potential

aberrations involving ROS1 that have been oncogenic drivers with other RTKs. One of these consists of overexpression of ROS1. A few studies have been performed in various cancer types to propose situations where ROS1 may contribute to oncogenesis. The highest percentage of ROS1 overexpression in a single cancer type is 80 to 100% that was observed in metastatic oral squamous cell carcinoma (109). Two other studies noted increased *ROS1* gene expression in *KRAS*-mutant mouse models of lung cancer, suggesting a positive feedback loop between RAS activity and *ROS1* gene expression (110,111). Another potential source of ROS1 overexpression comes from copy-number amplification (CNA) of *ROS1*. These are found in several cancers in the AACR Genie database like lung cancer and breast cancer (112-114). What is important to note is that CNA of *ROS1* has not been shown to be a driver alteration. Considering that CNA involving other RTKs promote oncogenesis (115), functional validation of *ROS1* CNA should be performed to assess the potential utility of ROS1-TKI against cancers with these alterations.

The one study that showed ROS1 overexpression to be a potential driver alteration was a 2018 study by Bajrami et al. that found that ROS1 is upregulated in E-cadherin-deficient breast cancers and importantly, the ROS1-TKI crizotinib demonstrates synthetic lethality with knockout of *CDH1* in preclinical models of these cancers (116). Based on these findings, a clinical trial is currently underway testing the combination of crizotinib with fulvestrant (estrogen receptor antagonist) in *CDH1*-, ER+ lobular breast cancer (117).

Finally, gain-of-function missense mutations in *ROS1* represents another mode of ROS1 overactivation as these were demonstrated to be oncogenic drivers in several RTKs (118-122). Examination of AACR Genie database reveals numerous missense mutations in *ROS1* that do not cluster in a particular region of the gene (112-114). As none of these mutations have been characterized prior to my dissertation, these represent “variants of unknown significance” that require further functional analysis. As will be seen in a later chapter of my dissertation, my lab

and I discover that gain-of-function mutations in *ROS1* can indeed promote ROS1 overactivation and oncogenesis.

Tyrosine kinase inhibition of ROS1 in cancer

- Categories of tyrosine kinase inhibitors (TKIs) that target ROS1

ROS1-TKIs can be split into first-generation and second-generation TKIs. First-generation TKIs include the FDA-approved crizotinib and entrectinib, and other TKIs such as ceritinib and brigatinib that are not as widely used in the clinic. A defining characteristic of first-generation TKIs is that they primarily are effective against cancers that have yet to be treated with ROS1-TKIs (123); the primary cause for this is the emergence of ROS1-dependent resistance, which will be discussed later in this chapter. Second-generation TKIs include those in clinical trials like lorlatinib, repotrectinib, and taletrectinib. These TKIs can overcome much of the ROS1-dependent resistance that has emerged with first-generation TKIs but may be vulnerable to new modes of ROS1-dependent and the same mechanisms of ROS1-independent resistance that affect even first-generation TKIs (discussed later in this chapter). It is also important to note that some ROS1-TKIs bind in a type I conformation to the tyrosine kinase domains (crizotinib, entrectinib, ceritinib, brigatinib, lorlatinib, repotrectinib, and taletrectinib), while others bind in a type II conformation (cabozantinib) (124,125). The type I conformation is considered the active shape of the kinase, defined by the “DFG motif” of the kinase domain placed in a position to facilitate ATP binding to the kinase, while the type II conformation is considered inactive, where the “DFG motif” is displaced, causing steric clashes with ATP (**Fig. 1.4B-D**). Recent work from my lab has challenged this categorization, arguing that crizotinib and entrectinib can act as dual type I/II inhibitors and that these function separately to the second-generation TKIs (lorlatinib,

repotrectinib, taletrectinib) that function more as true type I inhibitors (126). This will be discussed in greater depth later in the chapter with regards to ROS1-dependent resistance, as type I inhibitors and type II inhibitors have separate resistance profiles that can be countered with the alternative category.

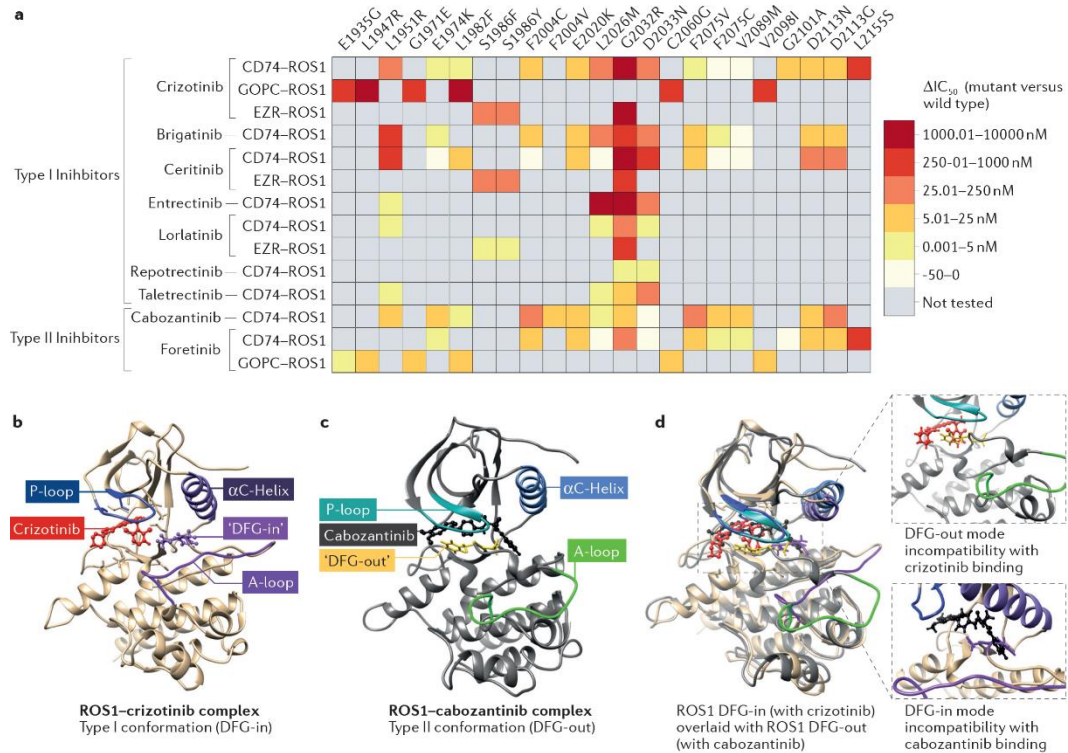


Figure 1.4: Pre-clinical activity and binding modes of ROS1-TKIs. **A.** Heatmap of IC₅₀ values for type I and type II ROS1-TKIs against various resistant kinase domain mutations. Measured in Ba/F3 CD74-ROS1 or GOPC-ROS1 cells. **B.** Crystal structure complex shows crizotinib binds preferentially in type I conformation to ROS1 kinase domain. **C.** Crystal structure complex shows cabozantinib binds preferentially in type II conformation to ROS1 kinase domain. **D.** Superimposed molecular docking simulations of crizotinib in type II binding mode and cabozantinib in type I binding mode. *Reproduced with permission from: Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. ROS1-dependent cancers — biology, diagnostics and therapeutics. Nature Reviews Clinical Oncology 2021;18(1):35-55 doi 10.1038/s41571-020-0408-9 PMID - 32760015.*

- What are the proteins that ROS1-TKIs target in addition to ROS1 (off-target activity)?

TKIs in general are known to target many different types of kinase, contributing to adverse effects seen in patients. ROS1-TKIs are no different: they target RTKs with kinase domains closely related in sequence to ROS1 such as ALK, the NTRK family of receptors, and MET (127-131). In addition, ROS1-TKIs can target other types of protein kinases (127-131). The inverse is not necessarily true though: not all ALK TKIs can inhibit ROS1. A modeling study my lab performed found that subtle differences in the structure of the ALK and ROS1 kinase domain prevent certain ALK-TKIs like alectinib from binding to the ROS1 kinase domain (124). My lab was also able to observe the reverse, where some TKIs, like cabozantinib, could bind the ROS1 kinase domain but not the ALK kinase domain as effectively (124).

- Preclinical testing of ROS1-TKIs

Most preclinical studies of ROS1 TKIs, including those from my lab, involved the Ba/F3 CD74-ROS1 model (132-139). Ba/F3 is a pro-B murine cell line that in normal circumstances require recombinant IL-3 for survival when cultured in flasks; when oncogenes like *ROS1* fusions are transduced into these cells, they can survive without IL-3 while control cells cannot. CD74-ROS1 is the fusion protein that is most often utilized in these studies, although others are also used (124,128,129,138,140,141). For first-generation TKIs, the most potent were found to be entrectinib, foretinib (not in development anymore), and cabozantinib, with IC50 values found to range between 2 and 6 nM (**Figure 1.3**). Less potent TKIs included crizotinib (IC50-15.4 nM), brigatinib (IC50-15.5 nM), and ceritinib (IC50-68 nM). Finally, the next-generation inhibitors lorlatinib, repotrectinib and taletrectinib are more potent than first-generation TKIs in the Ba/F3

CD74-ROS1 model (IC₅₀ 0.4 nM, 0.1 nM and 0.8 nM, respectively) (**Figure 1.3**) (79,81,133,136,142).

In vivo testing of ROS1-TKIs of both first and second-generation ROS1-TKI were also performed prior to clinical trial initiation. In general, all ROS1-TKIs discussed in this chapter have shown efficacy against *ROS1*-fusion-driven cancers in mouse models. Examples include crizotinib in genetically engineered CD74-ROS1-driven or SDC4-ROS1-driven mouse models of NSCLC (143), foretinib in a GOPC-ROS1-driven mouse cholangiocarcinoma allograft model (81), entrectinib in a mouse Ba/F3 TEL-ROS1-driven allograft cancer model (144), repotrectinib in a mouse Ba/F3 CD74-ROS1-driven allograft cancer model (134) and taletrectinib in a patient-derived xenograft model of CD74-ROS1-driven NSCLC (137).

Clinical activity of first-generation ROS1-TKIs in non-small-cell lung cancer

Most patient data of ROS1-TKI efficacy has been collected in NSCLC (145). The most studied ROS1-TKI in NSCLC is crizotinib (**Table 1.1**), which is approved for first-line treatment of *ROS1*-fusion-positive NSCLC by regulatory agencies in many countries (not limited to but including the USA (146), Europe (147), China (148), Taiwan (149), Israel (150), and Australia (151)). The objective response rate (ORR) measured in various clinical trials of crizotinib range between 65-72% (**Table 1.1**). In the two largest prospective clinical trials of crizotinib in *ROS1*-fusion NSCLC, the observed median progression-free survival (PFS) ranged between 16 and 19 months; this exceeds the PFS of 11 months measured in *ALK* fusion-positive NSCLCs treated with crizotinib (152-154). Importantly, trials also found crizotinib to be more effective than

chemotherapy in retrospective studies in *ROS1*-fusion-driven NSCLC (**Table 1.1**). Thus, crizotinib represents an effective therapy for *ROS1*-positive NSCLC.

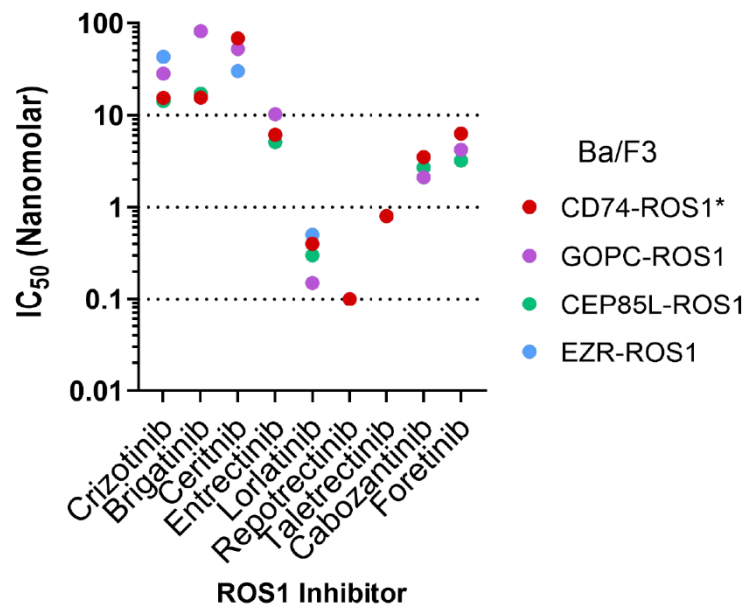


Figure 1.1: .3: Ba/F3 IC₅₀ values for listed ROS1-TKIs in Ba/F3 ROS1-fusion-positive cell lines. Reproduced with permission from: Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. *ROS1-dependent cancers — biology, diagnostics and therapeutics*. *Nature Reviews Clinical Oncology* 2021;18(1):35-55 doi 10.1038/s41571-020-0408-9 PMID - 32760015.

ROS1 TKI	Study (phase)	Overall outcomes				Intracranial outcomes	
		ORR (n)	Median DoR	Median PFS	Median OS	ORR (n)	Other
ROS1 TKI-naïve setting							
Crizotinib	PROFILE 1001 (REF.(153)) (Ib)	72% (38/53)	24.7 months	19.3 months	51.4 months	–	–
	OxOnc(152) (II)	72% (91/127)	19.7 months	15.9 months	–	–	–
	EUCROSS(155) (II)	70% (21/30)	19.0 months	20.0 months	–	–	–
	AcSe(156) (II)	69% (25/36)	–	5.5 months	17.2 months	–	–
	METROS(157)(II)	65% (17/26)	21.4 months	22.8 months	–	–	–
Entrectinib	Drilon et al.(158) (I/II)	77% (41/53)	24.6 months	19.0 months	–	55% (11/23)	DoR: 12.9 months PFS: 7.7 months
Ceritinib	Lim et al.(159) (II)	67% (20/30)	21.0 months	19.3 months	24.0 months	25% (2/8)	–
Brigatinib	Gettinger et al.(160) (I)	100% (1/1)	–	–	–	–	–
Lorlatinib	Shaw et al.(161) (I/II)	62% (13/21)	25.3 months	21.0 months	–	64% (7/11)	–
Repotrectinib	Cho et al.(162) (I/II)	91% (10/11)	–	–	–	100% (3/3)	–
Taletrectinib	Fujiwara et al.(163) (I)	67% (6/9)	–	–	–	–	–
ROS1 TKI pre-treated setting							
Entrectinib	Drilon et al.(164) (I/II)	0% (0/6)	–	–	–	–	–
Ceritinib	Lim et al.(159) (II)	0% (0/2)	–	–	–	–	–
Brigatinib	Gettinger et al.(160) (I)	0% (0/2)	–	–	–	–	–
Lorlatinib	Shaw et al.(161) (I/II)	35% (14/40)	13.8 months	8.5 months	–	50% (12/24)	PFS: not reached
Repotrectinib	Cho et al.(162) (I/II)	39% (7/18)	–	–	–	75% (3/4)	–
Taletrectinib	Fujiwara et al.(163) (I)	33% (1/3)	–	–	–	–	–

Table 1.1: Trials of ROS1 TKIs in patients with ROS1 fusion-positive NSCLC. Reproduced with permission from: Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. ROS1-dependent cancers — biology, diagnostics and therapeutics. *Nature Reviews Clinical Oncology* 2021;18(1):35-55 doi 10.1038/s41571-020-0408-9 PMID - 32760015.

Entrectinib is another FDA-approved inhibitor for ROS1-rearranged NSCLC that has similar measured ORR (77%) and median PFS (19 months) values relative to crizotinib (**Table 1.1**) (158,165). The important difference between these two TKIs is that entrectinib has intracranial activity, while this has not been measured for crizotinib (158,165). This is important as one of the most common sites of metastasis for NSCLC is the brain, predicating the need for TKIs that can cross the blood brain barrier (125). While **Table 1.1** has clinical trial data from other first-generation TKIs such as brigatinib and ceritinib, observation of significant GI side effects led to these drugs not being approved for official use by regulators (125).

Clinical activity of second-generation ROS1-TKIs in non-small-cell lung cancer patients not yet treated with TKI

Second-generation TKIs have been tested both in patients that have yet to be treated with TKI in *ROS1*-driven NSCLC and patients that have already been treated with TKI. In TKI-naïve tumors, lorlatinib (ORR 62% and median PFS 21 months with intracranial ORR of 64%, **Table 1.2**) (161), repotrectinib (ORR of 91% with intracranial *ROS1*-fusion-driven NSCLC), although they may provide some advantages against metastases to the brain due to the intracranial activity of in particular lorlatinib and repotrectinib.

Second-generation TKIs become more relevant when discussing the treatment of patients that have stopped responding to a first-generation TKI, this will be expanded upon later in this chapter.

Study	Crizotinib	Platinum-based chemotherapy	P-value
Retrospective (n=77)(166) ORR PFS	86.7% 18.4 months	44.7% 8.6 months	<0.001 <0.001
Retrospective (n=102)(167) ORR PFS	83.9% 14.9 months	56.5% 8.5 months	0.002 0.001
Retrospective (n=51)(168) ORR PFS	80.0% 9.4 months	40.8% 3.5 months	<0.05 <0.05

Table 1.2: Activity of crizotinib vs. chemotherapy in ROS1-fusion-positive lung cancers. ORR = objective response rate; PFS = progression-free survival. *Reproduced with permission from: Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. ROS1-dependent cancers — biology, diagnostics and therapeutics. Nature Reviews Clinical Oncology 2021;18(1):35-55 doi 10.1038/s41571-020-0408-9 PMID - 32760015.*

Efficacy of ROS1-TKIs in other cancer types.

ROS1 TKIs have had efficacy in other ROS1 fusion-positive cancer types. However, ROS1-fusion-driven cancers are rare outside of NSCLC, which means no clinical trial data exists but rather anecdotal case reports. **Table 1.3**, reproduced from a recent review, summarizes the instances where a response was recorded to ROS1-TKI. Considering the efficacy of this therapy in NSCLC, an opportunity exists to expand ROS1-TKI treatment to other forms of cancer. The hope is that enough data can be collected in the coming years in two phase II basket clinical trials, one for adult and the other for pediatric refractory cancers, that are being conducted in a cancer-agnostic fashion, while basing treatment decisions solely on clinical sequencing data (169,170).

Drug	Age Group	Histology	Fusions	Duration
Crizotinib(171)	Pediatric	Atypical meningioma	TFG-ROS1	14+ months
Crizotinib(172)	Adult	Biphasic pulmonary blastoma	CD74-ROS1	3 months
Crizotinib(173)	Adult	ER/PR+ HER2-breast cancer	GOPC-ROS1	not reported
Crizotinib(174)	Pediatric	Pediatric IMT	TFG-ROS1	4+ months
Ceritinib(175)	Adult	Adult IMT	TFG-ROS1	8+ months
Ceritinib(173)	Adult	ER/PR+ HER2-breast cancer	GOPC-ROS1	6+ months
Entrectinib(176)	Pediatric	IMT	TFG-ROS1	13+ months
Entrectinib(164)	Adult	Melanoma	GOPC-ROS1	12+ months
Entrectinib(177)	Pediatric	High grade glioma	EEF1G-ROS1	9+ months
Entrectinib(177)	Pediatric	High grade glioma	GOPC-ROS1	6+ months

Table 1.3: Response to ROS1 inhibition in ROS1 fusion-positive cancers other than lung cancer. ER/PR+ = estrogen and progesterone receptor positive; PR = partial response; IMT = inflammatory myofibroblastic tumor. *Reproduced with permission from: Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. ROS1-dependent cancers — biology, diagnostics and therapeutics. Nature Reviews Clinical Oncology 2021;18(1):35-55 doi 10.1038/s41571-020-0408-9 PMID - 32760015.*

Mechanisms of Resistance to ROS1-TKI Treatment

Like other forms of TKI therapy, resistance to treatment of ROS1-fusion positive lung cancers is mediated by either ROS1-dependent or independent mechanisms. Both mechanisms of resistance are observed clinically; however, ROS1-dependent mechanisms of resistance, consisting of point mutations in the ROS1 kinase domain (KD), appear more often and have been studied more extensively.

ROS1-dependent mechanisms of resistance

Multiple pre-clinical studies have been conducted to predict ROS1 KD point mutations that confer resistance to TKI (**Fig. 1.5A-B**). To discover these resistant mutations, groups have primarily performed saturated mutagenesis screening with the mutagen N-ethyl-N-nitrosourea (ENU) and Ba/F3 ROS1-fusion cells sensitive to TKI. The initial study on this front, performed by Davare et al. in 2013, identified six crizotinib-resistant point mutations: V2098I, C2060G, L1982F, G1971E, L1947R, and E1935G (81). A study performed by Katayama et al. in 2015 further discovered the crizotinib-resistant G2032R, L2026M, L1982F, and L1951R mutations (142). The same study also probed for resistant point mutations towards another first-generation TKI, ceritinib, and found the F1994L and E1990G mutations to confer resistance along with the G2032R and L1951R mutations (142). A third study in 2015 then found that the L2155S mutation also confers crizotinib resistance; this mutation appeared while culturing an established ROS1 fusion non-small-cell lung cancer (NSCLC) cell line (HCC78) with low-dose crizotinib (178). Two studies also utilized ENU mutagenesis screens to find resistant point mutations to the second-generation, type II TKI, cabozantinib, and discovered the following mutations specific to cabozantinib: M2134I, D2113N/G, V2089M, F2075V/C, E2020K, F2004V/C, and E1974K (133,179). Interestingly, some of these mutations remain sensitive to crizotinib; however, cells

with compound mutations involving a crizotinib-resistant mutant (like G2032R) and a cabozantinib-resistant mutant (like D2113N) display resistance to both TKIs (133). Finally, a recent study from members of my lab conducted a thorough analysis (both with structural modeling and functional validation) of resistance profiles from different categories of TKIs (first-

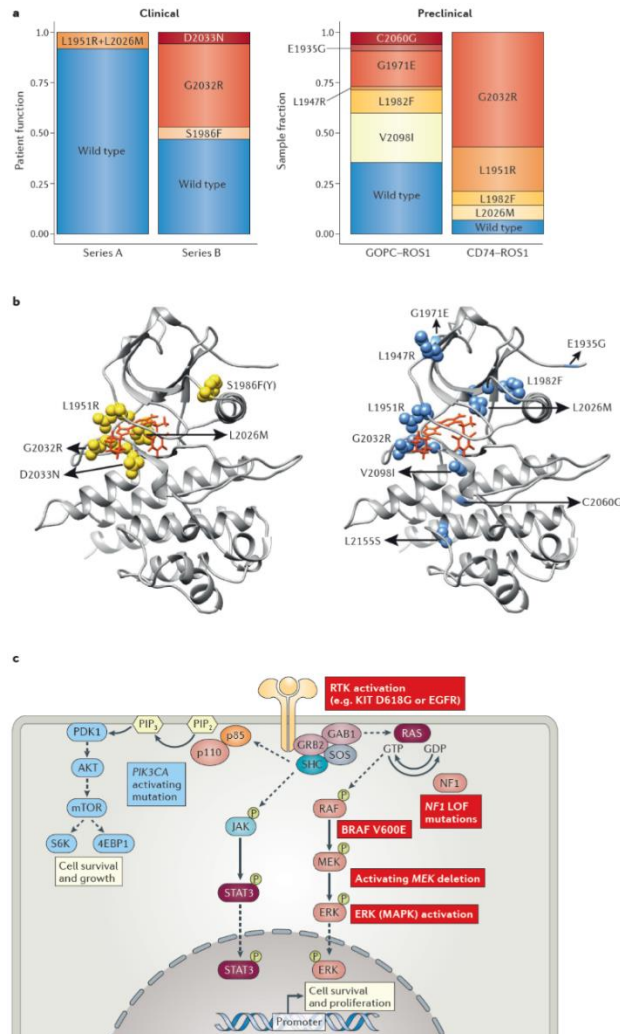


Figure 1.5: Mechanisms of resistance to ROS1-TKIs. A. Frequencies of ROS1-dependent and -independent resistance in clinical (left) and pre-clinical (right) studies. **B.** Crystal structure of ROS1 tyrosine kinase domain with labels for crizotinib-resistant kinase domain point mutations, where clinical mutations listed on left and pre-clinical mutations on right. *Reproduced with permission from: Drilon A, Jenkins C, Iyer S, Schoenfeld A, Keddy C, Davare MA. ROS1-dependent cancers — biology, diagnostics and therapeutics. Nature Reviews Clinical Oncology 2021;18(1):35-55 doi 10.1038/s41571-020-0408-9 PMID - 32760015.*

generation and second-generation TKIs) and determined a novel mutation, L2086F, that can confer resistance to all ROS1-TKIs that bind in a type I conformation (crizotinib, entrectinib, lorlatinib, repotrectinib, and taletrectinib) (126). This mutation indeed is an important finding as it confers resistance to all second-generation ROS1-TKIs, precipitating the need for a third generation of inhibitors.

To combat this form of resistance, studies have been performed in the pre-clinical setting to overcome resistance mutations with the use of alternative ROS1-TKIs. The G2032R mutation has proven to be a particularly problematic resistant mutation as it is largely insensitive to the first-generation inhibitors, crizotinib, ceritinib, and entrectinib (**Fig. 1.5A**). Studies were performed with second-generation TKIs to overcome this mutation. No ROS1-TKI was found to be as effective against G2032R as wild-type, but it was found that repotrectinib (IC₅₀-10 nM) and taletrectinib (IC₅₀-13.5 nM) in Ba/F3 CD74-ROS1^{G2032R} cells retain IC₅₀ values less than 20 nM (126,129). By contrast, the IC₅₀ of lorlatinib against Ba/F3 CD74-ROS1^{G2032R} increased from 0.7 nM for wild-type ROS1 fusions to 43.1 nM, suggesting less of an ability to overcome the G2032R resistant mutation (126). Alongside repotrectinib and taletrectinib, cabozantinib, a type II ROS1-TKI, may also effectively target G2032R (IC₅₀ of 21 for Ba/F3 CD74-ROS1^{L2086F} vs. IC₅₀ of 5.5 for Ba/F3 CD74-ROS1^{WT}) (126). In addition to G2032R, L2086F is likely to be another problematic resistant mutation due to its ability to confer resistance to both first- and second- generation ROS1-TKIs. Importantly however, it was found that cabozantinib, the type II TKI, can overcome this mutation (IC₅₀ of 1.1 for Ba/F3 CD74-ROS1^{L2086F} vs. IC₅₀ of 5.5 for Ba/F3 CD74-ROS1^{WT}) (126).

In the clinical setting, several ROS1 KD mutations induce therapeutic resistance to crizotinib, with some overlap and some notable differences to those found pre-clinically. The best-described mutation to date is the G2032R mutation, discovered by Awad et al. in 2013 (180).

The authors detected this mutation in RT-PCR products from tissue samples of a patient with CD74-ROS1 NSCLC experiencing acquired crizotinib resistance (180). Subsequently, they demonstrated the G2032R mutation to confer a >10-fold increase in crizotinib IC₅₀ relative to CD74-ROS1^{WT} in HEK293T cells (180). Another study confirmed that both crizotinib and the second-generation TKI, lorlatinib (both type I TKIs) could not successfully attenuate ROS1-positive NSCLC in patients that contained the G2032R mutation (181). Later reports discovered several other mutations in patients with crizotinib-resistant ROS1 NSCLC including D2033N, L2026M, S1986F/Y (not seen preclinically), and L1951R (135,136,182,183).

Notably, cabozantinib and lorlatinib were able to overcome the crizotinib resistance caused by the D2033N and S1986Y/F mutations, respectively, in the original patients (135,136). Finally, cabozantinib was also found to overcome the G2032R and L2086F mutations in a recent study in a patient pre-treated with crizotinib and lorlatinib, confirming the findings seen in the pre-clinical studies (181).

To characterize how ROS1 KD point mutations confer resistance to TKI, several studies generated crystal structures of the ROS1 kinase domain in complex with ROS1-TKIs. The Awad et al. paper that detailed the G2032R mutation also characterized the crystal structure of the ROS1 KD in complex with crizotinib; they inferred that a switch from glycine to arginine at position 2032 likely allows for continued ATP binding but leads to steric conflict with the piperidine ring of crizotinib, preventing its binding (180). Subsequent studies utilized this crystal structure to determine structural causes of other resistant ROS1 KD mutations. Katayama et al. in 2015 determined from the crystal structure that the L1951R, like G2032R, is in the solvent-front region (next to where crizotinib interacts with the KD), while L2026M is a gatekeeper mutation, analogous to L1196M in ALK (142). Fachinetti et al. in 2016 also used the crystal structure with molecular dynamics simulation to determine that the S1986Y/F mutations hinder crizotinib

binding by inducing a positional change in the α C helix rather than affecting the solvent front like previous mutations (136). Finally, a recent study found that the L2086F mutation, which confers resistance pre-clinically to all ROS1-TKIs with type I binding capability, causes steric hindrance due to the replacement of a small side chain in leucine with a phenyl side chain in phenylalanine; this bulky side chain prevents type I ROS1-TKIs from occupying the ATP-binding pocket (181).

In parallel to the crystal structure studies, Davare et al. in 2015 used molecular dynamics simulations to analyze not only mutation effects on crizotinib but also cabozantinib binding (133). They first validated that the simulation predicted the same crizotinib binding pose as the crystal structure and found that the L1951R and G2032R mutations do lead to steric clash between the arginine side chain and piperidine ring, as predicted previously by the crystal structure (133). Next, they simulated the docking of cabozantinib to ROS1 G2032R and saw that the quinolone group on this TKI does not trigger the same steric clash as the piperidine group on crizotinib as it binds at a different location in the open area between DFG motif of A-loop and α C-helix (referred to as the “specificity site”) (133). The simulation then showed that cabozantinib preferentially binds to the inactive conformation of the kinase (type II) while crizotinib prefers the active conformation (type I) (133). Finally, the authors simulated the D2113N cabozantinib-resistant mutation and found that the mutation displaces the residue R2107 outside the α C-helix, which leads to the loss of a normal interaction between E1997 and R2107 (133). This allows the E1997 side chain to partially obstruct the specificity site where cabozantinib binds but not affect the site where crizotinib binds (133). The authors in a later study combined the molecular dynamics simulations with the crystal structure from the Awad et al. 2013 paper and determined that the crizotinib-resistant, cabozantinib-sensitive D2033N mutation disrupts an interaction between the negatively charged D2033 and positively charged piperidine ring on crizotinib (similar to G2032R), whereas cabozantinib binding at the specificity site is unaffected (135).

Ultimately, these structural findings correlate to observations of IC50s, where certain mutations affect type I TKI IC50 values more greatly than type II TKIs and vice versa. To functionally validate this hypothesis, members of my lab conducted a recent thorough study with further structural modeling and discovered that both crizotinib and entrectinib actually bind equally to the type I and type II conformations and that this is and this renders both TKIs susceptible to type I and type II resistant kinase domain mutations like F2004C (126). This means that future treatment almost certainly will need to use alternative ROS1-TKIs to the current FDA-approved options that can combat the inevitable ROS1-dependent resistance mutations that may arise.

Thus, studies on the resistant point mutation over the last several years established a paradigm by which TKIs that bind to different sites will be effective against certain mutations and ineffective against others. I summarized these findings in **Figure 1.4A**, which shows divergence in the resistance profiles between type I and type II ROS1-TKIs. Other members of my lab contributed to **Figure 1.4B**, which highlights the different binding modes of type I and type II TKIs to the ROS1 TKD, illustrating structural observations described above. Moving forward, clinicians should consider using a combination of type I and type II ROS1-TKIs during treatment to attenuate the occurrence of subclonal tumor cells that contain resistant TKD mutations against either type.

ROS1-independent mechanisms of resistance

ROS1-independent mechanisms of resistance are less studied than ROS1-dependent resistance, despite forming greater than 60% of the resistance cases observed clinically (135,136,181-183). Nonetheless, a few studies have characterized certain aberrations that cause ROS1-independent resistance to TKI (**Fig. 1.5C**). Davies et al. in 2013 created a ROS1-TKI

resistant cell line by culturing HCC78 cells (contains SLC34A2-ROS1 oncogene) with low dose TKI and discovered that increased EGFR pathway activation compensated for TKI-mediated loss of constitutive ROS1 signaling; combined treatment with gefitinib (reversible EGFR inhibitor) sensitized these cells to the ALK-TKI, TAE-684 (184). This finding was reproduced by Song et al. in 2015, with this group demonstrating synergistic inhibition of resistant HCC78 cells with crizotinib and afatinib or dacomatinib (both irreversible EGFR inhibitors) (178). It is important to note that neither study could find a specific mutation or alteration that led to increased EGFR pathway activity (178,184). Afatinib was also found to restore crizotinib sensitivity in a different resistant ROS1-fusion cell line (CUTO23) that was found to have increased HER2 phosphorylation (183). Finally, a different study discovered that the activating mutation D816G in the receptor tyrosine kinase KIT also serves as a mechanism of ROS1-independent resistance (185). Transducing KIT D816G into either HCC78 or CUTO-2 cells (derived from NSCLC biopsy, contains SDC4-ROS1 fusion (184)) promoted crizotinib resistance and treatment with combination of crizotinib and KIT inhibitor, ponatinib, resensitized cells to crizotinib inhibition (185). Finally, a series of alterations related to the MAPK pathway sequenced in patients (activating in-frame deletions of *MEK1* and *MEKK1*, loss-of-function mutations in the tumor-suppressor NF1) were found to constitutively activate MAPK signaling downstream of *ROS1*-fusion oncogene signaling suppressed with TKI; addition of MEK inhibition successfully resensitized *ROS1*-fusion-positive cells engineered with the activating MEK deletions (186).

A few studies also described alterations that potentially mediate ROS1-independent resistance. Watanabe et al. in 2018 detailed a case of crizotinib resistance in a patient with ROS1-rearranged NSCLC, where the tumor was found to have acquired a BRAF V600E mutation post-crizotinib treatment with no ROS1 KD point mutation(187). McCoach et al. in 2018 also discovered an activating point mutation in β -catenin (S45F) upon targeted sequencing of a

crizotinib-resistant tumor sample with no ROS1 KD mutation (183). While the BRAF V600E and β -catenin S45F mutations were not functionally validated, prior literature concerning both alterations strongly suggest that these mutants compensate for loss of constitutive RTK signaling (188-191). Finally, a study by Lin et al. in 2021 performed targeted genomic sequencing of cohort of patients that had stopped responding to crizotinib (42 samples) and/or lorlatinib (28 samples); this study revealed potential ROS1-independent alterations such as *MET* copy-number amplification (a known resistant alteration in other subsets of NSCLC) and *MYC* copy-number amplification among others (181). Interestingly, we noticed that roughly 20% of the lorlatinib-resistant samples contained *MYC* copy-number amplification without any concurrent *ROS1* resistant mutation in the Lin et al. dataset (181). As discussed in a later chapter, I was able to functionally validate that this alteration indeed represents a mechanism of ROS1-independent resistance to TKI.

Side effects of ROS1-TKI treatment in patients

Common side effects observed clinically across all ROS1-TKIs include fatigue and gastrointestinal toxicities (greater than 10% of patients), while less common side effects (less than 5% of patients) include QT prolongation and pneumonitis with or without interstitial lung disease (125,192). Finally, different ROS1-TKIs also have side effects that are not seen in other ROS1-TKIs. These toxicities include hypertension with brigatinib (193), vision impairment with crizotinib, brigatinib, entrectinib and lorlatinib (194,195), hyperlipidemia with lorlatinib (196), and central adult male hypogonadism with crizotinib (197). In addition, severe liver toxicity can occur with patients that simultaneously take crizotinib with immune checkpoint inhibitors (198).

Objective of dissertation

In this dissertation, I have investigated several key questions pertaining to targeting ROS1-driven cancers. Specifically, I first elucidated a novel mechanism of therapeutic resistance to ROS1 tyrosine kinase inhibitors (ROS1-TKIs), overexpression of the proto-oncogenic transcription factor MYC, and proposed several treatment strategies to re-sensitize ROS1-positive cancer cells with MYC overexpression to ROS1-TKIs. The second question that I investigated with my lab was to determine if activating point mutations in ROS1 can drive oncogenesis. My lab and I conducted studies in multiple *in vitro* and *in vivo* models and discovered that the variant ROS1^{D2113N} exhibits increased kinase autophosphorylation, promotes oncogenic cell proliferation *in vitro* and *in vivo*, and can be targeted with ROS1-TKIs. Ultimately, the overall goal of my work was to increase the durability and expand the scope of ROS1-TKIs in the treatment of ROS1-driven cancers.

Chapter 2: *MYC* promotes tyrosine kinase inhibitor resistance in *ROS1* fusion-positive lung cancer

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Published in Molecular Cancer Research, 2022

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Respective Contributions

SI wrote the manuscript and contributed to the figure generation and creation. MD edited the manuscript and contributed to figure creation throughout the manuscript.

Figure 2.1: SI generated the data and panels apart from A and B. Panels A and B were generated with the help of the OHSU Clinical Cytogenetics core.

Figure 2.2: SI generated the data and panels.

Figure 2.3: SI generated the data and panels.

Figure 2.4: SI generated the samples while the RPPA data collection was done at MD Anderson Cancer Center. Resulting data analysis was done by SI and MD while figure creation was done by MD. SI generated the data for and created panel C.

Figure 2.5: SI generated panels A-D while panels E and F were generated by IK, EDS, and RS.

Figure 2.S1: SI created the panels from publicly available datasets.

Figure 2.S2: SI created panel A. Panels B, D and E were generated by MD. Panel C was generated by IO.

Figure 2.S3: SI generated the data and panels.

Figure 2.S4: IO generated the data and panels.

Figure 2.S5: SI generated the data and panels.

Figure 2.S6: SI generated the data and panels.

Figure 2.S7: SI generated the data and most panels while MD created panel A.

Figure 2.S8: SI generated the data and panels.

Figure 2.S9: SI generated panels A-D. IK, EDS, and RS generated panels E and F.

Figure 2.S10: SI generated the data and panels.

Appendix: SI and MD contributed to generating the panels and tables.

Abstract

Targeted therapy of ROS1 fusion-driven non-small cell lung cancer (NSCLC) has achieved notable clinical success. Despite this, resistance to therapy inevitably poses a significant challenge. *MYC* amplification was present in ~19% of lorlatinib-resistant ROS1-driven NSCLC. We hypothesized that *MYC* overexpression drives ROS1-TKI resistance. Using complementary approaches in multiple models, including a *MYC*-amplified patient-derived cell line and xenograft (LUAD-0006), we established that *MYC* overexpression induces broad ROS1 TKI resistance. Pharmacological inhibition of ROS1 combined with *MYC* knockdown were essential to completely suppress LUAD-0006 cell proliferation compared to either treatment alone. We interrogated cellular signaling in ROS1-TKI resistant LUAD-0006 and discovered significant differential regulation of targets associated with cell cycle, apoptosis, and mitochondrial function. Combinatorial treatment of mitochondrial inhibitors with crizotinib revealed inhibitory synergism, suggesting increased reliance on glutamine metabolism and fatty-acid synthesis in chronic ROS1-TKI treated LUAD-0006 cells. *In vitro* experiments further revealed that CDK4/6 and BET bromodomain inhibitors effectively mitigate ROS1 TKI resistance in *MYC*-overexpressing cells. Notably, *in vivo* studies demonstrate that tumor control is regained by combining ROS1 TKI and CDK4/6 inhibition. Our results contribute to the broader understanding of ROS1-TKI resistance in NSCLC.

Implications:

This study functionally characterizes *MYC* overexpression as a novel form of therapeutic resistance to ROS1 tyrosine kinase inhibitors in non-small-cell lung cancer and proposes rational combination treatment strategies.

Introduction

Chromosomal alterations involving the receptor tyrosine kinase (RTK) ROS1 gene yield chimeric ROS1 fusion proteins that activate proliferation and survival pathways, and serve as oncogenic drivers in a subset of non-small cell lung cancer (NSCLC) (199). Previous preclinical studies establish that oncogenic ROS1 fusion proteins can be targeted effectively with tyrosine kinase inhibitors (TKIs) (140,200). Notably, robust clinical responses were achieved with the TKIs crizotinib and entrectinib in ROS1-fusion-positive NSCLC, which led to their FDA-approval for the treatment of ROS1 fusion-driven NSCLC (164,201).

Akin to experience with other targeted kinase inhibitors (202,203), emergence of therapeutic resistance hampers long-term durability of ROS1-TKI treatment. ROS1-dependent resistance resulting from point mutations in the kinase domain that reduce or prevent ROS1-TKI binding account for up to half of all resistance cases (204-206). These include the recurrent mutation, ROS1 G2032R, which confers high level of resistance to various ROS1 inhibitors (206,207). To combat ROS1-dependent resistance, inhibitors such as cabozantinib were repurposed, and next-generation TKIs such as lorlatinib, repotrectinib and Ds6051b have been developed (208-210). Although ROS1-independent resistance, involving alterations in other genes that compensate for loss of ROS1 kinase signaling, accounts for half or more of all therapeutic resistance cases, less is understood about these mechanisms (204-206). Previous studies concerning ROS1-independent resistance show a convergence onto compensatory MAPK pathway up-regulation, either through activation of alternative RTK or mutations in genes encoding MAPK pathway regulators. Implicated RTKs include EGFR and KIT (D816G/V) (206,211-213); mutations in MAPK regulators include KRAS G12D (214), BRAF V600E (215,216), deletions in MEK1 (delE41_L54), MEKK1 (delH907_C916), and NF1 loss (217).

PIK3CA mutations (L531P and E545K) (206,215,218) were also found in patients with crizotinib and lorlatinib resistance, although PIK3CA mutation was found not to confer resistance to ROS1-TKIs *in vitro* (217). Thus, no alterations outside of the canonical RTK signaling pathway have been functionally validated as a mechanism of resistance to ROS1-TKIs.

In this study, we functionally characterized the role of MYC overexpression as a mechanism of ROS1-independent resistance to targeted ROS1-TKIs. We demonstrate this with loss- and gain-of-function approaches. We also show efficacy of inhibitor combination strategies that simultaneously target ROS1 catalytic activity and cell signaling associated with MYC expression and/or resistance to long-term TKI treatment as an approach to mitigate drug resistance in tumors that have concurrent ROS1 fusion and MYC overexpression. Our data demonstrate that amplification of transcription factors such as MYC can confer resistance to ROS1-TKIs in ROS1-driven NSCLC.

Materials and Methods

Generation of patient-derived cell line

Patient-derived cell lines and xenografts were developed under institutional review board-approved biospecimen protocols (06-107, 14-091) and written informed consent was obtained from patients for collection of tumor material in accordance with the Belmont report. A tumor biopsy was obtained from the left chest wall of lung cancer patient with metastatic disease. The sample was minced and then mixed with 50% matrigel and implanted into a subcutaneous flank of NSG mouse to generate a patient-derived xenograft model which was named LUAD-0006pdx. A corresponding cell line was generated from the PDX tissue (first transplant) as we have described previously (219) by cutting fresh PDX tumor tissue into small pieces and then digesting in a cocktail of tumor dissociation enzymes obtained from Miltenyl Biotec (130-095-929) in 5 mL serum-free DME:F12 media for one hour at 37 °C, with vortexing every 5-10 min. Digested samples were resuspended in 45 mL growth media (DME:F12 high glucose supplemented with 1% antibiotics and 10% FBS) to inactivate the dissociation enzymes and then cells were pelleted by centrifugation. Finally, cells were plated in growth media (DME:F12 high glucose supplemented with 1% antibiotics and 2% FBS) and allowed to propagate over multiple generations, trypsinized when necessary to subculture and eventually only single cells remained. After ten passages, cells were grown in DMEM (high glucose) supplemented with 1% antibiotics and 10 % FBS. The Mice were cared for, and experiments conducted in accordance with a protocol approved by the Memorial Sloan Kettering Cancer Center Institutional Animal Care and Use Committee and Research Animal Resource Center. All cell lines were tested for mycoplasma

every 6 months and novel cell lines were checked frequently for ROS1 fusion oncogene to verify identity.

Growth and propagation of cell lines

All cell lines were grown in a tissue culture incubator with 5% CO₂ at 37 °C. Cells were maintained in 75-cm² flasks and subcultured when approaching 75% confluence. LUAD-0006 cells were maintained in DMEM medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) L-glutamine solution and detached using 0.05% Trypsin/0.53 mM EDTA. L-glutamine deprivation of LUAD-0006 cells was performed with DMEM supplemented with 10% FBS without any L-glutamine in original DMEM formulation or via later supplementation. HEK-293T/17, Platinum-A, and Platinum-E cells were maintained in DMEM medium supplemented with 10% (vol/vol) BGS and 1% (vol/vol) L-glutamine solution and detached using 0.05% Trypsin/0.53 mM EDTA. Ba/F3 and HCC78 cells were maintained in RPMI-1640 medium supplemented with 10% (vol/vol) FBS, 2 µg/mL recombinant murine IL-3, and 1% (vol/vol) L-glutamine solution. HBEC-ER1 and ER2 cells were maintained in DME-F/12 media supplemented with 10% (vol/vol) FBS and 1% (vol/vol) L-glutamine solution and detached using 2X TrypLE™ in 1X Versene. Every cell line was subject to two to three passages after thawing prior to initiating experiments. No cell line authentication was performed in this study. All cell lines were routinely tested for mycoplasma contamination every 3 months (Lonza MycoAlert™ PLUS Mycoplasma Detection Kit) and verified to be free of contamination.

Immunoblotting

Lysates were prepared from cells using a standard cell lysis buffer as described before (220). Protein quantitation of cleared cell lysates was performed using BCA assay, and 25 µg of total protein was loaded on pre-cast 4-12% Bolt™/NuPAGE™ Bis-Tris Protein Gels. Proteins were transferred to nitrocellulose membranes, and probed with relevant antibodies. All antibodies were used at dilutions recommended by manufacturer. Blots were imaged using a Bio-Rad Laboratories (Hercules, CA, USA) ChemiDoc™ imaging station according to the manufacturer's protocol for immunoblot detection with use of horseradish peroxidase-conjugated secondary antibodies. Spectra Multicolor Broad Range Protein Ladder was used to determine relative molecular weights of protein bands after imaging. Phospho-ROS1 detection required the SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher). Densitometry was performed using Image Lab™ Software (RRID:SCR_014210, Bio-Rad Laboratories, Hercules, CA, USA).

Cell proliferation, drug treatment, and viability assays

LUAD-0006 cells were cultured (n=4) in 96 well plates and treated with varying compounds and/or doxycycline (2 µg/mL unless otherwise specified). The final DMSO % was kept at ≤ 0.1%. 5000 cells were seeded and final working volume was 200 µL. Doxycycline was used in experiments involving the pTRIPZ or pLIX vectors. Cell proliferation was assessed using the IncuCyte ZOOM imaging platform with a 20X objective at 4- or 24-hour imaging intervals (Essen BioScience Inc., Ann Arbor, MI, USA). Resulting data analysis was performed and plotted using GraphPad Prism v9.0 (GraphPad Software, RRID:SCR_002798, San Diego, CA, USA).

For cell viability assays, LUAD-0006, HBEC-ER1, HBEC-ER2, or Ba/F3 cells were seeded at a density of 500 cells per well in 50 μ L onto 384-well plates, while HCC78 cells were seeded at a density of 5000 cells per well in 200 μ L in 96-well low-attachment plates with a Multidrop Combi Reagent Dispenser (ThermoFisher). Inhibitors, vehicle, and death controls were dispensed in 50 μ L volume with an HP D300e Digital Dispenser at increasing concentrations in triplicate or in a synergy grid with multiple inhibitors, where replicates were placed on different plates. Death control consisted of an equimolar mix of flavopiridol, staurosporine, and YM155. The final DMSO % was kept at $\leq 0.1\%$. After 3 days or 10 days of incubation, cell viability was measured using an MTS (methanethiosulfonate)-based assay and 490 nm absorbance was read at 4 hours post addition of MTS reagent using a BioTek Synergy™ H1 plate reader (BioTek Instruments, Inc., Winooski, VT, USA). MTS absorbances of inhibitor-treated wells were normalized to vehicle or death control-treated wells. Non-linear regression of the normalized data was then performed using GraphPad Prism to determine IC₅₀ values (RRID:SCR_002798). Synergy analysis was performed using SynergyFinder 2.0 (RRID:SCR_019318) (221).

To assess apoptosis and cell viability, cells were stained with Annexin V-Alexa Fluor 350 and 7-AAD, a cell impermeant dye, and then analyzed via flow cytometry to identify live, early apoptotic, and dead cells. Flow cytometry was performed with a BD FACSymphony Flow Cytometer (BD Biosciences-US, San Jose, CA, USA). Annexin V signal was quantified with a 355 nm laser and 450/50 bandpass filter while 7-AAD signal was quantified with a 561 nm laser and 670/30 bandpass filter. Resulting data analysis and flow cytometry plots were generated using FlowJo v10 (RRID:SCR_008520, BD Biosciences-US). All experiments were performed in triplicate.

Reverse-phase protein array (RPPA) studies

Reverse-phase protein array analysis was performed by the RPPA core at the MD Anderson Cancer Center (Houston, TX) as described previously (222). LUAD-0006 cells were treated with vehicle, 250 nM crizotinib for 4 hours, or 250 nM crizotinib for 10 days in triplicate in a 6 well plate. Cells were then lysed according to RPPA core protocol, quantified via BCA assay, normalized to 1.5 µg/µL and then sent to MD Anderson for analysis. GraphPad Prism was then used to calculate final statistical tests and produce plots (RRID:SCR_002798). Supplementary Data 1 contains list of antibodies on the RPPA panel and additional raw datasets.

***In vivo* efficacy studies.**

Ten million cells were mixed with matrigel (50%) and implanted into a subcutaneous flank of female NSG mice to generate xenografts. After randomizing into groups of 5, tumor-bearing animals were treated with vehicle, crizotinib (30 mg/kg, QD) ribociclib (75 mg/kg, QD) or crizotinib (30 mg/kg, QD) + ribociclib (75 mg/kg, QD). Treatment was initiated 12 days after implantation when tumors were approximately 185 mm³. This is indicated as day 0 for treatment time on the graphs. Crizotinib (Selleckchem, Houston, TX) was resuspended in deionized water and administered by oral gavage. Ribociclib (Selleckchem, Houston, TX) was resuspended in 0.5% methycellulose and administered by oral gavage. Tumor size and body weight were measured twice weekly and tumor volume was calculated using the formula: length x width² × 0.52. The percent change in tumor volume was calculated using the formula ((V2-V1)/V1 x 100) where V1 is the starting volume and V2 is the final volume.

Statistical analysis

Mean $\pm$ SEM are shown unless otherwise stated. Student's t-test, one-way, or two-way ANOVA with either Dunnett's T3 (one-way) or Tukey's (two-way) multiple comparisons tests were used. P values < 0.05 were deemed statistically significant. Asterisks used in figures correspond with descriptions in figure legends that denote level of statistical significance. For animal studies, area under curve (AUC) analysis was used to compare the average tumor volume between groups. Briefly, area under curve values and their standard errors were computed as an estimation of the surface area between baseline values (mean value of the tumor volumes at the beginning of the treatment) and growth curves for vehicle and each treatment conditions. Groups were compared using Brown-Forsythe and Welch ANOVA tests and Dunnett's T3 multiple comparisons test. All data were plotted and analyzed using GraphPad Prism v9 (RRID:SCR_002798).

Cell lines, compounds, and plasmids

HBEC-ER1 and ER2 were generated from human broncho-epithelial cells immortalized with dominant-negative TP53 as described previously (217). Briefly, the EZR-ROS1 fusion oncogene was formed by using two CRISPR-Cas9 guide RNAs to target intron 9 of EZR and intron 33 of ROS1 and by assessing monoclonal cell lines after FACS. Ba/F3 (ATCC Cat# HB-283, RRID:CVCL_1963), HEK-293T/17 (ATCC Cat# CRL-11268, RRID:CVCL_1926), and H1792 (ATCC Cat# CRL-5895, RRID:CVCL_1495) cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Platinum-A and Platinum-E cells were purchased from Cell BioLabs, Inc. (San Diego, CA, USA). HCC78 cells were purchased from DSMZ (DSMZ # ACC 563, RRID:CVCL_2061; Braunschweig, Germany). Primary antibodies used in the study are as follows: phospho-ROS1 (Cell Signaling Technology, Inc. (CST; Danvers,

MA, USA); Y2274; #3078; RRID:AB_2180473), ROS1 (CST; #3287; RRID:AB_2797603), phospho-MTOR (CST; S2448; #5536; RRID:AB_10691552), MTOR (CST; #2983; RRID:AB_2105622), phospho-STAT3 (CST; Y705; #9145; RRID:AB_2491009), STAT3 (CST; #9139; RRID:AB_331757), phospho-ERK (CST; T202/Y204; #9101; RRID:AB_331646), ERK (CST; #4696; RRID:AB_390780), α -TUBULIN (Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA, USA); 12G10; RRID:AB_1157911), C-MYC (CST; #5605 and #13987; RRIDs:AB_1903938 and AB_2631168), ACTIN (DSHB; JLA20; RRID:AB_528068), GAPDH (Santa Cruz Biotechnology, Inc.; Dallas, TX, USA; sc-47724; RRID:AB_627678), phospho-S6 (CST; S235/236, #4858; RRID:AB_916156), and S6 (CST; #2317; RRID:AB_2238583).

DMEM, DME-F/12, L-glutamine, and antibiotics were purchased from Genesee Scientific (San Diego, CA, USA). Fetal bovine serum (FBS) was procured from Neuromics (CA3 Biosciences, Inc., Edina, MN, USA). Bovine growth serum (BGS) was procured from VWR International (Radnor, PA, USA). 0.05% Trypsin/0.53 mM EDTA was procured from Corning Inc. (Corning, NY, USA) while TrypLE™ was purchased from ThermoFisher Scientific (Waltham, MA, USA). Recombinant murine IL-3 was purchased from Peptrotech, Inc (Rocky Hill, NJ, USA).

Crizotinib, cabozantinib, and staurosporine were purchased from LC Laboratories® (Woburn, MA, USA), lorlatinib, entrectinib, repotrectinib, flavopiridol, and YM155 from Selleck Chemicals Inc. (Houston, TX, USA), ribociclib, palbociclib, gamitinib, and ARV-825 from MedChemExpress LLC (Monmouth Junction, NJ, USA), and birabresib (OTX-015), venetoclax, SB 204990, ND-646, telaglenastat, SphK1 5C, AZD4320, and AZD5991 were gifts from Jeffrey Tyner and Brian Druker. Doxycycline (10 mg/mL) was procured from Alfa Aesar (Haverhill, MA, USA). Annexin V Alexa Fluor 350 and MitoTracker™ Green were purchased from ThermoFisher. 7-AAD and Annexin V binding buffer were purchased from BioLegend (San Diego, CA, USA). TransIT-LT1 transfection reagent was purchased from Mirus Bio LLC

(Madison, WI, USA). Gateway LR clonase was purchased from ThermoFisher. Protease and phosphatase inhibitors were purchased from Bimake (Houston, TX, USA). Pre-cast Bolt™ and NuPAGE™ 4-12% Bis-Tris protein gels and 4X LDS sample buffer were purchased from ThermoFisher. Pierce™ BCA Protein Assay Kit was purchased from ThermoFisher. MTS reagent was purchased from BioVision, Inc. (Milpitas, CA, USA). pTRIPZ shNT (5' ATCTCGCTTGGGCGAGAGTAAG 3') and pTRIPZ shMYC-1 (5' CCGAGAACAGTTGAAACACAAA 3') were purchased from GE Healthcare Dharmacon, Inc. (Lafayette, CO, USA). pMXs-c-Myc-T58A was a gift from Shinya Yamanaka (Addgene plasmid # 13372; <http://n2t.net/addgene:13372>; RRID:Addgene_13372) (223). pLIX_403 was a gift from David Root (Addgene plasmid # 41395; <http://n2t.net/addgene:41395>; RRID:Addgene_41395). psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260; <http://n2t.net/addgene:12260>; RRID:Addgene_12260). pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454; <http://n2t.net/addgene:8454>; RRID:Addgene_8454) (224). pLenti-GIII-CMV-MYC-RFP-2A-Puro was ordered from Abmgood (Applied Biological Materials, Richmond, BC, CA). Human AXIN1 cDNA was purchased from Arizona State University (Tempe, AZ, USA). Oligonucleotides were ordered from Integrated DNA Technologies Corp. (Newark, NJ, USA) and Eurofins Genomics (Eurofins Scientific, Luxembourg).

Cytogenetics

FISH to detect presence of ROS1 fusion and MYC copy number amplification was performed by the Oregon Health and Science University (OHSU) Knight Diagnostic Laboratories (Portland, OR, USA). LUAD-0006 cells cultured with vehicle or 200 nM crizotinib were provided to the clinical diagnostic core in triplicate.

CRISPR-Cas9 Genome Editing

Genome editing of HBEC-ER1 and ER2 cell lines with CRISPR-Cas9 was performed using synthetic guide RNAs purchased from Synthego. The following guide RNA sequence for the AXIN1 gene was used: 5' GTTCCCTAGGTATGTGCAGG 3'. Manufacturer protocols were followed to obtain desired monoclonal cell lines. Genome edits were verified using ICE analysis and results can be found in Supplementary Data 3 (ice.synthego.com; Synthego Corporation, Menlo Park, CA, USA) (225).

RT-PCR of EZR-ROS1 mRNA

Reverse-transcriptase PCR to detect EZR-ROS1 mRNA in LUAD-0006, HBEC-ER1, and HBEC-ER2 cell lines (Supplementary Fig. S1B) was performed as described previously (217).

Cloning

The retroviral constructs pCX4 CEP85L-ROS1 and pCX4 GOPC-ROS1-L were cloned as described previously (133). pLIX AXIN1 was cloned by performing a Gateway LR clonase reaction between pDONR221 AXIN1 and pLIX_403 (RRID:Addgene_127696).

Generation of stable isogenic cell lines

Replication incompetent, infectious ecotropic and amphotropic retroviral particles were generated using Platinum-E and Platinum-A cells (Cell Biolabs), respectively. To generate stable Ba/F3 GOPC-ROS1 and CEP85L-ROS1 cell lines, Ba/F3 cells were maintained at a density between 0.5×10^6 to 1×10^6 /mL, transduced with ecotropic retrovirus, selected and validated as

previously described (133). Amphotropic retrovirus was used to generate stable, transduced HBEC-ER1, HBEC-ER2, and HCC78 cell lines that overexpress MYC^{T58A}. Lentivirus was generated by transfecting a plasmid of interest (pLenti-GIII-CMV-MYC-RFP-2A-Puro, pTRIPZ shNT, pTRIPZ shMYC-1, or pLIX AXIN1), psPAX2, and pVSV.G in a 1.3 to 0.72 to 1.64 picomolar ratio. Supernatant was collected at 48 and 72 hours post transfection and then concentrated via ultracentrifugation or ultrafiltration. Concentrated virus was then added along with 2 µg/mL polybrene to LUAD-0006 cells and incubated for 72 hours. Cells were then selected with 1 µg/mL puromycin or used directly in downstream assays.

IL-3 withdrawal assay

Oncogenic potential of the ROS1-fusions was assessed with an IL-3 independent growth assay (IL-3 withdrawal) as described previously (79). Briefly, stable cell lines were washed with R10 medium to remove IL-3. Cells were counted every 2-4 days, and those that exhibited sustained outgrowth were considered transformed. Transformed Ba/F3 cells were expanded and used for inhibitor testing via dose-response proliferation assays.

MitoTracker™ Green Quantitation

LUAD-0006 cells were treated with vehicle or 250 nM crizotinib for 10 days in triplicate. Cells were then pelleted and stained with MitoTracker™ Green using the manufacturer protocol. Flow cytometry was then performed with a BD FACSymphony Flow Cytometer (BD Biosciences-US). MitoTracker™ Green signal was quantified with a 488 nm laser and 530/30 bandpass filter.

Resulting data analysis and flow cytometry plots were generated using FlowJo v10 (RRID:SCR_008520) and GraphPad Prism v9 (RRID:SCR_002798).

Amplicon sequencing studies

LUAD-0006 cells were treated with vehicle or 250 nM lorlatinib for 10 days in triplicate. Cells were then collected and then harvested for genomic DNA (PureLink™ Genomic DNA Mini Kit, ThermoFisher) or total RNA (QIAGEN RNeasy Mini Kit; QIAGEN, Hilden, Germany). Total RNA was converted to cDNA with the All-in-One cDNA Synthesis SuperMix (Bimake). A region of the AXIN1 gene around exon 9 and of the AXIN1 transcript between amino acids 672 and 759 was then amplified from gDNA and cDNA, respectively, using Q5® Hot Start 2X PCR Master Mix (NEB). Partial illumina adapters were appended to the 5' ends of PCR primer to facilitate efficient NGS library preparation. Amplified PCR product was then sent to GENEWIZ, Inc. for sequencing (South Plainfield, NJ, USA). DNA amplicon was indexed and enriched by limited cycle PCR. The DNA library was validated using TapeStation (Agilent Technologies, Palo Alto, CA, USA), and was quantified using Qubit 2.0 Fluorometer and real time PCR (Applied Biosystems, Carlsbad, CA, USA). The pooled DNA libraries were loaded on the Illumina instrument according to manufacturer's instructions. The samples were sequenced using a 2x 250 paired-end (PE) configuration. Image analysis and base calling were conducted by the Illumina Control Software (HCS) on the Illumina instrument. Paired amplicon sequences from each sample were trimmed with Trimmomatic (RRID:SCR_011848) (226) and mapped to human genome reference GRCh38 using BWA-MEM (RRID:SCR_010910) (227). Alignments were visualized in Integrative Genomics Viewer v2.5.1 (RRID:SCR_011793) (228).

Results

ROS1-TKI-resistant ROS1 fusion patient sample has acquired MYC copy-number amplification

In order to study the ability of *MYC* CNA to potentially confer resistance to ROS1-TKIs, we acquired a patient sample from an *EZR-ROS1*-fusion-positive NSCLC patient. The patient experienced a short-lived response to crizotinib (6.5 months vs. progression-free-survival of 19.2 months) and eventual progression after second-line treatment with both lorlatinib and cabozantinib (Patient ID MSKCC 10; Supplementary Fig. 2.S2B) (206,229). A sample was obtained from a biopsy of this patient at progression on crizotinib; this sample was sequenced using the MSK-IMPACT platform (Supplementary Fig. 2.S1B and 2.S2A) and utilized to establish a patient-derived xenograft (PDX) and cell line (LUAD-0006) (Supplementary Fig. 2.S1B and 2.S2B).

Sequencing data from the resistant tumor sample revealed the following concurrent aberrations: *TP53* and *TERT* promoter missense mutations, copy-number amplification (CNA) of *MYC* (fold change: 6.5) and *CCND3* (fold change: 6.9), homozygous copy-number loss of *CDKN2A* (fold change: -1.6), *CDKN2B* (fold change: -1.6), and an *AXIN1* frame shift mutation resulting in a premature stop codon (Supplementary Fig. 2.S2A). *MYC* CNA posed as a compelling putative mechanism of ROS1 TKI resistance because the amplification was present prior to crizotinib treatment, the patient had short-lived response to crizotinib, and subsequently had no response to either cabozantinib or lorlatinib; moreover, the *MYC* CNA increased from 4.5-fold to 6.5-fold after crizotinib treatment (Supplementary Fig. 2.S2A). Hence, we hypothesized that *MYC* CNA and resulting putative overexpression of the *MYC* protein contributed to ROS1-TKI resistance in the patient.

Figure 1

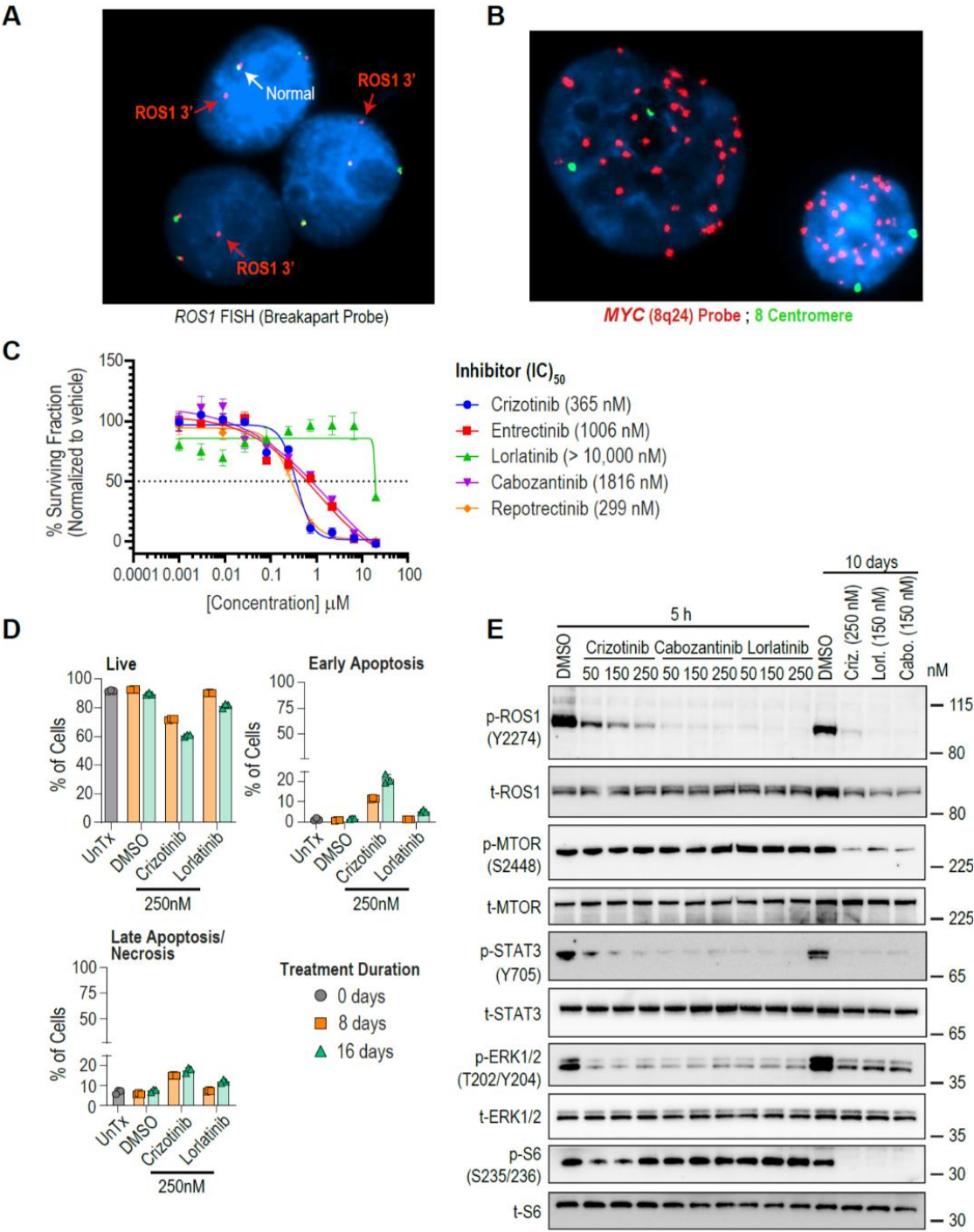
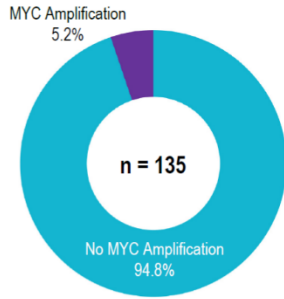


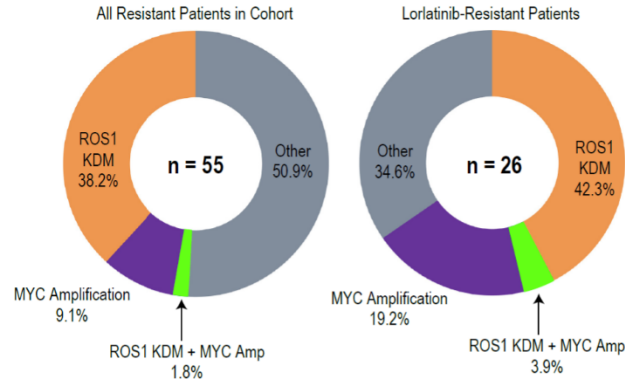
Figure 2.1. The patient-derived xenograft ROS1-fusion tumor cell line, LUAD-0006, is resistant to all generations of ROS1-TKIs and has copy-number amplification of MYC. A. Representative FISH graphic that demonstrates presence of EZR-ROS1 rearrangement in LUAD-0006 cells after 250 nM crizotinib treatment for 10 days. **B.** Representative FISH graphic that demonstrates presence of MYC copy-number amplification in LUAD-0006 cells after 250 nM crizotinib treatment for 10 days. **C.** Dose-response proliferation assay of LUAD-0006 cells after 10-day exposure to ROS1-TKIs crizotinib, entrectinib, lorlatinib, cabozantinib, or repotrectinib that shows LUAD-0006 cells are insensitive to TKI treatment. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3). **D.** Percentage of live, early apoptotic, and late apoptotic or necrotic LUAD-0006 cells as a function of treatment with vehicle, 250 nM crizotinib, or 250 nM lorlatinib after 8 and 16 days, measured with flow cytometric analysis of Annexin V and 7-AAD staining. Untreated LUAD-0006 cells at 0 days were also quantified. Live cells are defined as Annexin V-/7-AAD-, early apoptotic cells as Annexin V+/7-AAD- and late apoptotic or necrotic cells as Annexin V+/7-AAD+. Two-way ANOVA $P < 0.05$. **E.** Immunoblot analysis of LUAD-0006 cells shows that treatment with crizotinib, cabozantinib, and lorlatinib at various concentrations (50, 150, 250 nM) attenuate ROS1 autophosphorylation (phospho-ROS1, total-ROS1) and downstream signaling (phospho/total-MTOR, phospho/total-STAT3, phospho/total-ERK1/2, phospho-S6) at 5 hours and 10 days.

Figure S1**A**

AACR Genie (NSCLC ROS1-Fusion Cases)



ROS1-TKI Resistant NSCLC Cases (Lin et al. 2021)

**B**

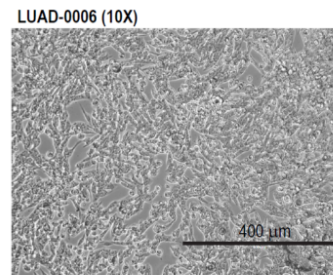
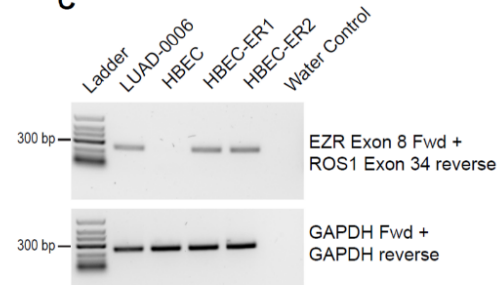
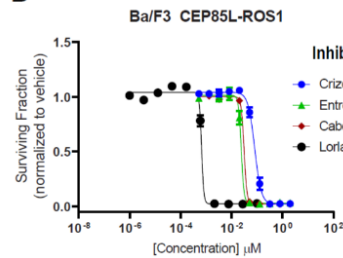
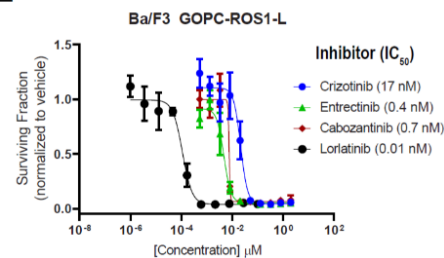
Data from Supplementary Table 1 of Lin et al. 2021

Patient ID	TKI(s)	Other Mutations
MGH0021	Lorlatinib	Point Mutations: PIK3CA E726K, MSH6 Q4*, EPCAM I193V, KAT6A D1216Y, KIF1B A930S, KIF1B T1106M, MRE11A R520H, PRKDC C1176S, TSHR D120N, WHSC1 G67S Low Copy-Number Gain: MCL1, GBA, RIT1, NTRK1, PVRL4, SDHC, DDR2, CDC73, UBE2T, PIK3C2B, MDM4, PTPN14, H3F3A, EGLN1, FH, EXO1, AKT3, SDHA, TERT, IL7R, RICTOR, CARD11, PMS2, RAC1, ETV1, JAZF1, IKZF1, EGFR, CDK6, SLC25A13, NRG1, WHSC1L1, FGFR1, KAT6A, POLB, PRKDC, MYBL1, TCEB1, NBN, RUNX1T1, RAD54B, EXT1, RECQL4, GATA3, DCLRE1C, RET, ERCC6, TET1, PRF1, KAT6B, DIS3, ERCC5, LIG4, NFKBIA, NKX2-1, FOXA1, FANCM, MAX, MLH3, TSHR, SERPINA1, DICER1, BCL11B, TRAF3, XRCC3, AKT1, MTA1, NTHL1, TSC2, TRAF7, SLX4, CREBBP, CITA, SOCS1, ERCC4, PALB2, FUS, CYLD, CBF3, CTCF, CDH1, MAF, MYC Copy-Number Gain: RSPO2 (6 copies), RAD21 (9 copies), MDM2 (52 copies), ZNF217 (15 copies) One-Copy Deletion: MAP3K1, PIK3R1, XRCC4, RASA1, RAD50, CTNNA1, ROS1, RSPO3, BRAF, PRSS1, EZH2, RHEB, XRCC2, PAXIP1, GATA4, NEIL2, NKX3-1, PTK2B, WRN, BMP1A, KLLN, PTEN, FAS, SUFU, NTSC2, SMC3, TCF7L2, HABP2, FGFR2, CBF2A2T3, FANCA, RPA1, TP53, AURKB, MAP2K4, FLCN, CRLF2 Two-Copy Deletion: APC
MGH0028	Lorlatinib	Point Mutations: TP53 G245S, AR Q67L, CD798 R141*, CSF1R R294Q, FGFR1 Q89R, RICTOR Q1154P Copy-Number Gain: KRAS, MYC
MGH0030	Crizotinib (1st) Lorlatinib (2nd)	Point Mutations: TP53 R280G Copy-Number Gain: MYC
MGH0038	Crizotinib (1st) Lorlatinib (2nd)	Point Mutations: TP53 R280G, TP53 V197fs, GNAS R201H, NRAS G60E Copy-Number Gain: MYC
MSKCC10 *LUAD-0006 generated from this case	Crizotinib (1st) Lorlatinib (2nd)	Point Mutations: TP53 R248W, AXIN1 Q732_E733delinsR*, TERT g.1295228C>A Copy-Number Gain: MYC , CCND3 Copy-Number Loss: CDKN2A, CDKN2B

Supplementary Figure 2.1. MYC amplification recurs in ROS1-positive non-small-cell lung cancer (NSCLC). **A.** Donut charts that quantify the percentage of ROS1-positive non-small-cell lung cancer with concurrent MYC copy-number amplification (CNA) in either the AACR Genie dataset or ROS1-positive NSCLC cases that are TKI-resistant (Lin et al. 2021). For the Lin et al. 2021 dataset, MYC CNA is compared to ROS1-dependent resistance (KDM = kinase domain mutation) and other forms of ROS1-independent resistance. Also for the Lin et al. 2021 dataset, the first donut chart consists of all patients treated with either crizotinib, lorlatinib, or both, while the second donut chart only consists of patients treated with lorlatinib alone or crizotinib and lorlatinib. **B.** Table that summarizes other known variants in MYC-amplified, TKI-resistant cases from Lin et al. 2021 dataset.

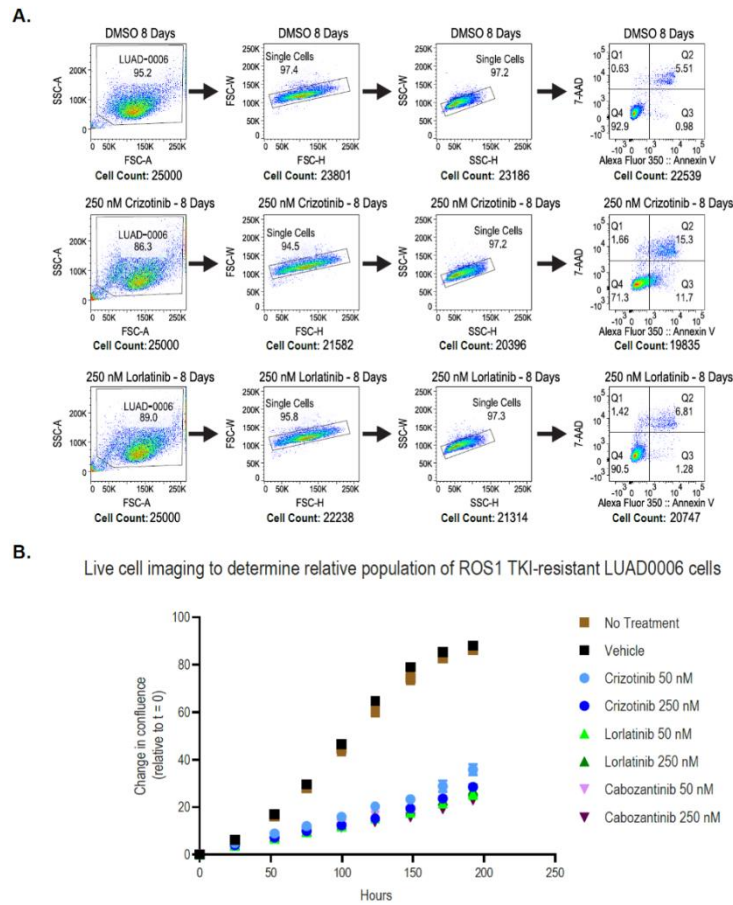
Figure S2**A**

ALTERATION	MSK-IMPACT 3/2015	MSK-IMPACT 11/2015
EZR-ROS1 Rearrangement	c.1090+142C:EZR_c.5366+794T:ROS1del	c.1090+142C:EZR_c.5366+794T:ROS1del
TP53 (NM_000546)	R248W (missense)	R248W (missense)
MYC (NM_002467 - 8q24.21)	Amplification (Fold Change: 4.5)	Amplification (Fold Change: 6.5)
AXIN1 (NM_003502)	Q732_D862delinsR	Q732_D862delinsR
TERT (NM_198253)	Promoter variant (g.1295228C>A)	Promoter variant (g.1295228C>A)
CCND3 (NM_001760 - 6p21.1)		Amplification (Fold Change: 6.9)
CDKN2A p16INK4A (NM_000077 - 9p21.3)		Loss (Fold Change: -1.6)
CDKN2A p14INK4A (NM_058195 - 9p21.3)		Loss (Fold Change: -1.6)
CDKN2B (NM_004936 - 9p21.3)		Loss (Fold Change: -1.6)

B**C****D****E**

Supplementary Figure 2.2. Characterization of LUAD-0006 NSCLC cell line. **A.** Table summarizes MSK-IMPACT targeted sequencing of lung tumor biopsy samples of patient prior to (3/2015) and post crizotinib (11/2015) treatment. **B.** Brightfield microscopy image depicting growth of LUAD-0006 patient-derived xenograft cells in vitro. **C.** Reverse transcriptase polymerase chain reaction shows expression of EZR-ROS1 fusion transcript in cDNA of LUAD-0006, HBEC-ER1, and HBEC-ER2 cells. Expected amplicon size is ~250 bp. **D-E.** Dose-response cell proliferation assay of Ba/F3 CEP85L-ROS1 (**D**) and GOPC-ROS1-L (**E**) cells after 3-day exposure to ROS1-TKIs crizotinib, entrectinib, cabozantinib, or lorlatinib that shows sensitivity of these cells to TKI treatment. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM ($n = 3$).

Figure S3



Supplementary Figure 2.3. Assessment of LUAD-0006 cell proliferation and apoptosis after ROS1-TKI treatment. **A.** Gating strategy used for flow cytometry of cells treated with vehicle, 250 nM crizotinib, or 250 nM lorlatinib to assess Annexin V (Alexa Fluor 350) and 7-AAD staining. Representative plots taken at 8 days post initiation of treatment. Number of cells included in gate listed below each plot. **B.** Proliferation assay of LUAD-0006 cells treated with vehicle, 25 nM, or 250 nM of crizotinib, lorlatinib, or cabozantinib that shows that TKI treatment does not stop cell proliferation over time. Cells were imaged using the Incucyte Zoom live-cell imaging platform at 24-hour intervals. % confluence was measured at each time point and change in confluence was calculated as follows: % confluence – % confluence at t=0. Values shown are mean $\pm$ SEM (n = 6).

Intriguingly, a recent study by Lin et al. reported sequencing data from a cohort of patient tumors that were clinically resistant to crizotinib or lorlatinib, and observed alterations in genes that are not traditionally implicated in canonical RTK signaling pathways (206). While several alterations that fit this criterion were found, a noteworthy observation is the recurrence of copy

number amplification (CNA) of the proto-oncogenic transcription factor, *MYC*. Specifically, 9.1% (5/55) of all of the resistant tumors and 19.2% (5/26) of lorlatinib-resistant tumors were found to have *MYC* CNA; only one of these cases had a concurrent kinase domain point mutation that could explain the emergence of resistance (206) (Supplementary Fig. 2.S1). We found that over 5% of ROS1-positive NSCLC in the AACR Genie dataset also contained *MYC* amplification (114). As a well-studied proto-oncogene and transcription factor, *MYC* promotes expression of a wide spectrum of target genes that cause pleiotropic effects on cellular function, proliferation, and survival. Therefore, we reasoned that overexpression of this protein could reduce the effectiveness of ROS1-TKI treatment.

We first validated the presence of the *EZR-ROS1* fusion oncogene in LUAD-0006 using RT-PCR, via successful amplification of *EZR-ROS1* fusion transcript using an *EZR* forward primer and *ROS1* reverse primer at the predicted size (Supplementary Fig. S2.2C). Additional confirmation of the chromosomal rearrangement that produced the *EZR-ROS1* fusion was conducted via fluorescence in-situ hybridization assay (FISH) using a *ROS1* break-apart probe (**Fig. 2.1A**). FISH also confirmed *MYC* CNA that was reported by MSK-IMPACT and revealed an amplified *MYC* (8q24) signal pattern with greater than twenty *MYC* signals per cell (two centromere signals per cell) in all cells imaged and counted (**Fig. 2.1B**). Thus, the LUAD-0006 genome has both the *EZR-ROS1* fusion oncogene and robust *MYC* CNA.

Next, we tested if LUAD-0006 retained crizotinib-resistance as observed in the patient at time of sample acquisition (**Fig. 2.1C and D**; Supplementary Fig. 2.S3). Specifically, treatment of LUAD-0006 cells with multiple ROS1 TKIs revealed relative resistance with high-nanomolar to micromolar inhibitor concentration needed to suppress the viability of 50% of the cells (IC_{50}) (**Fig. 2.1C**). This is in stark contrast to TKI-sensitive cell lines such as transformed Ba/F3 ROS1-fusion-positive cells that exhibit low nanomolar to picomolar IC_{50} values (Supplementary Fig. 2.S2D and

S2E), albeit lineage differences may account for some variation in relative sensitivity. Live-cell imaging further confirmed that LUAD-0006 cell proliferation was reduced but not eliminated with these inhibitors, at 25 nM or 250 nM concentrations, as compared to vehicle (DMSO) treatment (Supplementary Fig. 2.S3B); cells continued to proliferate in the presence of 250 nM crizotinib, lorlatinib or cabozantinib, a concentration that should induce complete and robust cell death. Flow cytometric analysis with annexin V and 7-AAD showed that while apoptosis is induced in a fraction of LUAD-0006 cells after prolonged (> 2 week) treatments with crizotinib (250 nM) or lorlatinib (250 nM), most cells (60% for crizotinib, 82% for lorlatinib at 16 days) were not affected (**Fig. 2.1D**; Supplementary Fig. 2.S3A).

We then determined if ROS1-TKI-resistance is a consequence of inefficient on-target inhibition of *EZR-ROS1* due to kinase domain point mutation(s) or other cellular changes that affect intracellular accumulation of TKI. First, no point mutations in the ROS1 kinase domain of the *EZR-ROS1* were noted in the MSK-IMPACT targeted sequencing data (Supplementary Fig. 2.S1A). Secondly, immunoblot analysis of LUAD-0006 cells treated with varying concentrations of crizotinib, cabozantinib, and lorlatinib after 5 hours and after 10 days showed robust attenuation of ROS1 autophosphorylation (surrogate measure for catalytic activity); inhibition was consistent with previously published potencies of these inhibitors and occurred in a dose-dependent manner (**Fig. 2.1C**) (210). Furthermore, we observed concurrent attenuation of ROS1 effector pathways, including phosphorylated status of MTOR, MAPK (ERK1/2), STAT3, and S6 signaling proteins after ROS1 TKI treatment (**Fig. 2.1C**). As a result, we determined that LUAD-0006 cells are resistant to ROS1-TKI treatment despite successful on-target inhibition of ROS1 and reduction of downstream signaling. These data establish LUAD-0006 as a ROS1-TKI-resistant, *EZR-ROS1* expressing NSCLC cell line.

Knockdown of MYC in combination with ROS1-TKI treatment leads to loss of LUAD-0006 cell proliferation.

We first examined if inhibition of ROS1 affects MYC protein expression in LUAD-0006 cells. LUAD-0006 cells treated with 250 nM of crizotinib, lorlatinib, and cabozantinib exhibited reduction in MYC expression after 10 days of treatment but not acute (hours) treatment (Fig. 2.2A). We postulated that even accounting for the reduction in MYC protein level after 10 days of ROS1 TKI treatment, the extent of MYC overexpression due to *MYC* CNA might still be at

Figure 2

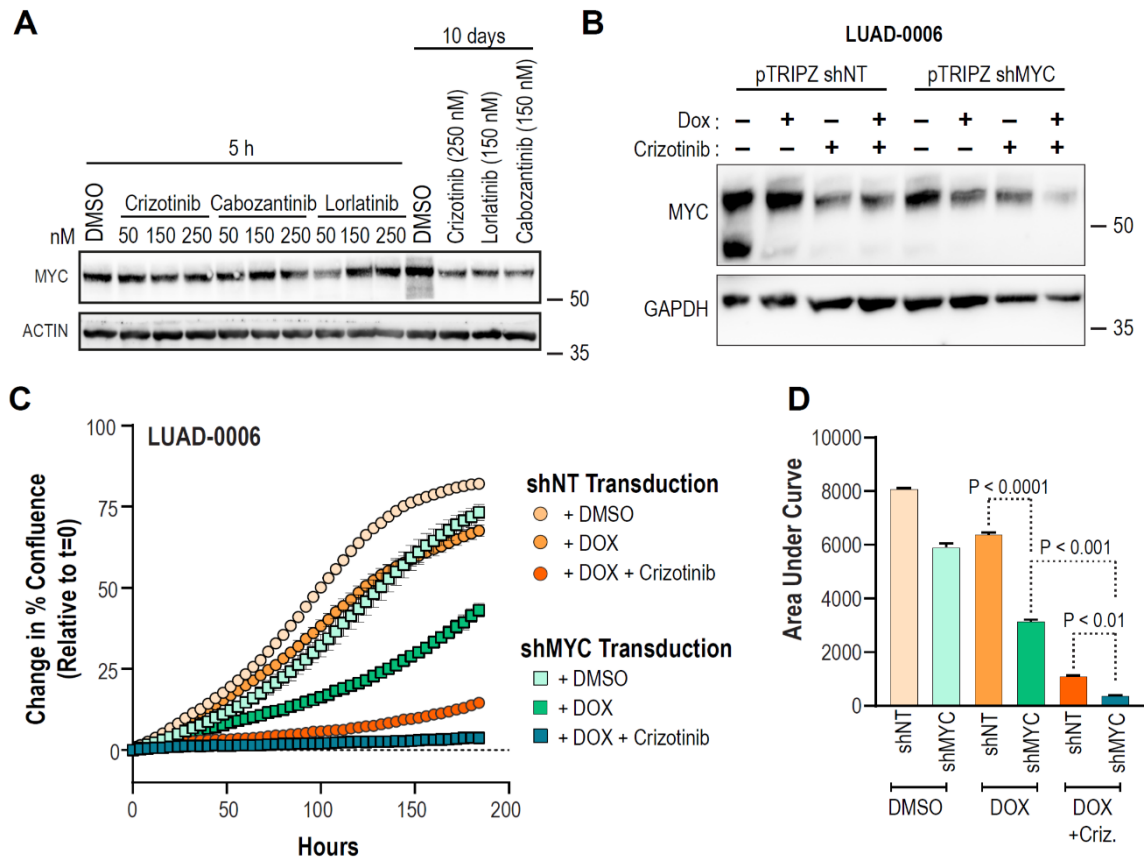


Figure 2.2. Knockdown of MYC resensitizes LUAD-0006 cells to crizotinib. **A.** Immunoblot analysis of LUAD-0006 cells demonstrates that ROS1-TKI treatment reduces MYC protein expression after 10 days of treatment. **B.** Immunoblot analysis of LUAD-0006 cells transduced with doxycycline-inducible shRNA vectors that either contain non-targeting shRNA (pTRIPZ shNT) or shRNA targeting MYC (pTRIPZ shMYC-1). Transduced cells were treated with vehicle, 2 µg/mL doxycycline, 250 nM crizotinib, or both doxycycline and crizotinib and then MYC protein expression was assessed. MYC expression is lowest in cells transduced with pTRIPZ shMYC-1 and treated with both doxycycline and crizotinib. **C.** 8-day cell proliferation assay of LUAD-0006 cells transduced with pTRIPZ shNT or pTRIPZ shMYC-1 and treated with vehicle, 2 µg/mL doxycycline, 250 nM crizotinib, or both doxycycline and crizotinib shows that attenuation of both ROS1 activity and MYC expression leads to shutdown of LUAD-0006 cell proliferation. Cells were imaged using the Incucyte Zoom live-cell imaging platform at 4-hour intervals. % confluence was measured at each time point and change in confluence was calculated as follows: % confluence – % confluence at t=0. Values shown are mean ± SEM (n = 6). **D.** Area-under-the-curve (AUC) analysis of the replicate curves from **C.** One-way ANOVA (Brown-Forsythe and Welch tests) $P < 0.05$. P values on graph specify results of multiple comparison tests (Dunnett's T3).

greater than general basal level of this protein and thus sufficient for driving ROS1 TKI resistance. To test this, LUAD-0006 cells were transduced with doxycycline-inducible lentiviral plasmids encoding either non-targeting shRNA (shNT) or shRNA targeting *MYC* mRNA (shMYC) to further knockdown MYC levels. Doxycycline treatment successfully induced downregulation of MYC protein in shMYC- as compared to shNT-transduced cells (Fig. 2.2B). We then assessed if combination of *MYC* knockdown and crizotinib treatment together decreased LUAD-0006 cell proliferation to a greater extent than either treatment alone. Live-cell imaging of LUAD-0006 shNT and shMYC transduced cells, over an eight-day period, showed near-total inhibition of cell proliferation when *MYC* knockdown is concurrent with crizotinib treatment as compared to *MYC* knockdown alone (Fig. 2.2C). Area-under-the-curve analysis showed that the LUAD-0006 shMYC-1 cells treated with both doxycycline and crizotinib had significantly lower cell proliferation rate relative to every other experimental group (Fig. 2.2D). These data support the hypothesis that overexpression of MYC decreases ROS1-TKI sensitivity that is observed in LUAD-0006 cells.

Overexpression of MYC in ROS1-TKI sensitive cell lines leads to therapeutic resistance.

We determined the sufficiency of MYC overexpression in the induction of ROS1-TKI resistance by utilizing two recently developed human bronchial epithelial cell lines, HBEC-ER1 and HBEC-ER2, that were engineered to endogenously express the EZR-ROS1 fusion oncogene and, importantly, are highly sensitive to ROS1-TKIs (Supplementary Fig. 2.S2C) (217). Ectopic expression of wildtype MYC (MYC^{WT}) did not result in measurable overexpression (Fig. 2.3A).

Figure 3

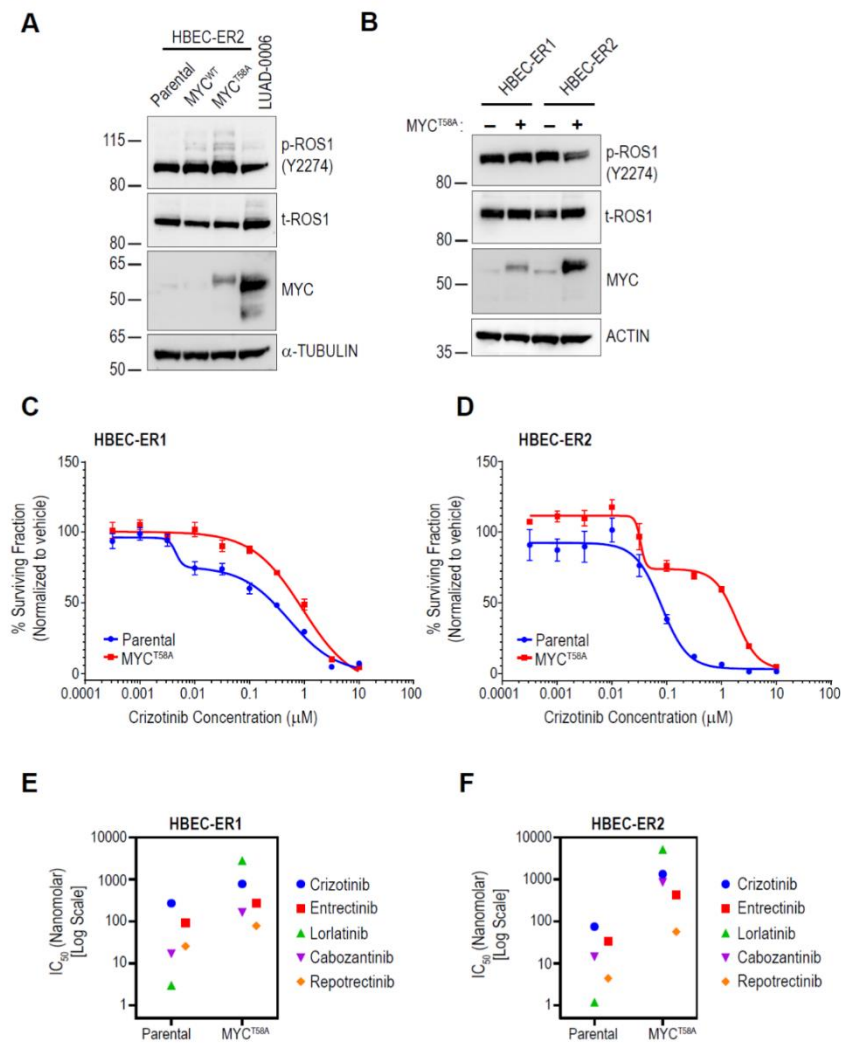
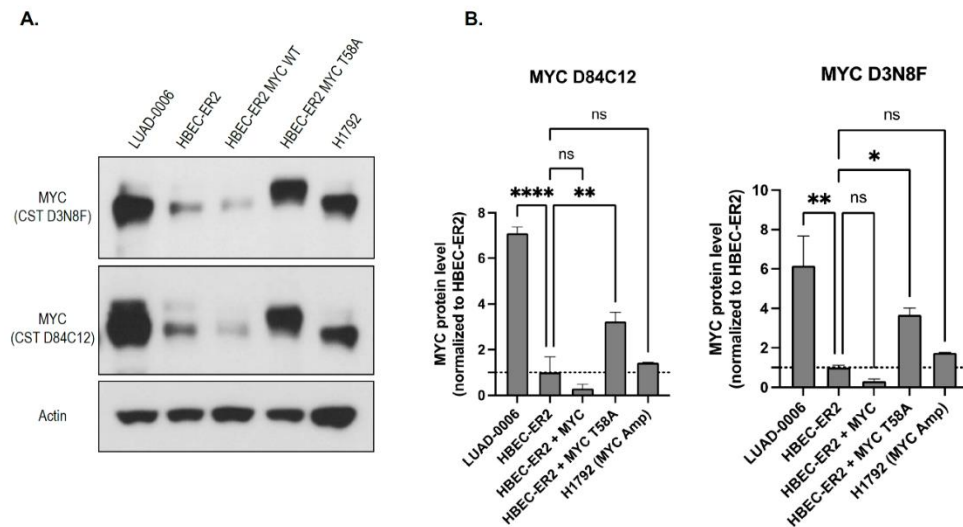


Figure 2.3. Overexpression of MYC in ROS1-TKI sensitive cell lines confers resistance to ROS1-TKIs. **A.** Immunoblot analysis of HBEC-ER2 cells transduced with either wild-type or mutant MYC^{T58A} compared to parent HBEC-ER2 and LUAD-0006 cells. ROS1 activity (phospho/total-ROS1) and MYC expression was assessed. While ROS1 activity was comparable across all conditions, only mutant MYC came close to recapitulating high MYC overexpression in LUAD-0006. **B.** Immunoblot analysis of HBEC-ER1 and HBEC-ER2 cells with or without MYC^{T58A} overexpression. MYC expression was higher in cells transduced with MYC^{T58A} in both cell lines but overexpression was notably higher in HBEC-ER2. **C.** Dose-response proliferation assay of HBEC-ER1 cells after 72-hour exposure to crizotinib. Cells transduced with MYC^{T58A} are less sensitive to crizotinib treatment relative to parent HBEC-ER1 cells. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3). **D.** Dose-response proliferation assay of HBEC-ER2 cells after 72-hour exposure to crizotinib. Cells transduced with MYC^{T58A} are less sensitive to crizotinib treatment relative to parent HBEC-ER2 cells. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3). **E.** Chart of IC50 values derived from 72-hour dose-response proliferation assays described in figures 3C and S3A, demonstrating loss of ROS1-TKI sensitivity with overexpression of MYC (MYC^{T58A}) in HBEC-ER1 cells. **F.** Chart of IC50 values derived from 72-hour dose-response proliferation assays described in figures 3D and S3B, demonstrating loss of ROS1-TKI sensitivity with overexpression of MYC (MYC^{T58A}) in HBEC-ER2 cells.

Hence, to achieve robust overexpression of MYC that is relatively comparable to LUAD-0006 cells, we expressed the MYC^{T58A} mutant that is resistant to proteasomal degradation and thus accumulates at relatively high levels in cells as compared to wildtype MYC (**Fig. 2.3A**) (230,231). MYC^{T58A} has been used in previous studies to overexpress MYC and achieve high protein levels (232). Immunoblotting from stably transduced cells confirms that MYC^{T58A} provides substantially higher protein expression that is more comparable to the MYC levels detected in LUAD-0006 cells (**Fig. 2.3A**). We also compared MYC expression between LUAD-0006, HBEC-ER2 MYC^{T58A} and H1792, a well-characterized MYC-amplified, KRAS G12C NSCLC cell line (233), and found that MYC expression was 6-fold higher in LUAD-0006, while HBEC-ER2 MYC^{T58A} had comparable expression to H1792 (Supplementary Fig. 2.S4). Additional immunoblotting on HBEC-ER1 and ER2 cells with or without MYC^{T58A} revealed robust overexpression in both cell lines, albeit with higher levels achieved in HBEC-ER2 as compared to ER1 (**Fig. 2.3B**). Dose-response cell viability assays using the HBEC-ER MYC^{T58A} cell lines challenged with crizotinib, entrectinib,

cabozantinib, lorlatinib and repotrectinib consistently demonstrate acquired resistance to these TKI as compared to the parental HBEC-ER cell lines (**Fig. 2.3C-F**; Supplementary Fig. 2.S5). Notably, the greatest IC₅₀ increases were observed in cells treated with lorlatinib, an ALK/ROS1-TKI with a more selective kinase inhibition profile than other TKIs (**Fig. 2.3E and F**) (234). Finally, we repeated this same experiment in a well-characterized ROS1-fusion NSCLC cell line, HCC78, and found that overexpression of MYC in these cells also confers resistance (200) (Supplementary Fig. 2.S6). The significant loss of ROS1-TKI efficacy in HBEC-ER1, HBEC-ER2, and HCC78 cells with MYC overexpression demonstrates that enhanced *MYC*-mediated cell signaling sufficiently compensates for the loss of oncogenic signals from ROS1.

Figure S4

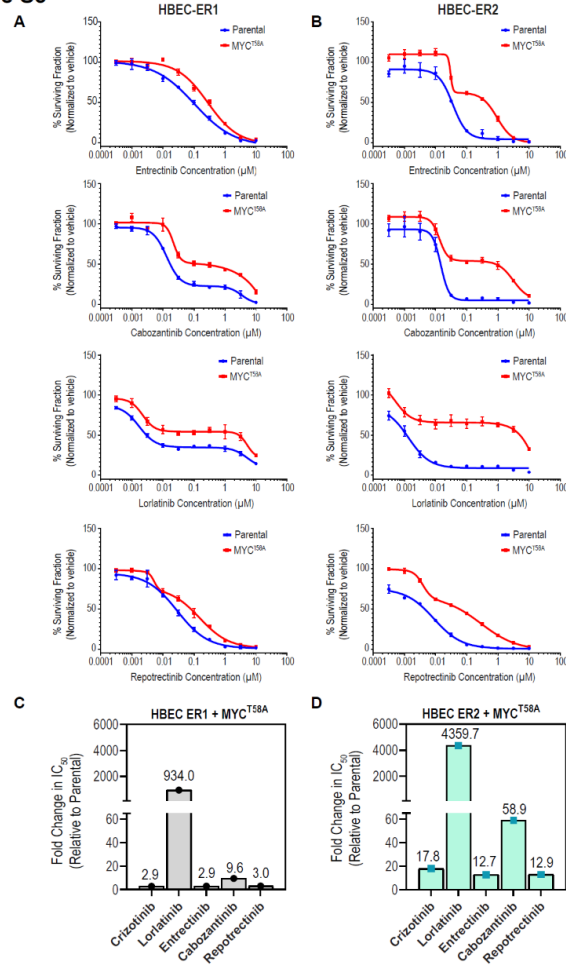


Supplementary Figure 2.4. MYC protein expression in LUAD-0006 is comparable to the established MYC-amplified NSCLC cell line, H1792. **A.** Representative immunoblot of LUAD-0006, HBEC-ER2, HBEC-ER2 MYC^{WT}, HBEC-ER2 MYC^{T58A}, and H1792 cells. MYC and actin protein expression was assessed, with two separate antibodies used to probe for MYC expression. MYC protein expression in LUAD-0006 is the highest, while HBEC-ER2 MYC^{T58A} has comparable protein expression to H1792. **B.** Densitometry analysis of replicate immunoblots, with representative image shown in **(A)**. Intensity of MYC band was quantified, divided by the intensity of the actin band, and then normalized to MYC expression seen in HBEC-ER2 cells. Values shown are mean $\pm$ SEM (n = 3). Statistical significance was assessed with Student's t-test (*, p < 0.05; **, p < 0.01; ****, p < 0.0001).

Chronic crizotinib treatment reprograms signaling pathways and protein expression in LUAD-0006

Next, we wanted to assess how signaling changes in LUAD-0006 cells to allow them to withstand long-term TKI treatment. For quantitative assessment of these changes at the protein level, we employed a reverse phase protein array (RPPA) that has been previously shown to be an effective method for studying cell signaling and tumor evolution (222,235). RPPA is a quantitative, antibody-based assay that permits measurement of multiple protein markers, as well as post-translational modifications such as phosphorylation. As expected, RPPA analysis of LUAD-0006 cells treated for 10 days with crizotinib demonstrated significant downregulation of canonical kinase-dependent cell growth, survival, and proliferation pathways downstream of RTK activation, including the RAS/MAPK pathway and PI3K/AKT/mTOR axes (**Fig. 2.4A-C**; Supplementary Data 2.1). Normalized protein levels of key targets within these pathways, that were significantly altered, are shown in **Fig. 2.4B**, and pathway analyses scores are shown in Supplementary Fig. 2.S7A. Intriguingly, expression of a subset of insulin family receptor subunits (IR β and IGF1R β) are upregulated (modest but statistically significant), while adaptor proteins in this signaling family, IRS2 and PKM2 are downregulated. Cell cycle associated proteins are also differentially altered in vehicle (DMSO) or crizotinib treated cells; proteins involved in G2/M progression are significantly downregulated (CDC25C, PLK1, CCNB1) while subset of proteins involved in G1/S phase transition are significantly upregulated (BRD4, TP53BP1). Other pathways that were significantly altered include the downregulation of YAP1 (Notch pathway), and modest but significant upregulation of immune pathway targets, including LCK kinase, and checkpoint proteins B7-H4 and PD-1.

Figure S5



Supplementary Figure 2.5. Overexpression of MYC in ROS1-TKI sensitive cell lines confers resistance to ROS1-TKIs. A. Dose-response cell proliferation assays of HBEC-ER1 cells after 72-hour exposure to the ROS1-TKIs entrectinib, lorlatinib, cabozantinib, or repotrectinib. Cells transduced with MYC^{T58A} are less sensitive to ROS1-TKI treatment relative to parent HBEC-ER1 cells. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM ($n = 3$). **B.** Dose-response proliferation assays of HBEC-ER2 cells after 72-hour exposure to the ROS1-TKIs entrectinib, lorlatinib, cabozantinib, or repotrectinib. Cells transduced with MYC^{T58A} are less sensitive to ROS1-TKI treatment relative to parent HBEC-ER2 cells. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM ($n = 3$). **C-D.** Bar charts showing fold change in IC₅₀ values with overexpression of MYC (MYC^{T58A}) for the ROS1-TKIs crizotinib, lorlatinib, entrectinib, cabozantinib, and repotrectinib in either **(C)** HBEC-ER1 or **(D)** HBEC-ER2 cells compared to cells without MYC overexpression.

Figure 4

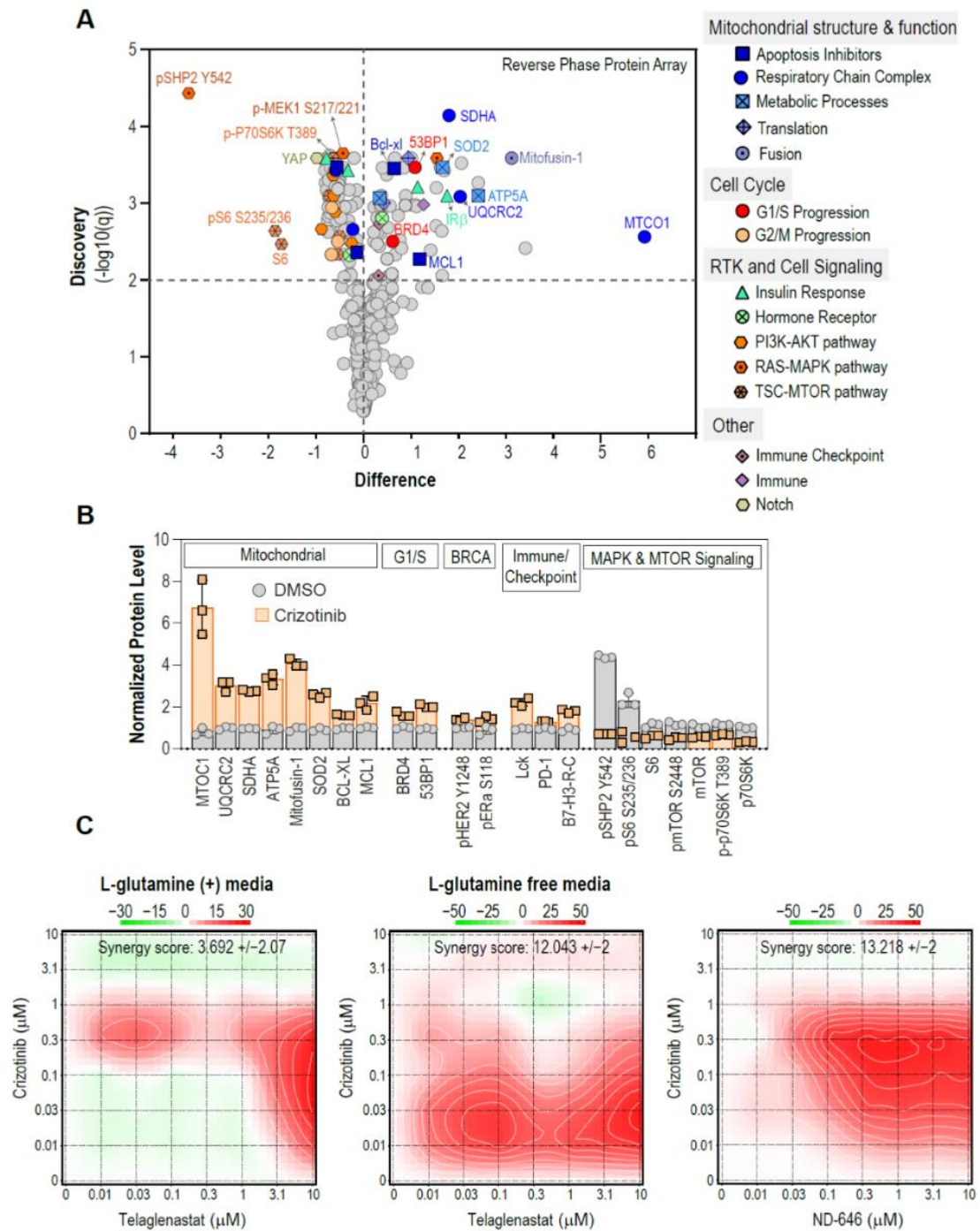
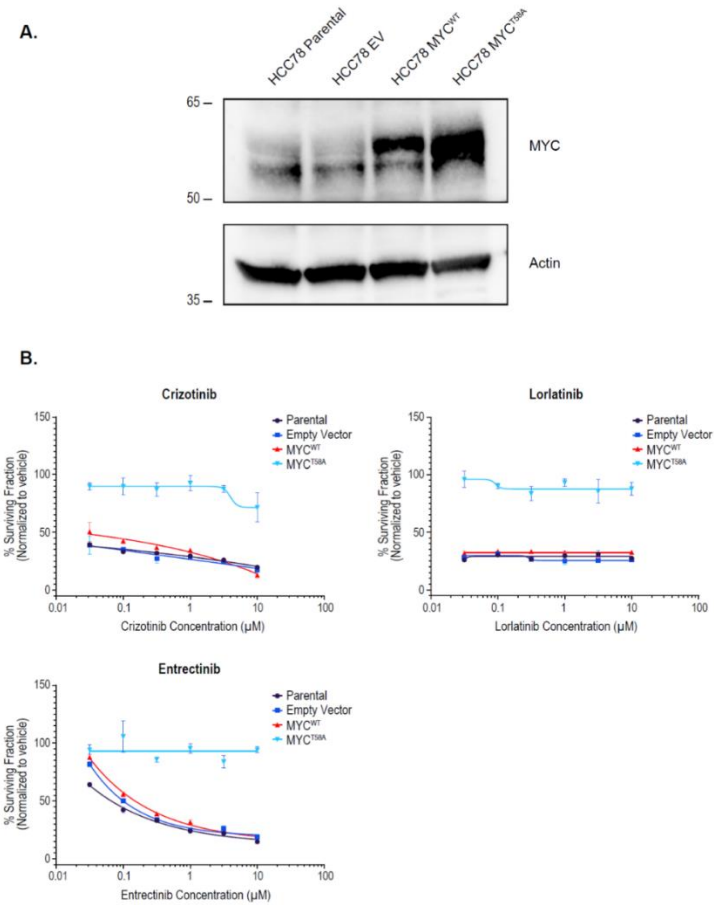
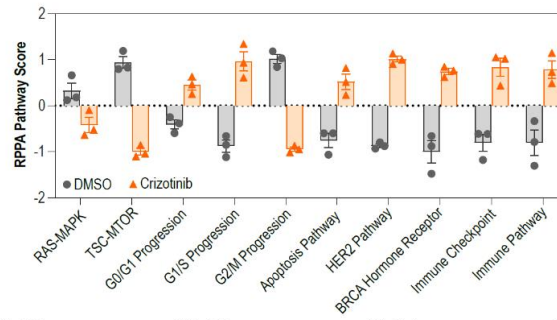
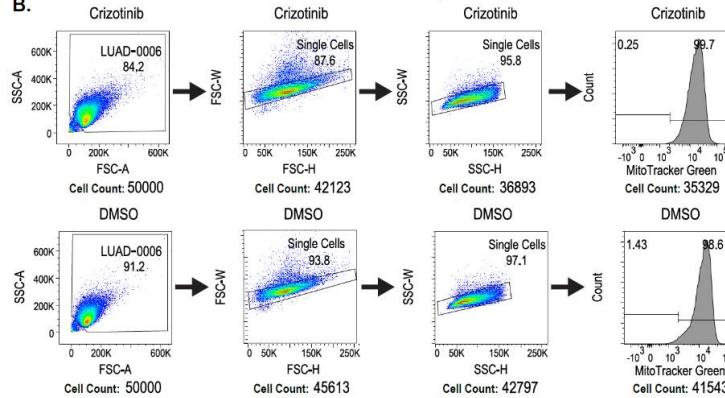
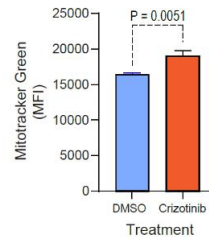


Figure 2.4. Reverse-phase protein array (RPPA) analysis of LUAD-0006 cells treated long-term with crizotinib shows that MAPK and MTOR signaling are downregulated while proteins that inhibit apoptosis and promote mitochondrial biogenesis are upregulated. A. Volcano plot of RPPA results that shows downregulation of key mediators of MAPK and MTOR signaling and upregulation of mitochondrial proteins involved with inhibition of apoptosis and mitochondrial biogenesis are upregulated. Normalized linear (NormLinear) values for each protein target (relative protein levels determined from antibody-lysate signal amplification and normalized for protein loading per sample) were compared between LUAD-0006 cells treated with vehicle vs. 250 nM crizotinib for 10 days. Difference in protein expression between lysates of cells treated with crizotinib vs. vehicle was calculated as follows for each antibody: Average of NormLinear[crizotinib] – Average of NormLinear[vehicle] (n = 3). Student's t-test (unpaired, two-tailed) and false discovery rate (FDR) were used to determine statistical significance ($q < 0.01$; $-\log_{10}(q) > 2$). **B.** Bar chart containing selected protein expression values from RPPA analysis that were found to be statistically significant between vehicle and 250 nM crizotinib (Student's t-test; $p < 0.05$). The average of NormLinear values from both vehicle and 250 nM crizotinib samples along with each replicate value (n = 3) are plotted. **C.** Synergy plots of 10-day treatment of LUAD-0006 cells with crizotinib in combination with telaglenastat and ND-646. Crizotinib and telaglenastat were tested in both L-glutamine-positive and L-glutamine-negative media. Synergy analysis and results were generated with SynergyFinder and the zero interaction potency (ZIP) model was used to detect synergistic or antagonistic effects. The synergy scores summarize the average % response of the two drugs beyond expectation across all drug concentration combinations, where positive scores are likelier to be considered “synergistic” and negative scores are likelier to be considered “antagonistic.”

Figure S6

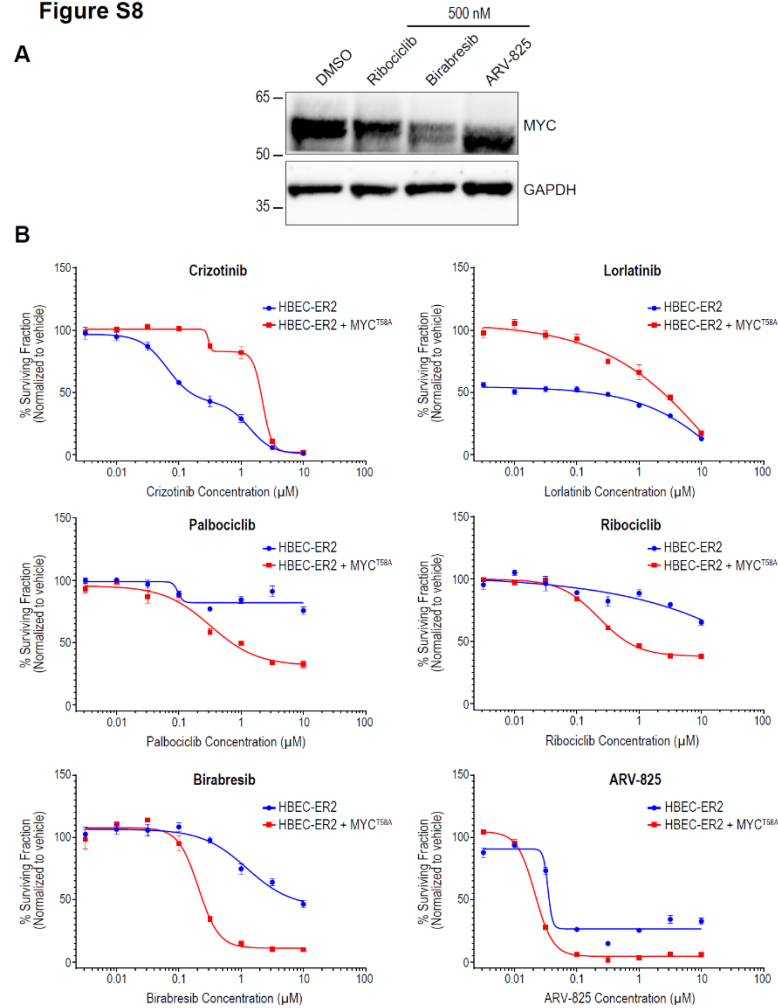


Supplementary Figure 2.6. MYC overexpression induces ROS1-TKI resistance in HCC78 cells. **A.** Immunoblot of MYC protein expression in HCC78 cells that are either untransduced (parental) or transduced with empty vector (EV), MYC^{WT}, or MYC^{T58A}. MYC and actin were assessed. Immunoblot shows that HCC78 cells with MYC^{T58A} have highest level of MYC protein expression. **B.** Dose-response cell proliferation assays of HCC78 cells after 72-hour exposure to the ROS1-TKIs crizotinib, lorlatinib, and entrectinib. Cells transduced with MYC^{T58A} are less sensitive to ROS1-TKI treatment relative to parent HCC78 cells and HCC78 cells transduced either with empty vector or MYC^{WT}. These assays were performed in 96-well low-attachment plates. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3).

Figure S7**A.****B.****C.**

Supplementary Figure 2.7. RPPA analysis reveals changes in multiple signaling pathways and MitoTracker Green staining demonstrates increased mitochondrial mass in LUAD-0006 cells treated long-term with crizotinib. **A.** Bar chart showing results of pathway enrichment analysis of RPPA data. RPPA pathway scores were calculated using NormLinear values transformed to a log2 scale (NormLog2) and inputted into a previously published algorithm. The average of pathway scores along with each replicate are plotted ($n = 3$) and pathways shown were found to be statistically significant between vehicle and 250 nM crizotinib (Student's t-test; $p < 0.05$). Positive RPPA pathway score indicates upregulation of pathway while negative score indicates downregulation. **B.** Flow cytometry was employed to assess relative intensity of MitoTracker Green™ staining in LUAD-0006 cells treated with vehicle or 250 nM crizotinib for 10 days. Gating strategy that was used to assess MitoTracker Green positive stained population is depicted. Representative plots from one replicate shown. Number of cells included in gate listed below each plot. **C.** Bar chart illustrating average mean fluorescence intensity (MFI) of LUAD-0006 cells treated for 10 days with vehicle or 250 nM crizotinib and stained with MitoTracker Green ($n = 3$). Statistical significance was assessed with Student's t-test (*, $p < 0.05$).

Figure S8



Supplementary Figure 2.8. Birabresib reduces MYC protein expression in LUAD-0006 cells and HBEC-ER2 cells with MYC overexpression become sensitized to CDK4/6 and BET bromodomain inhibition. **A.** Immunoblot of LUAD-0006 cells treated with vehicle, 500 nM ribociclib, 500 nM birabresib (OTX-015), or 500 nM ARV-825 for 96 hours to assess MYC protein expression. **B.** Dose-response proliferation assays of HBEC-ER2 cells after 72-hour exposure to ROS1-TKI (crizotinib and lorlatinib), CDK4/6 inhibition (palbociclib and ribociclib), or BET bromodomain inhibition (birabresib and ARV-825). Cells transduced with MYC^{T58A} are less sensitive to ROS1-TKI treatment but are more sensitive to either CDK4/6 or BET bromodomain inhibition relative to parent HBEC-ER2 cells. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3).

Figure 5

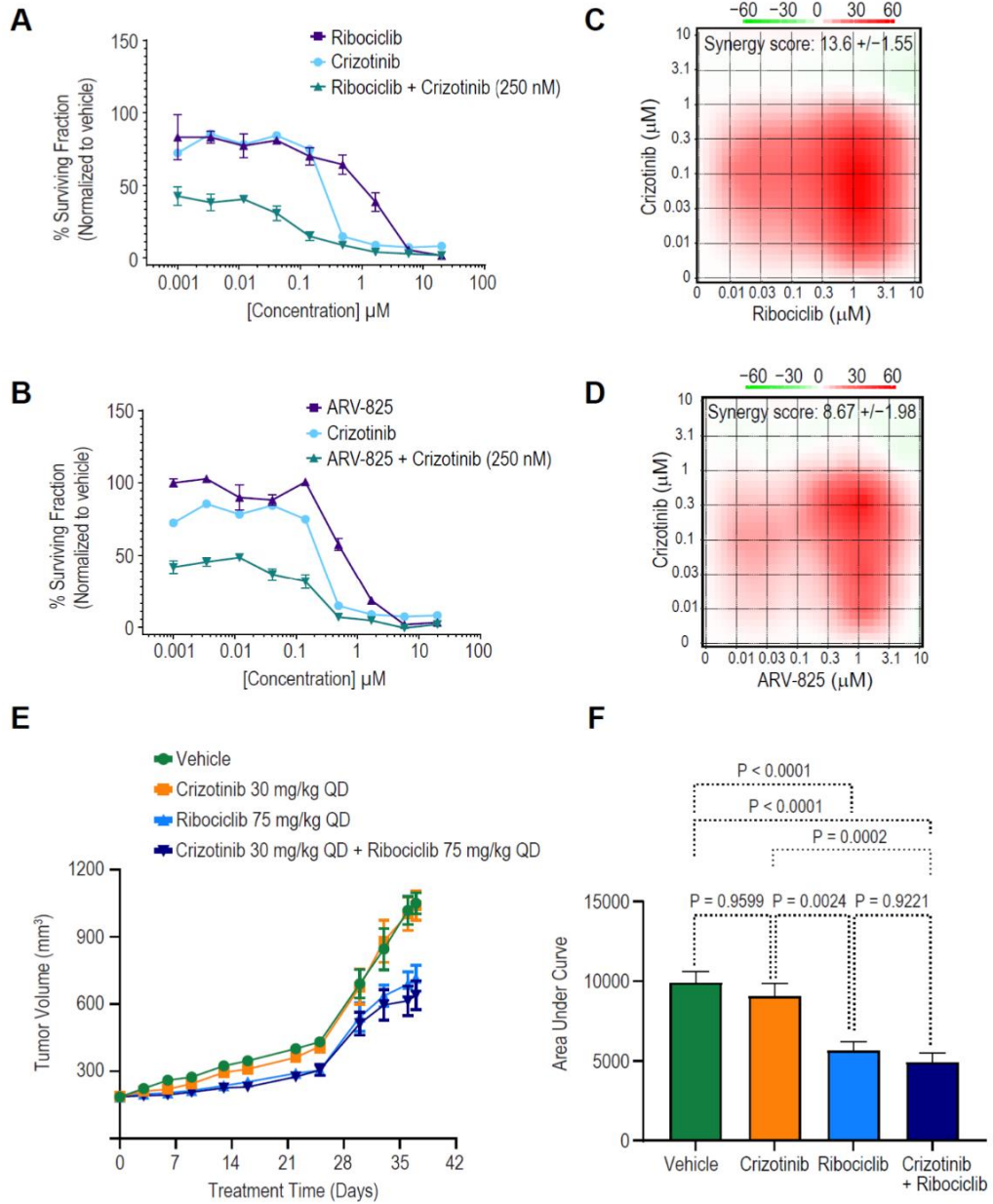


Figure 2.5. Combination treatment with CDK4/6 and BET bromodomain inhibitors and crizotinib effectively suppresses growth of LUAD-0006 cell and tumors in vitro and in vivo, respectively. **A.** Dose-response proliferation assay of LUAD-0006 cells after 10-day exposure to crizotinib, CDK4/6 inhibitor ribociclib, or ribociclib + 250 nM crizotinib. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3). **B.** Dose-response proliferation assay of LUAD-0006 cells after 10-day exposure to crizotinib, BET bromodomain inhibitor ARV-825, or ARV-825 + 250 nM crizotinib. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3). **C-D.** Synergy plots of 10-day treatment of LUAD-0006 cells with crizotinib in combination with ribociclib (**C**) and ARV-825 (**D**). Synergy analysis and results were generated with SynergyFinder and the zero interaction potency (ZIP) model was used to detect synergistic or antagonistic effects. The synergy scores summarize the average % response of the two drugs beyond expectation across all drug concentration combinations, where positive scores are likelier to be considered “synergistic” and negative scores are likelier to be considered “antagonistic.” **E.** Tumor volume. Animals bearing LUAD-0006 xenograft tumors were treated with vehicle, crizotinib, ribociclib or a combination of crizotinib and ribociclib. **F.** Area under curve (AUC) analysis. Adjusted P values for the indicated comparisons are shown above the bars. AUC values $\pm$ SEM are shown below the bars. There were five mice per group. Treatment commenced 12 days after implantation.

In addition, these data reveal statistically significant upregulation of multiple proteins associated with mitochondrial structure and function. These include those involved in respiratory chain function (MTCO1, UQCRC2), citric acid cycle and oxidative phosphorylation (SDHA), apoptosis inhibitors (BCL-XL and MCL1), metabolic processes and amino acid metabolism (ATP5A, SOD2, PARP, GLUD1), as well as mitochondrial translation and fusion (TUFM, TFAM, MFN1) (**Fig. 2.4A, B**; Supplementary Data 2.1). Mitochondrial mass measured using MitoTracker Green staining and flow cytometric analysis was modestly but significantly increased in LUAD-0006 cells that were chronically treated with crizotinib, thus corroborating that the significant upregulation of mitochondrial metabolic, translation and fusion proteins that was observed in the RPPA analysis translates into greater mitochondrial number and potentially enhanced function (Supplementary Fig. 2.S7B). To assess if these mitochondrial changes are druggable, we tested a panel of mitochondrial inhibitors that target various aspects of mitochondrial-related apoptosis and metabolism (Supplementary Data 2.2, Page 1). Interestingly, we found a significant degree of synergy between crizotinib and either telaglenastat (glutaminase inhibitor) or ND-646 (acetyl-CoA

carboxylase inhibitor) (236,237) (**Fig. 2.4C** and Supplementary Data 2.2), which suggests that the significant changes in mitochondrial mass and protein expression in LUAD-0006 treated long-term with ROS1-TKI can be targeted with these mitochondrial small molecule inhibitors.

Taken together, data shown in **Fig. 2.4**, Supplementary Fig. 2.S7 and Supplementary Data 2.1 and 2.2 demonstrate that chronic crizotinib treatment in LUAD-0006 cells results in widespread adaptive modulation of the homeostatic mitochondrial and metabolic, cell growth, survival, and cell cycle pathways that we postulate are permissive to cell proliferation and survival in the presence of the ROS1-TKI. Furthermore, the metabolic changes that occur with long-term TKI treatment in LUAD-0006 cells can be targeted with the use of targeted small molecule inhibitors of certain metabolic enzymes.

CDK4/6 and BET bromodomain inhibitors restore sensitivity of LUAD-0006 to crizotinib

As no robust pharmacological options currently exist to directly inhibit MYC, we used an unbiased small molecule inhibitor screen with a custom-curated library of 139 compounds to discover effective agents to inhibit LUAD-0006 cell viability. Interestingly, most compounds tested did not demonstrate an additive effect of reducing LUAD-0006 cell viability when combined with crizotinib for a treatment duration of 72 h (Supplementary Data 2.3), and we surmised that a longer duration of treatment may be required to observe a combination drug effect. We focused on testing the following classes of compounds: 1) CDK4/6 inhibitors to target genes upregulated by MYC to promote cell cycle progression via the G1 to S checkpoint and 2) BET bromodomain inhibitors to reduce transcription of MYC target genes and of MYC itself (238,239). Notably, RPPA data (**Fig. 2.4B and 2.4C**) show upregulation of proteins regulating G1 to S transition. We treated LUAD-0006 with a combination of crizotinib (250 nM, static dose) and varying concentrations of the FDA-

approved, CDK4/6 inhibitors ribociclib or palbociclib or the BET bromodomain inhibitors birabresib (OTX-015) or ARV-825 (240-243). After 10 days, we found that all 4 compounds, in concert with 250 nM crizotinib, reduced cell viability to a greater degree than any one inhibitor alone (**Fig. 2.5A-B**, Supplementary Fig. 2.S9A-B).

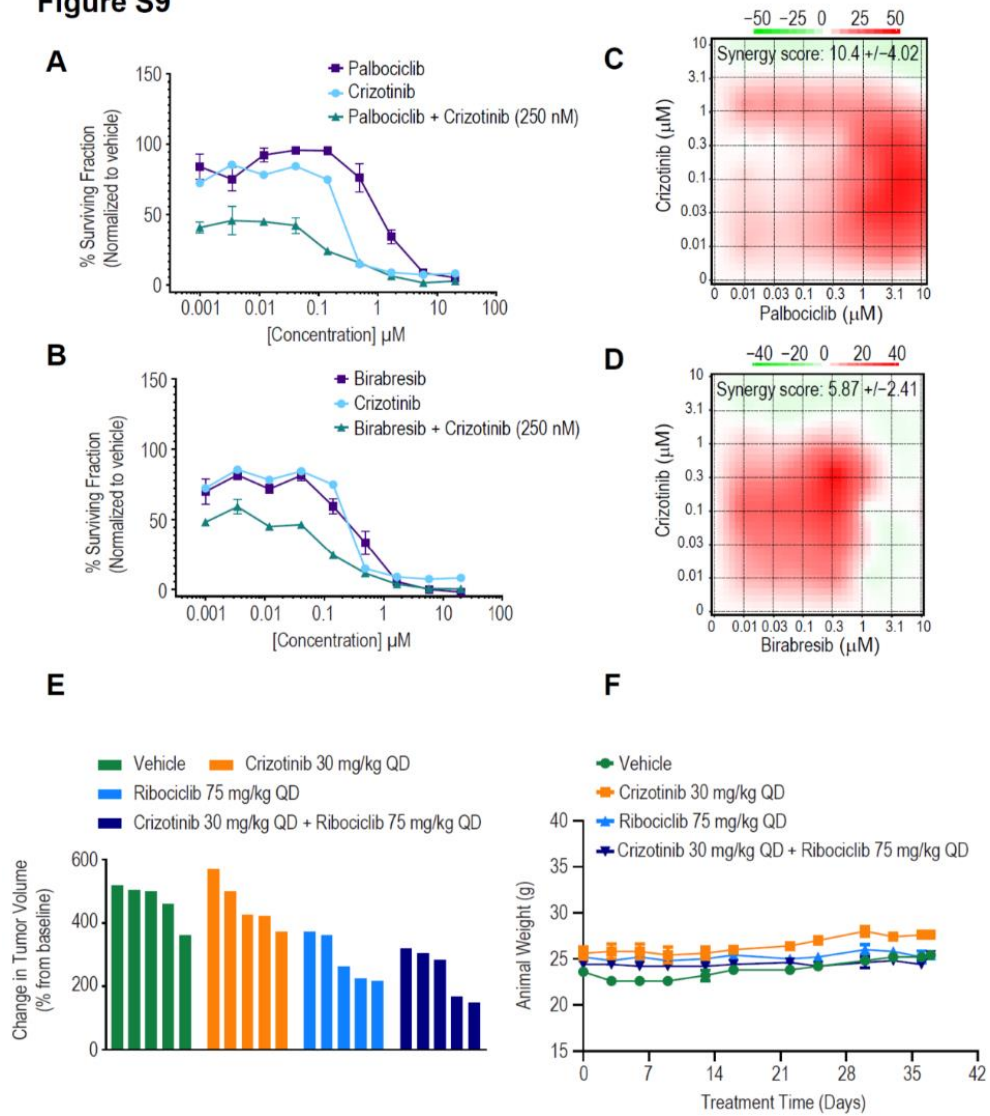
We next determined if combining CDK4/6 or BET bromodomain inhibitors with crizotinib reduced LUAD-0006 cell viability in a synergistic fashion. Analysis of combination inhibitor studies revealed that both CDK4/6 and BET bromodomain inhibitors exhibited synergistic interactions with crizotinib (**Fig. 2.5C-D**, Supplementary Fig. 2.S9C-D). Notably, the synergistic interactions for all compounds tested were evident even at sub-micromolar concentrations, which indicates that any synergy that we observed is unlikely to be driven by non-specific inhibitor protein interactions that can occur at higher drug concentrations. We also wanted to assess if any of the compounds synergistic with crizotinib decreased MYC protein levels. Ribociclib did not decrease MYC protein levels, which is expected as it targets CDK4/6, a downstream target of MYC; however, the BET inhibitor birabresib downregulated MYC protein relative to untreated cells (Supplementary Fig. 2.S8A). We also tested the CDK4/6 and BET bromodomain inhibitors in the HBEC-ER2 MYC^{T58A} cell model and crucially found that while HBEC-ER2 cells with MYC overexpression become resistant to ROS1-TKIs, they simultaneously become sensitized to both CDK4/6 and BET bromodomain inhibition; hence, these results concord with the LUAD-0006 inhibitor data in **Fig. 2.5** (Supplementary Fig. 2.S8B).

Finally, to determine if inhibition of CDK4/6 is an effective therapeutic strategy for lung tumors with *ROS1* fusion and *MYC* amplification, we treated mice bearing LUAD-0006 xenograft tumors as indicated in **Figures 2.5E-F** and Supplementary Figures 2.S9E-F. Treatment groups were compared using area under curve analysis (**Fig. 2.5F**). Crizotinib (30 mg/kg QD) caused a small but significant decrease in tumor volume compared to the vehicle-treated group (**Fig. 2.5E-F**);

however, the tumors continued to rapidly grow, which validated the crizotinib-resistance in vivo. Ribociclib (75 mg/kg QD) treatment resulted in a significantly larger decrease in tumor growth as compared to the vehicle- and crizotinib-treated groups (**Fig. 2.5E-F**). A combination of ribociclib (75 mg/kg QD) and crizotinib (30 mg/kg QD) also caused a significant reduction in growth of LUAD-0006 xenograft tumors (**Fig. 2.5E-F**). Although the combined treatment was more effective than crizotinib alone, it was not significantly different from single agent ribociclib (**Fig. 2.5E-F**). The change in volume of individual tumors is shown in Supplementary Figure 2.S9E. No treatment had an adverse effect on animal weight (Supplementary Figure 2.S9F).

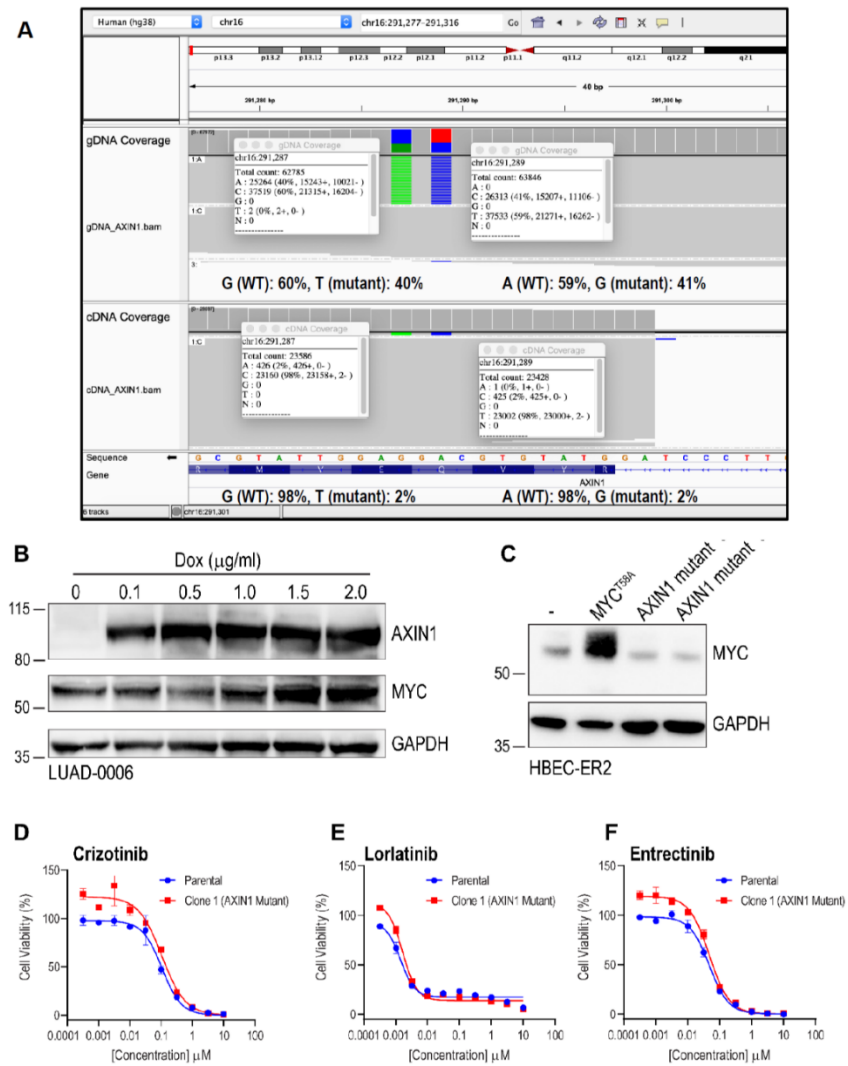
In conclusion, our in vitro data suggest that both CDK4/6 and BET bromodomain inhibition can resensitize ROS1-positive, MYC-overexpressed cells to crizotinib in a synergistic fashion and that CDK4/6 inhibition with ribociclib may offer an opportunity for disease control in the setting of crizotinib-resistance induced by MYC overexpression.

Figure S9



Supplementary Figure 2.9. Efficacy of CDK4/6 and BET bromodomain inhibitors in combination with crizotinib treatment in vivo and tolerability of CDK4/6 inhibitor ribociclib with crizotinib in vivo. **A.** Dose-response proliferation assay of LUAD-0006 cells after 10-day exposure to crizotinib, CDK4/6 inhibitor palbociclib, or palbociclib + 250 nM crizotinib. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3). **B.** Dose-response proliferation assay of LUAD-0006 cells after 10-day exposure to crizotinib, BET bromodomain inhibitor birabresib (OTX-015), or birabresib + 250 nM crizotinib. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3). **C-D.** Synergy plots of 10-day treatment of LUAD-0006 cells with crizotinib in combination with palbociclib (**C**) and birabresib (**D**). Synergy analysis and results were generated with SynergyFinder and the zero interaction potency (ZIP) model was used to detect synergistic or antagonistic effects. The synergy scores summarize the average % response of the two drugs beyond expectation across all drug concentration combinations, where positive scores are likelier to be considered “synergistic” and negative scores are likelier to be considered “antagonistic”. **E.** Change in volume (%) of individual tumors at the end of the study (day 37 of treatment). **F.** Animal weight. There was no significant change in animal weight in any of the experimental groups.

Figure S10



Supplementary Figure 2.10. The AXIN1 mutation in LUAD-0006 does not contribute to ROS1-TKI resistance. **A.** IGV diagram of aligned Amplicon-seq reads around the early part of exon 9 of AXIN1 on chromosome 16, where gDNA reads are on top, cDNA reads are in the middle, and reference sequence is on the bottom. Reference sequence shown is the reverse strand of the chromosome and sense strand of the AXIN1 gene. Text boxes in IGV specify anti-sense nucleotides while the bolded black text boxes specify the corresponding sense nucleotides and also specify WT and mutant nucleotides. **B.** Immunoblot of LUAD-0006 cells transduced with a doxycycline-inducible wild-type AXIN1 vector treated with varying concentrations of doxycycline to assess AXIN1, MYC, and GAPDH protein expression. This shows that increase in >2-fold increase AXIN1 protein expression does not reduce MYC protein expression and rather may increase it. **C.** Immunoblot of HBEC EZR-ROS1 #2 (ER2) cells either unchanged, transduced to overexpress MYC (MYC^{T58A}), or engineered with CRISPR-Cas9 to express a heterozygous premature stop codon in exon 9 of the AXIN1 gene (similar to the variant seen in LUAD-0006). This shows that reproduction of the AXIN1 mutant from LUAD-0006 in HBEC-ER2 cells does not affect MYC protein expression. **D-F.** Dose-response proliferation assays of HBEC-ER2 cells after 72-hour exposure to the ROS1-TKIs crizotinib (**D**), lorlatinib (**E**), and entrectinib (**F**). Cells genetically engineered to express heterozygous AXIN1 exon 9 premature stop codon (HBEC-ER2 AXIN1 Clone 1) retain similar sensitivity to ROS1-TKI treatment relative to parent HBEC-ER2 cells. Data are normalized to vehicle-treated control and values shown are the mean $\pm$ SEM (n = 3).

Discussion

Tyrosine kinase inhibitors (TKIs) such as crizotinib have demonstrated durability and effectiveness in targeting ROS1-driven non-small-cell lung cancer (NSCLC). However, therapeutic resistance adversely affects long-term outcomes in a significant number of patients. ROS1-TKI resistance falls into two categories: ROS1-dependent resistance, which consists of kinase domain point mutations that prevent competitive or non-competitive TKI binding, and ROS1-independent resistance that consists of mutations or rearrangements involving alternative proto-oncogenes. While much is now known about ROS1 kinase domain resistant mutations, ROS1-independent resistance is relatively understudied (210). Recent studies investigating ROS1-independent resistance mechanisms to ROS1-TKIs discovered alterations either in other receptor tyrosine kinases (RTKs) or in downstream effector pathways such as RAS-RAF-MEK-ERK. RTKs linked to ROS1-independent resistance include MET (copy number amplification), EGFR (pathway activation), and KIT (activating mutation) (178,184,185). Downstream mediators linked to ROS1-independent resistance include, NF1 (loss-of-function mutation), BRAF (V600E), MEK (activating deletion), and MAPK (pathway activation) (187,217). A recent study by Lin et al. that compiled molecular data from MSK-IMPACT and Dana Farber of ROS1-positive tumors resistant to crizotinib and/or lorlatinib found many other alterations that may mediate ROS1-independent resistance (206). This included mutations in *TSC1/2*, *SMAD4*, *CTNNB1*, *APC*, *NOTCH1*, and *PTCH1* and alterations in a variety of cell-cycle control genes such as *CDKN2A*, *CCNE1*, *ATM*, and *BRCA1/2* (206). Notably though, 19.2% (5/26) of all lorlatinib-resistant tumors contained copy-number amplification (CNA) of the proto-oncogenic, transcription factor *MYC* (206) (Supplementary Figure 2.S1). However, the role of any of these alterations in driving resistance to ROS1 TKI was not explored.

Our work adds to the understanding of ROS1-TKI resistance mechanisms by demonstrating that overexpression of the proto-oncogenic transcription factor MYC enables tumor cells to evade TKI treatment despite MYC not being directly related to canonical ROS1 signaling. Importantly, our work also establishes that overexpression of MYC can confer resistance to any generation of ROS1-TKI and to both type I and type II binding mode inhibitors, emphasizing the need to develop combination therapeutic strategies to prolong the duration of TKI response in clinic. Here we employed a ROS1-TKI resistant patient-derived xenograft cell line (LUAD-0006) from an *EZR-ROS1* fusion-positive NSCLC sample with concurrent MYC CNA (**Fig. 2.1-2**, Supplementary Figure 2.S1B-S2). Leveraging the fact that there were few concurrent alterations alongside the MYC CNA that increased by ~40% after patient received crizotinib therapy, we hypothesized that the MYC CNA drives MYC overexpression to cause adaptive resistance to ROS1-TKIs (Supplementary Fig. 2.S1). By using functional genomic and pharmacological approaches to reduce MYC levels in LUAD-0006 patient-derived cells, we establish that MYC overexpression induces ROS1-TKI resistance and reciprocally, loss of MYC re-sensitizes cells to ROS1-TKI inhibition (**Fig. 2.2**). For complementary validation studies, we also overexpressed MYC in two previously described ROS1-TKI-sensitive human broncho-epithelial cell lines that were engineered to harbor the endogenous *EZR-ROS1* fusion oncogene (HBEC-ER) and an established ROS1-positive NSCLC cell line (HCC78) (**Fig. 2.3** and Supplementary Fig. 2.S6) (200,217). Interestingly, we could only achieve robust overexpression of MYC using the mutant MYC^{T58A} variant; wildtype MYC expression was insufficient to approach the levels of MYC observed in LUAD-0006 cells. MYC^{T58A} expression results in highly abundant levels of the protein as compared to wildtype MYC as this phosphorylation defective mutant is unable to undergo ubiquitination and proteasomal degradation (230,231). Overexpression with MYC^{T58A} more closely recapitulated the MYC protein levels observed in

LUAD-0006 and was comparable with a previously characterized MYC-amplified NSCLC cell line, H1792 (233) (**Fig. 2.3A** and Supplementary Fig. 2.S4). The role of MYC in conferring ROS1-TKI resistance is further strengthened with the observation that the differing degree of MYC overexpression in HBEC-ER1 and ER2 correlated to the extent of ROS1-TKI resistance in these cell lines. The higher MYC expressing HBEC-ER2 cells exhibited a greater degree of resistance compared to those measured in ER1 cells (**Fig. 2.3E-F** and Supplementary Fig. 2.S3C-B). Overexpression of MYC in a well-characterized ROS1-fusion NSCLC cell line, HCC78, further validated the ability of ultra-high MYC protein expression to confer resistance to ROS1-TKIs (200). Future studies are required to assess if a certain threshold or magnitude of MYC overexpression is required to cause ROS1-TKI resistance. Ultimately, we postulate that overexpression of MYC leads to wider promoter and enhancer engagement that activates an additional subset of genes to promote sustained oncogenic transformation.

The homeostatic half-life of MYC protein is relatively short (~30 minutes), is intricately governed by various cell signaling pathways, and has been shown to involve a destruction complex that includes the scaffolding protein, Axin1 (231,244-246). While expression of degradation-resistant MYC^{T58A} significantly increased MYC protein levels relative to wildtype MYC, it was still significantly less than that observed in LUAD-0006. Intriguingly, the tumor specimen that LUAD-0006 cell line is derived from, also harbors a heterozygous Axin1 mutation (Q732_733delinsR*) that results in a premature stop codon in exon 9. We extensively interrogated the functional impact of Axin1 Q732_733delinsR* mutant using multiple approaches. However, our results, including amplicon sequencing of AXIN1 mRNA generated from these cells confirmed that this variant is not expressed, possibly due to nonsense-mediated decay of the mRNA (Supplementary Fig. 2.S10A). Furthermore, >2-fold overexpression of wild-type Axin1 protein in LUAD-0006 did not reduce MYC levels (Supplementary Fig. 2.S9B), and

reproduction of the heterozygous Axin1 mutation in HBEC-ER2 using CRISPR-Cas9 genome editing approach also did not affect MYC protein levels or TKI sensitivity (Supplementary Fig. 2.S10C-F and Supplementary Data 2.4). Thus, the Axin1 Q732_733delinsR* variant is likely not expressed at the protein level and does not have functional impact on MYC protein stability. Ultimately, it is possible that variants in other genes that are not picked up by MSK-IMPACT sequencing cooperate with the genomic *MYC* CNA to result in the high MYC expression observed in LUAD-0006. Thus, future studies will provide opportunities to better elucidate the spectrum of alterations both intrinsic and extrinsic to *MYC* that can cause its overexpression.

MYC has been investigated as a mediator of TKI resistance in cancers driven by other receptor tyrosine kinases. Most of these studies focused on resistant phenotypes mediated by an unaltered *MYC* gene, where the RTK-fusion oncogene itself, TKI treatment, dysregulated miRNAs or other concurrent alterations subsequently induced MYC upregulation that in turn decreased TKI sensitivity (247-249). Fewer studies have interrogated the role of CNA or other alterations of the *MYC* gene as a direct cause of resistance to TKI treatment. Clinical studies of EGFR-mediated NSCLC and a case report of ALK-fusion positive NSCLC identified *MYC* CNA as a potential mechanism of resistance; however, since only limited functional studies were performed therein, the causative role of *MYC* CNA as a mechanism of therapeutic resistance (primary or secondary) remained to be established (250,251). Hence, our findings in ROS1-fusion driven NSCLC definitively establish that *MYC* CNA drives forms of adaptive resistance, likely in different RTK-mediated cancers, and thus strategies targeting MYC alongside or sequential to TKI therapy may benefit a wide spectrum of patients.

Pharmacological inhibition of MYC activity currently represents a formidable bedside challenge, as there are no FDA approved or clinically validated direct inhibitors of MYC. Consequently, pre-clinical approaches have focused indirectly targeting MYC expression,

stability or downstream signaling. One strategy involves inhibiting cell cycle proteins that promote mitosis which are transcriptionally upregulated by MYC. Interestingly, analysis of our RPPA data showed a statistically significant increase in BRD4 and 53BP1 (**Fig. 2.4B** and Supplementary Fig. 2.S7A) that concords with ongoing mitosis in the presence of crizotinib by increasing G1 to S activity. Furthermore, multiple studies have established the link between MYC and cell cycle kinases like CDK4 and 6 that accelerate the transition from G1 to S (252,253). In our study, we demonstrated that the CDK4/6 inhibitors palbociclib and ribociclib act synergistically with crizotinib to robustly inhibit LUAD-0006 cell growth (**Fig. 2.5A and 2.5C**, Supplementary Fig. 2.S9A and 2.S9C). We also showed that HBEC-ER2 cells are more sensitive to CDK4/6 inhibitors when MYC is overexpressed (Supplementary Fig. 2.S8B). These data therefore suggest that cell cycle control represents one avenue by which to restrain MYC-mediated TKI resistance. Another approach to targeting MYC involves the use of BET bromodomain inhibitors, such as birabresib (OTX-015), JQ1, and their corresponding proteolysis targeting chimeras (PROTACs) ARV-825 and dBET6 (242,243,254,255). These inhibitors target proteins that recognize acetylated histones and recruit transcription factors and coactivators to promote gene expression (256). Several studies have shown the benefit of attenuating MYC directly or MYC target genes with the use of these compounds (239,257). Consistent with these previous findings, here we establish that in *MYC*-amplified ROS1 driven NSCLC, BET bromodomain inhibition (birabresib and ARV-825) synergistically acts with crizotinib to suppress cell growth (**Fig. 2.5B and 2.5D**; Supplementary Fig. 2.S9B and 2.S9D). Ultimately, our data suggest that CDK4/6 inhibition and BET bromodomain inhibition are worth further consideration in targeting TKI resistance mediated by MYC overexpression in ROS1-positive NSCLC. Other inhibitors that target MYC in the context of the cell cycle, such as CDK9 inhibitors, may also be worth further investigation.

Finally, we also show that small-molecule compounds that target metabolic enzymes can also be utilized in combating ROS1-TKI resistance. Specifically, the use of either glutaminase or acetyl-CoA carboxylase inhibition re-sensitized LUAD-0006 cells to crizotinib, leveraging the signaling changes that occur when these cells are able to survive long-term TKI treatment (**Fig. 2.4**, Supplementary Fig. 2.S7 and Supplementary Data 2.1 and 2.2). More studies will need to be performed to ascertain the effect of the MYC overexpression versus the loss of constitutive RTK signaling in allowing for the increased glutamine metabolism and fatty acid synthesis observed in the LUAD-0006 cells. Ultimately, such studies would provide greater insight into the degree that metabolism plays in ROS1-TKI resistance and may offer new approaches to combat a wide range of ROS1-independent resistance mechanisms.

Acknowledgements

We would like to thank the following colleagues: Drs. Steve Kurtz and Jeffrey Tyner for contributing the small-molecule inhibitor panel; Drs. Jacob Henderson and Sunil Joshi for resource sharing; Karina Ray, Dr. Suzanne Fei, Dr. Lina Gao, and Dr. Jessica Minnier for bioinformatics support; and Dr. Marilyne Labrie for help with RPPA analysis. This project was supported with funding from the National Cancer Institute of the National Institutes of Health (NRSA: F30CA247253, R01CA233495), OHSU Doernbecher Children's Hospital Foundation, and Memorial Sloan Kettering Cancer Center Support Grant (National Cancer Institute of the National Institutes of Health P30 CA008748).

Appendix

Raw data from reverse phase protein array (RPPA) experiment

Statistics of RPPA experiment for each antibody tested

Protein Name	Discovery?	P value	Mean of Chronic Crizotinib	Mean of DMSO	Difference	SE of difference	t ratio	df	q value	-log10(q)
MLKL	Yes	3.00E-06	0.303	1.022	-0.719	0.019	37.740	4.000	0.000	3.623
RIP3	Yes	4.00E-06	0.526	1.034	-0.507	0.015	34.290	4.000	0.000	3.587
c-Jun_pS73	Yes	8.00E-06	2.509	0.968	1.541	0.053	29.150	4.000	0.000	3.587
p70-S6K_pT389	Yes	8.00E-06	0.525	1.170	-0.644	0.022	29.370	4.000	0.000	3.587
TFAM	Yes	1.00E-05	1.930	0.992	0.938	0.034	27.520	4.000	0.000	3.587
FOXO1	Yes	1.20E-05	0.367	1.049	-0.682	0.026	26.360	4.000	0.000	3.587
Lasu1	Yes	1.20E-05	0.790	0.991	-0.201	0.008	26.400	4.000	0.000	3.587
ASNS	Yes	1.50E-05	0.280	1.053	-0.773	0.031	25.060	4.000	0.000	3.587
C-Raf	Yes	1.50E-05	1.588	0.930	0.658	0.026	24.970	4.000	0.000	3.587
RBM15	Yes	1.50E-05	2.285	0.878	1.407	0.056	25.270	4.000	0.000	3.587
MEK1_p_S217_S221	Yes	1.60E-05	0.513	1.025	-0.512	0.021	24.610	4.000	0.000	3.587
eEF2K	Yes	1.80E-05	0.278	1.046	-0.768	0.032	24.100	4.000	0.000	3.587
Lyn	Yes	1.80E-05	1.920	0.918	1.002	0.042	23.860	4.000	0.000	3.587
YAP	Yes	1.80E-05	0.264	1.047	-0.783	0.033	23.830	4.000	0.000	3.587
Mitofusin-1	Yes	1.90E-05	4.074	0.949	3.125	0.133	23.480	4.000	0.000	3.587
PMS2	Yes	2.00E-05	0.380	0.984	-0.605	0.026	23.360	4.000	0.000	3.587
Mnk1	Yes	2.20E-05	0.524	1.023	-0.499	0.022	22.670	4.000	0.000	3.587
BiP-GRP78	Yes	2.30E-05	0.517	1.164	-0.647	0.029	22.590	4.000	0.000	3.587
PKM2	Yes	2.30E-05	0.257	1.036	-0.779	0.034	22.620	4.000	0.000	3.587
PAX8	Yes	2.40E-05	0.430	1.046	-0.616	0.028	22.400	4.000	0.000	3.587
PERK	Yes	2.90E-05	3.007	0.950	2.057	0.097	21.310	4.000	0.000	3.520
XIAP	Yes	3.30E-05	0.484	1.049	-0.565	0.028	20.510	4.000	0.000	3.472
TRAP1	Yes	3.50E-05	2.562	0.947	1.615	0.080	20.210	4.000	0.000	3.465

SOD2	Yes	3.70E-05	2.567	0.912	1.655	0.083	20.020	4.000	0.000	3.465
53BP1	Yes	3.80E-05	2.034	0.953	1.081	0.055	19.820	4.000	0.000	3.465
Gys	Yes	4.30E-05	0.518	1.051	-0.533	0.028	19.190	4.000	0.000	3.461
Patched	Yes	4.30E-05	1.321	0.921	0.400	0.021	19.200	4.000	0.000	3.461
PKC-b-II_pS660	Yes	4.40E-05	1.796	0.950	0.847	0.044	19.160	4.000	0.000	3.461
p70-S6K1	Yes	4.50E-05	0.330	1.018	-0.688	0.036	19.040	4.000	0.000	3.461
FoxO3a_pS318_S321	Yes	4.60E-05	0.595	1.014	-0.419	0.022	18.950	4.000	0.000	3.461
Bcl-xL	Yes	4.80E-05	1.612	0.970	0.642	0.034	18.730	4.000	0.000	3.453
ARID1A	Yes	5.20E-05	0.481	1.009	-0.527	0.029	18.350	4.000	0.000	3.432
p38-MAPK	Yes	5.50E-05	0.220	1.028	-0.808	0.045	18.100	4.000	0.000	3.426
IRS2	Yes	5.70E-05	0.701	1.028	-0.328	0.018	17.910	4.000	0.000	3.426
eEF2	Yes	5.90E-05	0.371	1.036	-0.666	0.037	17.790	4.000	0.000	3.426
NDUFB4	Yes	5.90E-05	0.444	1.029	-0.585	0.033	17.790	4.000	0.000	3.426
CA9	Yes	6.00E-05	2.631	0.936	1.695	0.096	17.660	4.000	0.000	3.425
Tuberin_pT1462	Yes	6.20E-05	0.435	1.045	-0.610	0.035	17.520	4.000	0.000	3.423
FAK	Yes	7.20E-05	0.660	1.172	-0.512	0.030	16.870	4.000	0.000	3.370
Caspase-3-cleaved	Yes	7.40E-05	1.445	0.960	0.485	0.029	16.780	4.000	0.000	3.370
PDK1	Yes	7.70E-05	0.402	1.039	-0.637	0.038	16.590	4.000	0.000	3.361
PAI-1	Yes	8.20E-05	0.348	1.041	-0.693	0.042	16.320	4.000	0.000	3.343
p44-42-MAPK	Yes	8.60E-05	0.408	1.026	-0.619	0.038	16.160	4.000	0.000	3.334
CD44	Yes	9.20E-05	0.494	0.993	-0.500	0.031	15.870	4.000	0.000	3.313
Stat3_pY705	Yes	9.70E-05	0.331	1.062	-0.731	0.047	15.670	4.000	0.001	3.301
HSP60	Yes	1.08E-04	3.369	0.960	2.409	0.158	15.250	4.000	0.001	3.266
PLC-gamma1_pS1248	Yes	1.10E-04	0.343	1.141	-0.798	0.053	15.190	4.000	0.001	3.266
PEA-15_pS116	Yes	1.21E-04	0.535	1.059	-0.524	0.035	14.800	4.000	0.001	3.240
LDHA	Yes	1.23E-04	0.531	1.052	-0.521	0.035	14.740	4.000	0.001	3.240
Myosin-IIa	Yes	1.23E-04	0.327	1.099	-0.772	0.052	14.740	4.000	0.001	3.240
p53	Yes	1.32E-04	0.674	1.163	-0.489	0.034	14.490	4.000	0.001	3.219
VASP	Yes	1.38E-04	0.473	1.064	-0.591	0.041	14.340	4.000	0.001	3.210
IGFRb	Yes	1.40E-04	2.120	0.980	1.139	0.080	14.280	4.000	0.001	3.210
Smac	Yes	1.46E-04	2.466	0.934	1.532	0.108	14.120	4.000	0.001	3.199
GCLC	Yes	1.54E-04	0.551	1.090	-0.539	0.039	13.920	4.000	0.001	3.182
Smad4	Yes	1.62E-04	0.255	1.031	-0.776	0.056	13.740	4.000	0.001	3.173
Rb	Yes	1.67E-04	0.279	1.030	-0.751	0.055	13.650	4.000	0.001	3.173
U-Histone-H2B	Yes	1.68E-04	0.479	1.082	-0.603	0.044	13.620	4.000	0.001	3.173
TIGAR	Yes	1.69E-04	0.739	1.047	-0.308	0.023	13.600	4.000	0.001	3.173
Cdc42	Yes	1.72E-04	0.861	1.005	-0.144	0.011	13.550	4.000	0.001	3.173
TRIM25	Yes	2.08E-04	0.381	1.111	-0.730	0.057	12.900	4.000	0.001	3.097
ATP5A	Yes	2.12E-04	3.328	0.916	2.412	0.188	12.840	4.000	0.001	3.097
Rictor_pT1135	Yes	2.21E-04	0.341	1.051	-0.710	0.056	12.700	4.000	0.001	3.097

Sox17	Yes	2.23E-04	1.354	0.981	0.373	0.029	12.680	4.000	0.001	3.097
IR-b	Yes	2.26E-04	2.712	0.958	1.754	0.139	12.640	4.000	0.001	3.097
b-Actin	Yes	2.27E-04	1.797	0.912	0.885	0.070	12.620	4.000	0.001	3.097
PTEN	Yes	2.28E-04	0.411	1.037	-0.626	0.050	12.610	4.000	0.001	3.097
NAPSIN-A	Yes	2.32E-04	0.482	1.012	-0.530	0.042	12.550	4.000	0.001	3.095
UQCRC2	Yes	2.41E-04	3.016	0.988	2.029	0.163	12.430	4.000	0.001	3.085
Cyclin-D3	Yes	2.55E-04	0.350	1.071	-0.721	0.059	12.250	4.000	0.001	3.068
PARP	Yes	2.58E-04	1.338	1.000	0.338	0.028	12.220	4.000	0.001	3.068
DRP1	Yes	2.63E-04	0.389	0.985	-0.596	0.049	12.150	4.000	0.001	3.064
E-Cadherin	Yes	2.97E-04	1.379	0.960	0.418	0.036	11.780	4.000	0.001	3.017
ACVRL1	Yes	3.14E-04	0.880	1.024	-0.145	0.012	11.610	4.000	0.001	3.003
TUFM	Yes	3.15E-04	1.354	0.931	0.423	0.036	11.610	4.000	0.001	3.003
Vinculin	Yes	3.30E-04	0.380	1.088	-0.708	0.062	11.470	4.000	0.001	2.988
Lck	Yes	3.43E-04	2.220	0.957	1.263	0.111	11.350	4.000	0.001	2.982
Atg5	Yes	3.44E-04	0.344	1.045	-0.700	0.062	11.350	4.000	0.001	2.982
DNA-Ligase-IV	Yes	3.62E-04	2.011	0.924	1.087	0.097	11.200	4.000	0.001	2.978
Rab25	Yes	3.62E-04	1.315	0.929	0.385	0.034	11.200	4.000	0.001	2.978
MSH6	Yes	3.64E-04	0.355	1.039	-0.684	0.061	11.180	4.000	0.001	2.978
mTOR	Yes	3.66E-04	0.504	1.191	-0.687	0.062	11.170	4.000	0.001	2.978
A-Raf	Yes	3.69E-04	1.784	0.774	1.009	0.091	11.140	4.000	0.001	2.978
Glutamate-D1-2	Yes	3.79E-04	1.710	0.831	0.879	0.079	11.070	4.000	0.001	2.971
RSK1	Yes	3.93E-04	0.410	1.030	-0.620	0.057	10.970	4.000	0.001	2.962
MRAP	Yes	3.96E-04	1.544	0.988	0.556	0.051	10.940	4.000	0.001	2.962
PDH	Yes	4.11E-04	2.075	0.961	1.114	0.103	10.840	4.000	0.001	2.957
PI3K-p110-a	Yes	4.14E-04	0.739	1.002	-0.263	0.024	10.820	4.000	0.001	2.957
PLC-gamma2_pY759	Yes	4.14E-04	1.305	0.947	0.358	0.033	10.820	4.000	0.001	2.957
MSH2	Yes	4.20E-04	0.641	1.016	-0.376	0.035	10.780	4.000	0.001	2.956
Rictor	Yes	4.30E-04	0.703	1.089	-0.386	0.036	10.710	4.000	0.001	2.950
Gys_pS641	Yes	4.36E-04	0.357	1.057	-0.700	0.066	10.670	4.000	0.001	2.950
FABP5	Yes	4.40E-04	1.148	0.926	0.223	0.021	10.650	4.000	0.001	2.950
XPA	Yes	4.47E-04	0.356	1.097	-0.741	0.070	10.610	4.000	0.001	2.948
Cyclin-B1	Yes	4.56E-04	0.369	1.036	-0.667	0.063	10.550	4.000	0.001	2.943
eIF4G	Yes	5.29E-04	0.417	1.061	-0.645	0.063	10.160	4.000	0.001	2.885
Akt2	Yes	5.32E-04	0.395	0.993	-0.598	0.059	10.140	4.000	0.001	2.885
RSK	Yes	5.55E-04	0.345	1.043	-0.698	0.070	10.030	4.000	0.001	2.871
E2F1	Yes	5.69E-04	1.317	0.955	0.362	0.036	9.966	4.000	0.001	2.864
Cdc6	Yes	6.03E-04	0.530	1.076	-0.546	0.056	9.821	4.000	0.001	2.844
EphA2	Yes	6.25E-04	1.727	0.998	0.729	0.075	9.729	4.000	0.001	2.839
FASN	Yes	6.27E-04	0.379	1.053	-0.673	0.069	9.722	4.000	0.001	2.839
AMPKa	Yes	6.28E-04	0.559	1.007	-0.448	0.046	9.718	4.000	0.001	2.839

HMHA1	Yes	6.46E-04	0.649	1.385	-0.736	0.076	9.646	4.000	0.001	2.830
Hexokinase-II	Yes	6.86E-04	1.253	0.966	0.287	0.030	9.499	4.000	0.002	2.812
RIP	Yes	6.86E-04	1.371	0.997	0.374	0.039	9.498	4.000	0.002	2.812
TSC1	Yes	7.05E-04	0.559	1.036	-0.477	0.051	9.430	4.000	0.002	2.805
HER2	Yes	7.11E-04	1.400	1.004	0.396	0.042	9.409	4.000	0.002	2.805
PAK4	Yes	7.18E-04	0.503	1.067	-0.564	0.060	9.386	4.000	0.002	2.804
GSK-3B	Yes	7.82E-04	0.309	1.064	-0.755	0.082	9.180	4.000	0.002	2.773
RRM2	Yes	7.93E-04	0.521	1.087	-0.566	0.062	9.145	4.000	0.002	2.773
Gab2	Yes	7.96E-04	0.709	1.024	-0.315	0.035	9.138	4.000	0.002	2.773
Annexin-VII	Yes	8.00E-04	0.686	1.159	-0.473	0.052	9.124	4.000	0.002	2.773
PD-1	Yes	8.84E-04	1.299	0.968	0.330	0.037	8.892	4.000	0.002	2.733
Collagen-VI	Yes	9.29E-04	1.548	0.993	0.555	0.063	8.778	4.000	0.002	2.716
COG3	Yes	9.63E-04	2.478	0.956	1.522	0.175	8.696	4.000	0.002	2.704
VAV1	Yes	1.02E-03	1.388	0.873	0.515	0.060	8.561	4.000	0.002	2.681
MEK2	Yes	1.04E-03	0.753	1.040	-0.287	0.034	8.522	4.000	0.002	2.680
PTPN12	Yes	1.04E-03	0.621	1.077	-0.455	0.053	8.518	4.000	0.002	2.680
ATP5H	Yes	1.08E-03	1.325	0.970	0.355	0.042	8.443	4.000	0.002	2.669
Akt2_pS474	Yes	1.10E-03	0.226	1.108	-0.882	0.105	8.393	4.000	0.002	2.663
Cox-IV	Yes	1.12E-03	0.825	1.050	-0.225	0.027	8.353	4.000	0.002	2.658
A-Raf_pS299	Yes	1.16E-03	0.522	1.062	-0.539	0.065	8.289	4.000	0.002	2.649
VEGFR-2_pY1175	Yes	1.18E-03	0.639	1.029	-0.391	0.047	8.253	4.000	0.002	2.646
eIF4E_pS209	Yes	1.18E-03	0.435	1.075	-0.640	0.078	8.239	4.000	0.002	2.646
SLC1A5	Yes	1.22E-03	2.614	0.970	1.645	0.201	8.179	4.000	0.002	2.637
S6_pS235_S236	Yes	1.23E-03	0.456	2.314	-1.858	0.228	8.163	4.000	0.002	2.637
S100A4	Yes	1.28E-03	1.177	0.960	0.217	0.027	8.067	4.000	0.002	2.623
p38-MAPK-pT180_Y182	Yes	1.29E-03	0.342	1.085	-0.743	0.092	8.059	4.000	0.002	2.623
DNMT1	Yes	1.33E-03	0.604	1.022	-0.418	0.052	7.988	4.000	0.002	2.617
GAPDH	Yes	1.33E-03	1.181	0.930	0.251	0.031	7.988	4.000	0.002	2.617
PAX6	Yes	1.33E-03	1.208	0.918	0.290	0.036	7.986	4.000	0.002	2.617
ATM_pS1981	Yes	1.39E-03	0.651	1.021	-0.370	0.047	7.893	4.000	0.002	2.602
MMP14	Yes	1.41E-03	1.259	0.911	0.347	0.044	7.870	4.000	0.002	2.602
MERIT40_pS29	Yes	1.41E-03	0.719	1.003	-0.284	0.036	7.866	4.000	0.002	2.602
mTOR_pS2448	Yes	1.54E-03	0.570	1.066	-0.496	0.065	7.687	4.000	0.003	2.568
MTCO1	Yes	1.57E-03	6.720	0.800	5.919	0.774	7.647	4.000	0.003	2.562
RRM1	Yes	1.73E-03	0.865	1.008	-0.144	0.019	7.453	4.000	0.003	2.523
FAK_pY397	Yes	1.76E-03	1.240	0.976	0.264	0.036	7.424	4.000	0.003	2.520
MCT4	Yes	1.78E-03	1.622	0.858	0.764	0.103	7.401	4.000	0.003	2.518
GSK-3a-b	Yes	1.80E-03	0.749	1.042	-0.293	0.040	7.373	4.000	0.003	2.514
NF-kB-p65_pS536	Yes	1.87E-03	1.159	0.939	0.220	0.030	7.307	4.000	0.003	2.503
PLK1	Yes	1.88E-03	0.509	1.049	-0.540	0.074	7.294	4.000	0.003	2.503

BRD4	Yes	1.90E-03	1.634	1.020	0.614	0.084	7.277	4.000	0.003	2.502
PDK1_pS241	Yes	2.01E-03	0.757	1.004	-0.247	0.034	7.161	4.000	0.003	2.479
Paxillin	Yes	2.08E-03	1.261	0.974	0.286	0.040	7.099	4.000	0.003	2.468
Wee1_pS642	Yes	2.11E-03	0.533	1.017	-0.484	0.068	7.071	4.000	0.003	2.466
PEA-15	Yes	2.12E-03	0.819	0.999	-0.180	0.026	7.065	4.000	0.003	2.466
S6	Yes	2.13E-03	0.563	2.298	-1.735	0.246	7.055	4.000	0.003	2.466
ACSL1	Yes	2.28E-03	1.331	1.001	0.330	0.048	6.925	4.000	0.004	2.439
Aurora-ABC_pT288_pT232_pT198	Yes	2.41E-03	0.833	0.961	-0.128	0.019	6.829	4.000	0.004	2.419
Coup-TFII	Yes	2.45E-03	4.395	0.980	3.415	0.503	6.796	4.000	0.004	2.414
SCD	Yes	2.47E-03	1.677	0.876	0.801	0.118	6.782	4.000	0.004	2.413
TRIP13	Yes	2.50E-03	0.864	1.022	-0.158	0.023	6.760	4.000	0.004	2.411
c-IAP2	Yes	2.85E-03	0.891	1.030	-0.138	0.021	6.522	4.000	0.004	2.356
Beclin	Yes	2.97E-03	2.310	0.886	1.424	0.221	6.452	4.000	0.005	2.341
S6_pS240_S244	Yes	3.08E-03	0.588	1.131	-0.543	0.085	6.391	4.000	0.005	2.332
Chk1_pS345	Yes	3.09E-03	1.603	0.928	0.675	0.106	6.383	4.000	0.005	2.332
cdc25C	Yes	3.09E-03	0.464	1.137	-0.672	0.105	6.382	4.000	0.005	2.332
MLH1	Yes	3.11E-03	0.626	1.046	-0.420	0.066	6.371	4.000	0.005	2.332
PR	Yes	3.19E-03	0.736	1.028	-0.292	0.046	6.328	4.000	0.005	2.323
Bak	Yes	3.42E-03	1.517	0.953	0.564	0.091	6.213	4.000	0.005	2.297
Mcl-1	Yes	3.64E-03	2.180	1.006	1.173	0.192	6.106	4.000	0.005	2.271
Raptor	Yes	3.94E-03	0.749	1.043	-0.294	0.049	5.978	4.000	0.006	2.240
ACC1	Yes	5.72E-03	0.728	1.011	-0.283	0.052	5.393	4.000	0.008	2.081
CD5	Yes	5.98E-03	0.730	0.993	-0.263	0.049	5.328	4.000	0.009	2.064
TFRC	Yes	6.15E-03	2.572	0.919	1.653	0.313	5.284	4.000	0.009	2.057
B7-H4	Yes	6.15E-03	1.307	0.993	0.314	0.059	5.284	4.000	0.009	2.057
DAPK2	Yes	6.46E-03	1.457	0.958	0.499	0.096	5.212	4.000	0.009	2.038
Rad23A	Yes	6.51E-03	0.723	1.133	-0.410	0.079	5.201	4.000	0.009	2.037
Caspase-7-cleaved-	Yes	6.64E-03	1.447	0.908	0.538	0.104	5.172	4.000	0.009	2.031
PKC-a-b-II_pT638_T641	Yes	6.82E-03	1.608	1.011	0.597	0.116	5.134	4.000	0.010	2.022
ACLY_pS455	Yes	7.13E-03	1.209	0.966	0.243	0.048	5.070	4.000	0.010	2.005
p38-a	Yes	7.19E-03	1.208	0.959	0.249	0.049	5.058	4.000	0.010	2.004
LC3A-B	Yes	7.25E-03	1.408	0.957	0.451	0.089	5.047	4.000	0.010	2.003
c-Kit	Yes	8.03E-03	1.315	0.975	0.339	0.069	4.903	4.000	0.011	1.961
B7-H3	Yes	8.15E-03	1.173	0.979	0.194	0.040	4.881	4.000	0.011	1.957
UBQLN4	Yes	8.26E-03	1.239	1.010	0.229	0.047	4.863	4.000	0.011	1.953
DJ1	Yes	9.22E-03	0.618	1.079	-0.460	0.098	4.713	4.000	0.012	1.908
14-3-3-zeta	Yes	9.38E-03	0.873	0.998	-0.125	0.027	4.689	4.000	0.012	1.905
Cyclophilin-F	Yes	9.39E-03	2.194	0.966	1.228	0.262	4.688	4.000	0.012	1.905
CD49b	Yes	9.46E-03	0.853	0.972	-0.120	0.026	4.679	4.000	0.012	1.904
Glutaminase	Yes	9.66E-03	2.290	0.936	1.354	0.291	4.651	4.000	0.013	1.898

Cox2	Yes	9.83E-03	0.850	0.993	-0.143	0.031	4.627	4.000	0.013	1.894
GCN5L2	Yes	9.87E-03	1.265	0.979	0.286	0.062	4.622	4.000	0.013	1.894
Puma	Yes	9.89E-03	1.507	0.907	0.599	0.130	4.619	4.000	0.013	1.894
IGFBP3	Yes	1.00E-02	1.347	0.973	0.374	0.081	4.601	4.000	0.013	1.891
PAK1	Yes	1.04E-02	1.537	0.938	0.599	0.132	4.552	4.000	0.013	1.877
Atg7	Yes	1.08E-02	1.290	0.981	0.309	0.069	4.505	4.000	0.014	1.865
ERCC5	Yes	1.08E-02	0.769	0.984	-0.215	0.048	4.501	4.000	0.014	1.865
FRS2-alpha_pY196	Yes	1.09E-02	0.895	0.991	-0.097	0.022	4.496	4.000	0.014	1.865
CD29	Yes	1.15E-02	1.444	0.917	0.527	0.119	4.420	4.000	0.014	1.842
Porin	Yes	1.31E-02	1.323	0.937	0.386	0.091	4.252	4.000	0.016	1.788
GCLM	Yes	1.32E-02	1.268	0.958	0.310	0.073	4.249	4.000	0.016	1.788
Bax	Yes	1.34E-02	1.242	1.004	0.238	0.056	4.228	4.000	0.016	1.783
CD45	Yes	1.50E-02	1.198	1.000	0.198	0.048	4.087	4.000	0.018	1.735
Connexin-43	Yes	1.56E-02	1.088	0.950	0.138	0.034	4.043	4.000	0.019	1.722
Mitofusin-2	Yes	1.59E-02	1.757	0.928	0.829	0.206	4.017	4.000	0.019	1.715
PYGB	Yes	1.60E-02	0.883	1.010	-0.127	0.032	4.013	4.000	0.019	1.715
EGFR_pY1173	Yes	1.61E-02	1.172	0.947	0.225	0.056	4.005	4.000	0.019	1.715
p27-Kip1	Yes	1.69E-02	1.199	0.948	0.251	0.064	3.946	4.000	0.020	1.697
ADAR1	Yes	1.69E-02	1.117	1.032	0.085	0.021	3.944	4.000	0.020	1.697
Rad50	Yes	1.73E-02	1.247	0.962	0.285	0.073	3.915	4.000	0.020	1.689
PHLPP	Yes	1.74E-02	0.827	0.965	-0.137	0.035	3.911	4.000	0.020	1.689
Akt_pS473	Yes	1.77E-02	0.931	0.999	-0.068	0.017	3.890	4.000	0.021	1.683
Axl	Yes	1.78E-02	1.935	0.937	0.998	0.257	3.884	4.000	0.021	1.683
MIF	Yes	1.80E-02	0.895	0.992	-0.097	0.025	3.868	4.000	0.021	1.680
GATA3	Yes	1.88E-02	1.435	0.906	0.529	0.138	3.820	4.000	0.022	1.665
MAPK_pT202_Y204	Yes	1.88E-02	0.829	1.099	-0.270	0.071	3.818	4.000	0.022	1.665
PRC1_pT481	Yes	1.95E-02	1.119	0.945	0.174	0.046	3.775	4.000	0.022	1.651
ER-a	Yes	1.96E-02	1.411	0.882	0.528	0.140	3.768	4.000	0.022	1.651
TAZ	Yes	1.97E-02	0.776	1.019	-0.242	0.064	3.764	4.000	0.022	1.651
Gli1	Yes	2.00E-02	1.178	0.904	0.274	0.073	3.750	4.000	0.023	1.648
PRAS40	Yes	2.03E-02	1.113	0.967	0.147	0.039	3.727	4.000	0.023	1.641
IL-6	Yes	2.10E-02	1.161	0.977	0.184	0.050	3.689	4.000	0.024	1.628
Myt1	Yes	2.13E-02	1.589	0.973	0.616	0.168	3.677	4.000	0.024	1.627
ATR_pS428	Yes	2.13E-02	1.440	0.974	0.466	0.127	3.674	4.000	0.024	1.627
4E-BP1	Yes	2.21E-02	0.735	1.058	-0.323	0.089	3.633	4.000	0.024	1.613
Jagged1	Yes	2.23E-02	1.290	0.949	0.341	0.094	3.621	4.000	0.025	1.610
b-Catenin_pT41_S45	Yes	2.28E-02	0.840	1.029	-0.189	0.053	3.598	4.000	0.025	1.604
Calnexin	Yes	2.36E-02	1.137	0.964	0.172	0.048	3.561	4.000	0.026	1.591
Dvl3	Yes	2.43E-02	0.846	1.028	-0.181	0.051	3.526	4.000	0.026	1.580
c-Met_pY1234_Y1235	Yes	2.51E-02	1.093	0.988	0.105	0.030	3.493	4.000	0.027	1.570

PI3K-p110-b	Yes	2.51E-02	1.287	0.946	0.341	0.098	3.493	4.000	0.027	1.570
PKA-a	Yes	2.53E-02	0.654	1.010	-0.356	0.102	3.481	4.000	0.027	1.568
CD26	Yes	2.60E-02	1.380	1.015	0.365	0.106	3.453	4.000	0.028	1.558
Enolase-1	Yes	2.63E-02	0.859	0.970	-0.111	0.032	3.439	4.000	0.028	1.555
EphA2_pY588	Yes	2.67E-02	1.506	1.001	0.505	0.148	3.424	4.000	0.028	1.551
Src	Yes	2.91E-02	0.914	0.989	-0.075	0.023	3.329	4.000	0.031	1.514
Granzyme-B	Yes	2.94E-02	1.395	0.933	0.462	0.139	3.320	4.000	0.031	1.513
Melanoma-gp100	Yes	2.95E-02	1.197	0.934	0.263	0.079	3.316	4.000	0.031	1.513
CIITA	Yes	2.98E-02	1.255	0.983	0.272	0.082	3.304	4.000	0.031	1.510
Caspase-8	Yes	3.01E-02	1.269	0.992	0.277	0.084	3.293	4.000	0.031	1.507
CSK	Yes	3.05E-02	0.815	1.014	-0.199	0.061	3.281	4.000	0.031	1.504
Tau	Yes	3.26E-02	0.765	0.959	-0.194	0.060	3.209	4.000	0.033	1.476
ATRX	Yes	3.33E-02	0.844	0.999	-0.156	0.049	3.189	4.000	0.034	1.471
Stat5a	Yes	3.35E-02	0.816	0.971	-0.155	0.049	3.182	4.000	0.034	1.471
NDRG1_pT346	Yes	3.35E-02	1.137	0.965	0.171	0.054	3.182	4.000	0.034	1.471
Chk1_pS296	Yes	3.39E-02	1.136	0.973	0.163	0.051	3.169	4.000	0.034	1.467
NQO1	Yes	3.45E-02	1.369	1.011	0.358	0.114	3.152	4.000	0.035	1.462
ER-a_pS118	Yes	3.54E-02	1.178	0.956	0.222	0.071	3.125	4.000	0.035	1.453
HSP70	Yes	3.55E-02	0.827	1.008	-0.181	0.058	3.122	4.000	0.035	1.453
Src_pY527	Yes	3.56E-02	1.120	0.971	0.149	0.048	3.118	4.000	0.035	1.453
Merlin	Yes	3.63E-02	0.819	0.981	-0.162	0.052	3.097	4.000	0.036	1.447
CDKN2A	Yes	3.64E-02	0.783	0.965	-0.182	0.059	3.096	4.000	0.036	1.447
HLA-DR-DP-DQ-DX	Yes	3.71E-02	1.243	0.707	0.536	0.174	3.074	4.000	0.036	1.440
Creb	Yes	3.78E-02	0.819	0.967	-0.148	0.048	3.057	4.000	0.037	1.434
EphA2_pS897	Yes	3.80E-02	0.933	1.032	-0.099	0.033	3.051	4.000	0.037	1.434
INPP4b	Yes	3.90E-02	1.182	0.963	0.220	0.073	3.025	4.000	0.038	1.424
GATA6	Yes	4.02E-02	1.317	1.000	0.317	0.106	2.993	4.000	0.039	1.412
Chk1	Yes	4.19E-02	0.947	1.002	-0.056	0.019	2.951	4.000	0.040	1.396
Smad3	Yes	4.23E-02	1.151	1.020	0.131	0.045	2.942	4.000	0.040	1.394
IDO	Yes	4.26E-02	1.281	0.994	0.287	0.098	2.934	4.000	0.041	1.392
UBAC1	Yes	4.36E-02	1.296	1.004	0.292	0.100	2.911	4.000	0.041	1.384
H2AX_pS139	Yes	4.40E-02	1.463	0.962	0.501	0.172	2.903	4.000	0.041	1.383
Stat3	Yes	4.40E-02	0.850	0.954	-0.104	0.036	2.902	4.000	0.041	1.383
Akt_pT308	Yes	4.48E-02	0.747	0.960	-0.213	0.074	2.884	4.000	0.042	1.377
HER2_pY1248	Yes	4.56E-02	1.519	0.935	0.585	0.204	2.868	4.000	0.043	1.371
14-3-3-epsilon	Yes	4.73E-02	1.313	0.942	0.372	0.131	2.832	4.000	0.044	1.357
Akt1	Yes	4.76E-02	0.828	1.114	-0.285	0.101	2.826	4.000	0.044	1.356
PDHA1	Yes	5.00E-02	1.236	0.885	0.351	0.126	2.776	4.000	0.046	1.336
PAICS	Yes	5.02E-02	0.746	1.208	-0.462	0.167	2.773	4.000	0.046	1.336
c-Abl	Yes	5.10E-02	1.209	1.014	0.195	0.071	2.757	4.000	0.047	1.331

Sox2	Yes	5.22E-02	1.303	0.976	0.328	0.120	2.734	4.000	0.048	1.322
Rab11	Yes	5.24E-02	1.197	0.924	0.274	0.100	2.730	4.000	0.048	1.322
Akt	Yes	5.31E-02	1.267	1.023	0.245	0.090	2.719	4.000	0.048	1.320
PDHK1	Yes	5.32E-02	1.225	1.003	0.223	0.082	2.717	4.000	0.048	1.320
G6PD	Yes	5.33E-02	0.864	1.064	-0.200	0.074	2.714	4.000	0.048	1.320
JAB1	Yes	5.57E-02	1.224	0.901	0.323	0.121	2.672	4.000	0.050	1.303
DNA_POLG	Yes	5.59E-02	1.210	0.961	0.249	0.093	2.668	4.000	0.050	1.303
4E-BP1_pS65	Yes	5.60E-02	0.581	1.207	-0.626	0.235	2.667	4.000	0.050	1.303
JNK2	No	5.67E-02	1.102	0.946	0.156	0.059	2.656	4.000	0.050	1.300
ACC_pS79	No	5.77E-02	0.874	0.951	-0.077	0.029	2.638	4.000	0.051	1.294
p27_pT198	No	5.78E-02	0.835	0.981	-0.146	0.055	2.637	4.000	0.051	1.294
PAR	No	5.86E-02	1.159	1.007	0.152	0.058	2.623	4.000	0.051	1.290
CD38	No	5.98E-02	1.236	0.929	0.307	0.118	2.604	4.000	0.052	1.283
CHD1L	No	6.00E-02	1.185	0.998	0.187	0.072	2.601	4.000	0.052	1.283
CD31	No	6.03E-02	1.191	0.979	0.212	0.082	2.595	4.000	0.052	1.282
DUSP4	No	6.17E-02	1.371	1.015	0.356	0.138	2.574	4.000	0.053	1.274
DAPK1_pS308	No	6.19E-02	1.197	0.980	0.217	0.084	2.571	4.000	0.053	1.274
Smad1	No	6.28E-02	0.912	1.005	-0.093	0.036	2.558	4.000	0.054	1.269
Oct-4	No	6.42E-02	1.209	0.990	0.219	0.086	2.537	4.000	0.055	1.261
SF2	No	6.51E-02	1.133	0.979	0.154	0.061	2.523	4.000	0.055	1.256
Caveolin-1	No	6.54E-02	1.262	1.020	0.242	0.096	2.520	4.000	0.055	1.256
CD171	No	6.59E-02	1.403	1.016	0.387	0.154	2.512	4.000	0.056	1.254
XBP-1	No	6.61E-02	1.353	0.945	0.408	0.162	2.510	4.000	0.056	1.254
Tuberin	No	7.05E-02	0.900	0.991	-0.091	0.037	2.449	4.000	0.059	1.228
Twist	No	7.32E-02	1.202	0.970	0.232	0.096	2.414	4.000	0.061	1.213
Bid	No	7.59E-02	1.157	0.935	0.223	0.094	2.381	4.000	0.063	1.199
Bim	No	7.64E-02	0.853	0.945	-0.092	0.039	2.374	4.000	0.063	1.198
c-Abl_pY412	No	7.65E-02	1.096	0.977	0.119	0.050	2.374	4.000	0.063	1.198
Tyro3	No	7.70E-02	0.915	0.990	-0.075	0.032	2.368	4.000	0.064	1.197
MITF	No	7.76E-02	0.874	0.954	-0.080	0.034	2.361	4.000	0.064	1.195
TTF1	No	7.99E-02	0.907	1.032	-0.125	0.054	2.335	4.000	0.065	1.184
MelanA	No	8.04E-02	1.056	0.974	0.082	0.035	2.328	4.000	0.066	1.183
Notch1-cleaved	No	8.57E-02	0.922	1.001	-0.079	0.035	2.270	4.000	0.070	1.156
HER3	No	8.62E-02	0.933	1.008	-0.075	0.033	2.265	4.000	0.070	1.155
ATR	No	9.08E-02	0.805	0.940	-0.136	0.061	2.218	4.000	0.073	1.134
Wee1	No	9.11E-02	1.124	0.935	0.189	0.085	2.215	4.000	0.073	1.134
RPA32	No	9.32E-02	0.909	0.986	-0.076	0.035	2.195	4.000	0.075	1.126
MTSS1	No	9.38E-02	1.121	0.975	0.147	0.067	2.189	4.000	0.075	1.125
Transglutaminase	No	9.83E-02	0.854	0.993	-0.139	0.065	2.148	4.000	0.078	1.106
VEGFR-2	No	9.88E-02	0.964	1.007	-0.043	0.020	2.143	4.000	0.079	1.105

Chk2_pT68	No	1.00E-01	0.956	1.031	-0.075	0.035	2.130	4.000	0.079	1.100
PRAS40_pT246	No	1.03E-01	0.875	0.950	-0.075	0.036	2.109	4.000	0.081	1.091
C-Raf_pS338	No	1.03E-01	1.112	0.985	0.126	0.060	2.107	4.000	0.081	1.091
CD74	No	1.05E-01	0.864	1.010	-0.146	0.070	2.085	4.000	0.083	1.082
Histone-H3	No	1.06E-01	0.921	0.976	-0.054	0.026	2.078	4.000	0.083	1.080
SGK1	No	1.12E-01	1.141	1.020	0.121	0.060	2.035	4.000	0.087	1.060
IRF-3	No	1.12E-01	0.926	0.997	-0.071	0.035	2.029	4.000	0.087	1.059
Claudin-7	No	1.13E-01	1.072	1.019	0.053	0.026	2.025	4.000	0.087	1.058
Cyclin-D1	No	1.14E-01	1.215	0.932	0.283	0.140	2.016	4.000	0.088	1.055
cdc2_pY15	No	1.15E-01	1.176	1.017	0.159	0.079	2.009	4.000	0.089	1.053
PYGM	No	1.18E-01	1.211	1.091	0.121	0.061	1.987	4.000	0.090	1.044
MSI2	No	1.25E-01	1.250	1.018	0.232	0.120	1.934	4.000	0.096	1.019
RPA32_pS4_S8	No	1.26E-01	0.772	0.895	-0.122	0.063	1.932	4.000	0.096	1.019
YB1_pS102	No	1.26E-01	1.235	0.991	0.244	0.126	1.927	4.000	0.096	1.018
Heregulin	No	1.27E-01	1.145	1.026	0.119	0.062	1.925	4.000	0.096	1.018
HSP27	No	1.27E-01	1.398	0.987	0.411	0.214	1.918	4.000	0.096	1.016
MMP2	No	1.30E-01	1.033	0.990	0.043	0.023	1.904	4.000	0.097	1.012
Elk1_pS383	No	1.30E-01	1.064	1.008	0.057	0.030	1.904	4.000	0.097	1.012
BAP1	No	1.31E-01	1.051	0.937	0.114	0.060	1.895	4.000	0.098	1.008
EVI1	No	1.37E-01	0.947	1.047	-0.099	0.054	1.854	4.000	0.102	0.990
b-Catenin	No	1.38E-01	1.126	0.937	0.190	0.102	1.853	4.000	0.102	0.990
DUSP6	No	1.46E-01	1.077	0.987	0.090	0.050	1.800	4.000	0.109	0.964
Cyclin-E1	No	1.47E-01	1.082	0.973	0.109	0.061	1.796	4.000	0.109	0.964
GRB7	No	1.50E-01	1.099	0.942	0.157	0.088	1.778	4.000	0.110	0.957
MERIT40	No	1.50E-01	1.158	0.967	0.191	0.108	1.777	4.000	0.110	0.957
NRF2	No	1.53E-01	0.873	0.950	-0.077	0.044	1.759	4.000	0.112	0.949
Shc_pY317	No	1.55E-01	1.594	0.951	0.642	0.367	1.752	4.000	0.113	0.948
CD4	No	1.55E-01	0.905	0.981	-0.076	0.044	1.752	4.000	0.113	0.948
Hexokinase-I	No	1.55E-01	1.192	0.990	0.202	0.116	1.748	4.000	0.113	0.948
HSP27_pS82	No	1.58E-01	1.115	0.990	0.125	0.072	1.733	4.000	0.114	0.941
Notch3	No	1.60E-01	0.858	1.033	-0.175	0.101	1.724	4.000	0.115	0.938
Complex-II-Subunit	No	1.63E-01	1.109	0.981	0.128	0.075	1.707	4.000	0.117	0.931
ATM	No	1.65E-01	1.071	0.953	0.118	0.070	1.697	4.000	0.118	0.927
Rad51	No	1.66E-01	0.772	0.894	-0.122	0.072	1.694	4.000	0.118	0.926
EMA	No	1.69E-01	1.834	0.915	0.919	0.549	1.675	4.000	0.121	0.918
Hif-1-alpha	No	1.74E-01	0.938	1.007	-0.069	0.042	1.651	4.000	0.124	0.907
4E-BP1_pT37_T46	No	1.75E-01	0.650	1.066	-0.416	0.253	1.647	4.000	0.124	0.907
STING	No	1.80E-01	1.215	0.992	0.223	0.138	1.623	4.000	0.127	0.896
CD20	No	1.80E-01	1.105	0.972	0.132	0.082	1.622	4.000	0.127	0.896
FGF-basic	No	1.89E-01	1.108	0.988	0.119	0.075	1.583	4.000	0.133	0.877

Aurora-A	No	1.94E-01	0.923	1.018	-0.095	0.061	1.557	4.000	0.136	0.866
JNK_pT183_Y185	No	1.98E-01	1.569	0.885	0.684	0.444	1.541	4.000	0.139	0.858
Rheb	No	2.03E-01	1.116	1.027	0.090	0.059	1.519	4.000	0.142	0.849
LRP6_pS1490	No	2.04E-01	0.896	0.975	-0.080	0.053	1.517	4.000	0.142	0.849
Notch1	No	2.05E-01	1.054	0.998	0.056	0.037	1.511	4.000	0.142	0.847
DDB-1	No	2.07E-01	0.887	1.003	-0.115	0.077	1.506	4.000	0.143	0.845
CREB_pS133	No	2.12E-01	0.852	0.938	-0.087	0.058	1.486	4.000	0.146	0.836
HER3_pY1289	No	2.13E-01	0.967	1.004	-0.038	0.025	1.481	4.000	0.146	0.835
14-3-3-beta	No	2.26E-01	0.927	0.965	-0.039	0.027	1.431	4.000	0.155	0.810
AceCS1	No	2.28E-01	1.117	0.953	0.164	0.116	1.423	4.000	0.156	0.808
FN14	No	2.40E-01	1.274	1.011	0.262	0.190	1.379	4.000	0.163	0.787
PKC-delta_pS664	No	2.41E-01	0.987	1.076	-0.088	0.064	1.375	4.000	0.164	0.786
PARG	No	2.42E-01	1.782	0.996	0.785	0.572	1.373	4.000	0.164	0.786
WIP1	No	2.47E-01	0.994	0.938	0.056	0.041	1.355	4.000	0.167	0.778
Enolase-2	No	2.56E-01	0.997	0.965	0.032	0.024	1.325	4.000	0.172	0.764
XRCC1	No	2.69E-01	1.071	0.989	0.081	0.063	1.283	4.000	0.181	0.743
Stat1_pY701	No	2.73E-01	1.219	1.076	0.143	0.113	1.269	4.000	0.183	0.737
Jak2	No	2.76E-01	0.980	1.016	-0.036	0.029	1.260	4.000	0.185	0.734
Erk5	No	2.93E-01	0.896	1.016	-0.120	0.099	1.211	4.000	0.195	0.710
PKCa	No	3.00E-01	1.030	0.971	0.059	0.049	1.190	4.000	0.199	0.701
Fibronectin	No	3.05E-01	1.163	0.984	0.178	0.152	1.175	4.000	0.202	0.694
PI3K-p85	No	3.08E-01	1.035	0.984	0.051	0.043	1.166	4.000	0.204	0.691
p90RSK_pT573	No	3.26E-01	1.133	0.949	0.184	0.165	1.118	4.000	0.215	0.668
CD68	No	3.35E-01	1.131	1.042	0.089	0.081	1.095	4.000	0.220	0.657
CD134	No	3.37E-01	0.997	0.970	0.027	0.025	1.090	4.000	0.220	0.657
BCL2A1	No	3.37E-01	1.058	1.010	0.048	0.044	1.090	4.000	0.220	0.657
ERCC1	No	3.39E-01	0.884	0.993	-0.109	0.100	1.086	4.000	0.221	0.656
BMK1-Erk5_pT218_Y220	No	3.52E-01	0.817	0.923	-0.106	0.101	1.052	4.000	0.228	0.641
AMPKa_pT172	No	3.52E-01	1.040	0.974	0.066	0.063	1.052	4.000	0.228	0.641
HNRNPK	No	3.54E-01	0.902	0.989	-0.087	0.083	1.048	4.000	0.229	0.640
MYH11	No	3.55E-01	1.028	0.982	0.046	0.044	1.044	4.000	0.229	0.640
B-Raf	No	3.56E-01	1.025	0.995	0.030	0.028	1.043	4.000	0.229	0.640
Slfn11	No	3.60E-01	1.143	0.990	0.153	0.148	1.032	4.000	0.231	0.637
DDR1_pY513	No	3.61E-01	0.947	0.819	0.128	0.125	1.031	4.000	0.231	0.637
Aurora-B	No	3.64E-01	1.115	0.999	0.116	0.113	1.023	4.000	0.232	0.634
P-Cadherin	No	3.66E-01	1.001	0.970	0.031	0.030	1.018	4.000	0.233	0.632
CtIP	No	3.69E-01	0.881	0.972	-0.091	0.090	1.011	4.000	0.234	0.630
MR1	No	3.76E-01	0.970	1.049	-0.079	0.079	0.996	4.000	0.238	0.624
SHP2	No	3.76E-01	0.928	0.998	-0.071	0.071	0.995	4.000	0.238	0.624
ERRalpha	No	3.86E-01	0.997	0.967	0.030	0.030	0.973	4.000	0.243	0.615

ZEB1	No	3.88E-01	0.973	0.999	-0.026	0.027	0.969	4.000	0.243	0.614
Bad_pS112	No	3.93E-01	1.014	0.963	0.051	0.053	0.957	4.000	0.246	0.609
UVRAG	No	3.99E-01	1.073	1.015	0.058	0.061	0.942	4.000	0.250	0.603
Stathmin-1	No	4.02E-01	1.037	0.991	0.047	0.050	0.937	4.000	0.250	0.601
IRS1	No	4.23E-01	1.012	0.990	0.022	0.025	0.892	4.000	0.263	0.580
Rb_pS807_S811	No	4.28E-01	1.054	0.947	0.107	0.121	0.881	4.000	0.265	0.576
AR	No	4.29E-01	1.242	0.947	0.295	0.335	0.880	4.000	0.265	0.576
Ets-1	No	4.33E-01	0.943	0.982	-0.039	0.044	0.871	4.000	0.267	0.573
eIF4E	No	4.42E-01	1.058	0.994	0.064	0.075	0.853	4.000	0.272	0.565
CDK1_pT14	No	4.46E-01	0.974	0.989	-0.015	0.018	0.844	4.000	0.274	0.562
Pdcd4	No	4.49E-01	1.165	0.841	0.325	0.388	0.838	4.000	0.275	0.560
PCNA	No	4.53E-01	0.941	0.989	-0.048	0.058	0.831	4.000	0.276	0.558
DDR1	No	4.65E-01	1.074	1.033	0.042	0.052	0.807	4.000	0.283	0.548
D-a-Tubulin	No	4.71E-01	1.039	0.979	0.060	0.076	0.795	4.000	0.287	0.543
Annexin-I	No	4.90E-01	0.983	1.015	-0.032	0.042	0.759	4.000	0.297	0.527
Ambra1_pS52	No	5.01E-01	1.007	0.979	0.028	0.038	0.739	4.000	0.303	0.519
Atg3	No	5.04E-01	0.992	0.871	0.122	0.166	0.734	4.000	0.304	0.517
ZAP-70	No	5.12E-01	1.094	0.920	0.174	0.242	0.719	4.000	0.308	0.511
Syk	No	5.17E-01	0.775	0.947	-0.172	0.242	0.710	4.000	0.310	0.508
ULK1_pS757	No	5.18E-01	1.090	1.023	0.067	0.095	0.708	4.000	0.310	0.508
VHL-EPPK1	No	5.35E-01	0.988	1.031	-0.043	0.064	0.677	4.000	0.320	0.495
Myosin-IIa_pS1943	No	5.42E-01	1.168	1.079	0.089	0.133	0.666	4.000	0.323	0.491
p21	No	5.46E-01	0.887	0.824	0.063	0.095	0.659	4.000	0.324	0.489
ENY2	No	5.62E-01	1.028	0.974	0.054	0.086	0.631	4.000	0.333	0.477
LAD1	No	5.63E-01	0.820	0.928	-0.108	0.172	0.630	4.000	0.333	0.477
PLC-gamma1	No	5.78E-01	1.137	1.094	0.043	0.071	0.604	4.000	0.341	0.467
HLA-DQA1	No	5.80E-01	0.951	0.992	-0.041	0.068	0.602	4.000	0.341	0.467
Akt1_pS473	No	5.93E-01	1.020	0.965	0.055	0.095	0.580	4.000	0.348	0.458
EGFR	No	5.99E-01	1.010	0.992	0.017	0.030	0.570	4.000	0.351	0.455
N-Cadherin	No	6.02E-01	0.930	0.982	-0.052	0.092	0.566	4.000	0.351	0.455
IGF1R_pY1135_Y1136	No	6.02E-01	1.042	1.017	0.025	0.044	0.565	4.000	0.351	0.455
H2AX_pS140	No	6.04E-01	0.945	0.978	-0.033	0.059	0.563	4.000	0.351	0.455
DM-K9-Histone-H3	No	6.17E-01	1.029	0.993	0.036	0.066	0.541	4.000	0.358	0.446
Gli3	No	6.28E-01	1.052	0.999	0.052	0.100	0.525	4.000	0.363	0.440
PHGDH	No	6.37E-01	1.014	1.044	-0.030	0.058	0.511	4.000	0.368	0.435
AMPK-a2_pS345	No	6.46E-01	0.975	0.944	0.030	0.061	0.495	4.000	0.372	0.429
Histone-H3_pS10	No	6.49E-01	0.914	0.956	-0.042	0.086	0.491	4.000	0.373	0.428
PREX1	No	6.54E-01	0.821	0.936	-0.115	0.238	0.484	4.000	0.374	0.427
GGPS1	No	6.54E-01	0.841	0.902	-0.061	0.126	0.483	4.000	0.374	0.427
B-Raf_pS445	No	6.61E-01	1.016	1.003	0.013	0.028	0.473	4.000	0.376	0.424

Snail	No	6.61E-01	1.009	0.986	0.023	0.049	0.473	4.000	0.376	0.424
SOD1	No	6.76E-01	1.013	0.983	0.029	0.065	0.451	4.000	0.384	0.416
Pyk2_pY402	No	6.81E-01	0.872	0.827	0.045	0.102	0.442	4.000	0.386	0.413
p27_pT157	No	6.91E-01	0.970	0.987	-0.017	0.041	0.428	4.000	0.391	0.408
Atg4B	No	6.97E-01	0.905	0.942	-0.037	0.088	0.419	4.000	0.392	0.406
Bcl2	No	6.97E-01	1.043	1.020	0.023	0.055	0.418	4.000	0.392	0.406
MDM2_pS166	No	7.01E-01	1.050	1.032	0.018	0.044	0.413	4.000	0.393	0.405
CDT1	No	7.08E-01	0.980	0.929	0.051	0.128	0.402	4.000	0.397	0.402
YAP_pS127	No	7.13E-01	0.966	0.993	-0.027	0.067	0.395	4.000	0.398	0.400
N-Ras	No	7.20E-01	0.974	0.990	-0.016	0.041	0.385	4.000	0.401	0.397
MIG6	No	7.58E-01	0.942	0.968	-0.026	0.080	0.330	4.000	0.422	0.375
MEK1	No	7.72E-01	1.053	1.034	0.019	0.062	0.311	4.000	0.428	0.368
VHL	No	7.75E-01	0.932	1.127	-0.196	0.640	0.306	4.000	0.429	0.367
FOXO3	No	7.89E-01	0.996	1.010	-0.014	0.050	0.285	4.000	0.436	0.360
HES1	No	8.13E-01	1.080	1.109	-0.029	0.115	0.253	4.000	0.448	0.349
IGFBP2	No	8.15E-01	1.006	0.976	0.030	0.121	0.250	4.000	0.448	0.349
SFRP1	No	8.39E-01	0.995	1.011	-0.017	0.077	0.216	4.000	0.461	0.337
SGK3	No	8.56E-01	0.995	0.984	0.011	0.057	0.194	4.000	0.469	0.329
UGT1A	No	8.68E-01	0.934	0.948	-0.013	0.075	0.177	4.000	0.474	0.324
Src_pY416	No	8.75E-01	0.954	0.967	-0.012	0.073	0.168	4.000	0.477	0.322
c-Myc	No	8.77E-01	0.996	0.971	0.025	0.152	0.165	4.000	0.477	0.322
Chk2	No	8.96E-01	0.960	0.967	-0.007	0.052	0.140	4.000	0.486	0.314
BRCA1	No	8.97E-01	0.968	0.955	0.013	0.097	0.138	4.000	0.486	0.314
IRF-1	No	9.23E-01	0.993	0.997	-0.005	0.045	0.103	4.000	0.498	0.302
XPF	No	9.43E-01	0.904	0.910	-0.006	0.075	0.076	4.000	0.508	0.294
WIPI2	No	9.45E-01	0.923	0.935	-0.012	0.169	0.073	4.000	0.508	0.294
YES1	No	9.46E-01	1.000	1.005	-0.005	0.070	0.072	4.000	0.508	0.294
GSK-3a-b_pS21_S9	No	9.64E-01	1.023	1.020	0.003	0.062	0.048	4.000	0.516	0.287
SDHA	Yes	<0.000001	2.764	0.969	1.796	0.032	56.270	4.000	0.000	4.143
SHP-2_pY542	Yes	<0.000001	0.709	4.380	-3.671	0.046	79.200	4.000	0.000	4.432

Pathway analysis of RPPA data: List of pathways that contained proteins with statistically significant differential expression between DMSO and long-term crizotinib treatment

Negative Regulators of Apoptosis	PI3K-AKT Pathway	Mitochondrial Respiratory Chain
XIAP	PDK1_pS241	MTCO1
Bcl-xL	Akt2_pS474	Cox-IV
c-IAP2	Akt2	UQCRC2
Mcl-1	PTEN	NDUFB4
	PDK1	SDHA
Positive Regulators of Apoptosis	TSC-MTOR Pathway	Mitochondrial Translation
Cleaved Caspase 7	S6_pS240_S244	TUFM
Bak	S6	TFAM
DNA Damage Checkpoint	mTOR_pS2448	Mitochondrial Fusion
Wee1_pS642	S6_pS235_S236	Mitofusin-1
ATM_pS1981	Rictor_pT1135	
G2/M Progression (Cell Cycle)	p70-S6K_pT389	Mitochondrial Metabolic Processes
cdc25C	RAS-MAPK Pathway	PARP
PLK1	c-Jun_pS73	ATP5A
Cyclin-B1	MEK1_p_S217_S221	SOD2
G1/S Progression (Cell Cycle)	SHP-2_pY542	Amino Acid Metabolism
BRD4	Tumor Content	Glutamate-D1-2
53BP1	Collagen-VI	GCLC
Hormone Signaling	E-Cadherin	Insulin Response
PR	PAI-1	IR-b
HER2	RBM15	IGFRb
Immune	Notch	IRS2
Lck	YAP	PKM2
Immune Checkpoint		
B7-H4		
PD-1		

Pathway analysis of RPPA data: Calculated values and statistics

	DMSO Average	DMSO SEM	Crizotinib Average	Crizotinib SEM	p value (t-test)
HER2 Pathway	-0.869	0.040	1.014	0.066	1.69E-05
G2/M Progression	1.019	0.100	-0.944	0.043	5.43E-05
TSC-MTOR	0.940	0.127	-0.999	0.074	1.92E-04
G1/S Progression	-0.875	0.133	0.963	0.212	1.84E-03
Breast Cancer Hormone Receptor Signaling	-1.005	0.244	0.744	0.066	2.30E-03
Immune Checkpoint	-0.802	0.188	0.839	0.200	3.93E-03
G0/G1 Progression	-0.409	0.101	0.452	0.110	4.46E-03
Apoptosis Pathway	-0.754	0.156	0.524	0.169	5.11E-03
Immune Pathway	-0.808	0.280	0.791	0.193	9.29E-03
RAS-MEK-ERK Pathway	0.322	0.170	-0.417	0.164	3.52E-02
PI3K-Akt Pathway	-0.170	0.092	0.334	0.221	1.03E-01
BH3 Balance	0.247	0.166	-0.123	0.155	1.79E-01
Hormone Receptor Signaling	-0.371	0.270	0.190	0.218	1.81E-01
Tumor Markers	0.090	0.129	-0.089	0.069	2.86E-01
DNA Damage	-0.096	0.398	0.093	0.177	6.86E-01
RTK Pathway	0.061	0.326	0.220	0.223	7.07E-01
DNA Damage Checkpoint	-0.109	0.220	-0.006	0.177	7.32E-01
Notch Pathway	-0.152	0.115	-0.146	0.201	9.83E-01

List of mitochondrial drugs tested in combination with crizotinib and corresponding drug inhibition matrices

Mitochondrial Drugs Tested

Inhibitor Name	Target
Gamitrinib	Mitochondrial HSP90 Inhibitor
SB 204990	ATP Citrate Lyase (ACLY) - Step 1 of FAS
ND-646	Acetyl-CoA Carboxylase 1 (ACC) - Step 2 of FAS
Telaglenastat (CB-839)	Glutaminase
SphK1 5C	Sphingosine kinase
Venetoclax	Bcl-2
AZD4320	Bcl-2/Bcl-xL
AZD5991	Mcl-1

Calculation and Visualization of synergy scores for Drug Combinations

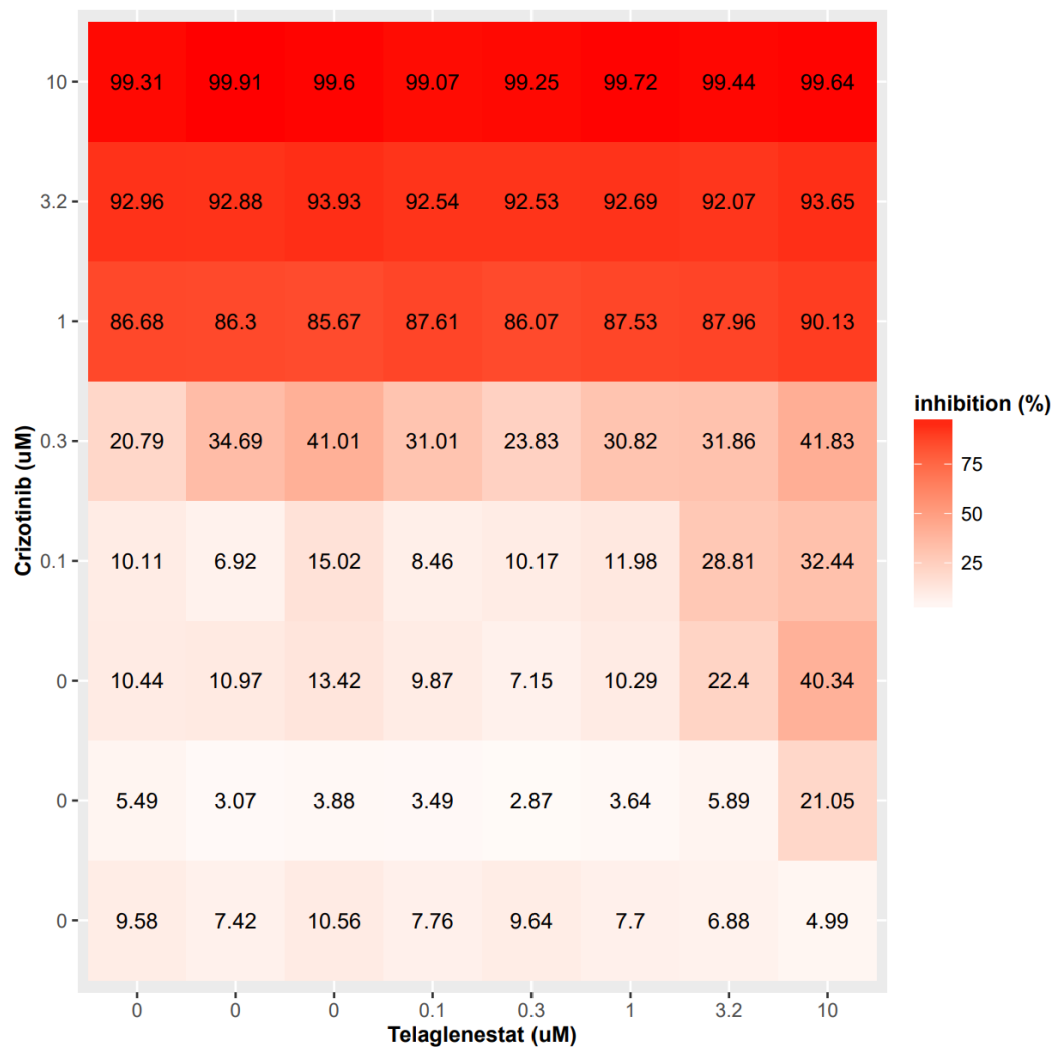
Drug combinations:

Drug combination	Synergy score	Most synergistic area score	Method
Telaglenestat - Crizotinib	3.69	14.98	ZIP
ND-646 - Crizotinib	13.22	35.80	ZIP
SB 204990 - Crizotinib	1.66	9.47	ZIP
AZD4320 - Crizotinib	1.60	5.49	ZIP
SphK1-5C - Crizotinib	0.98	5.82	ZIP
Telaglenestat No Glut - Crizotinib No Glut	12.04	28.50	ZIP

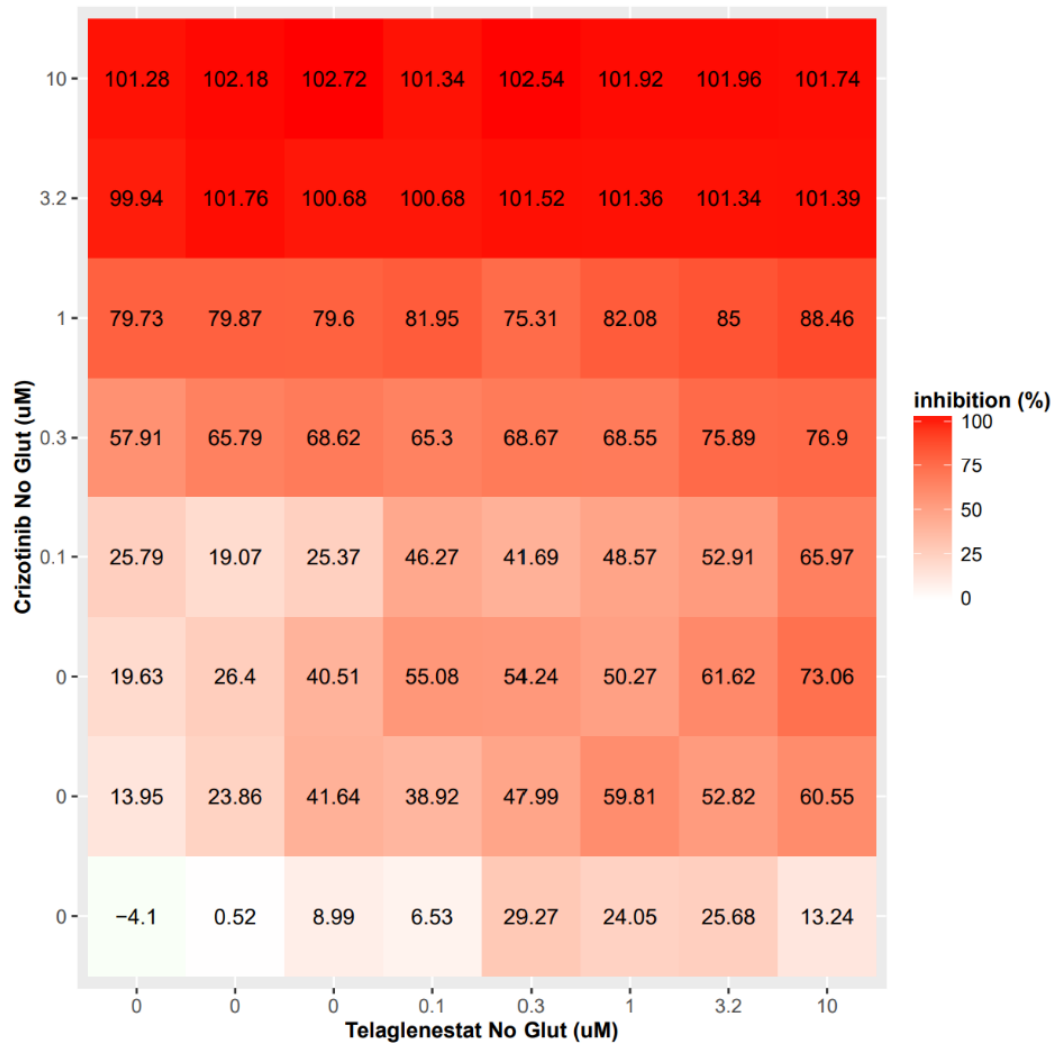
Chosen parameters:

Readout: viability ; Baseline correction: Yes ;

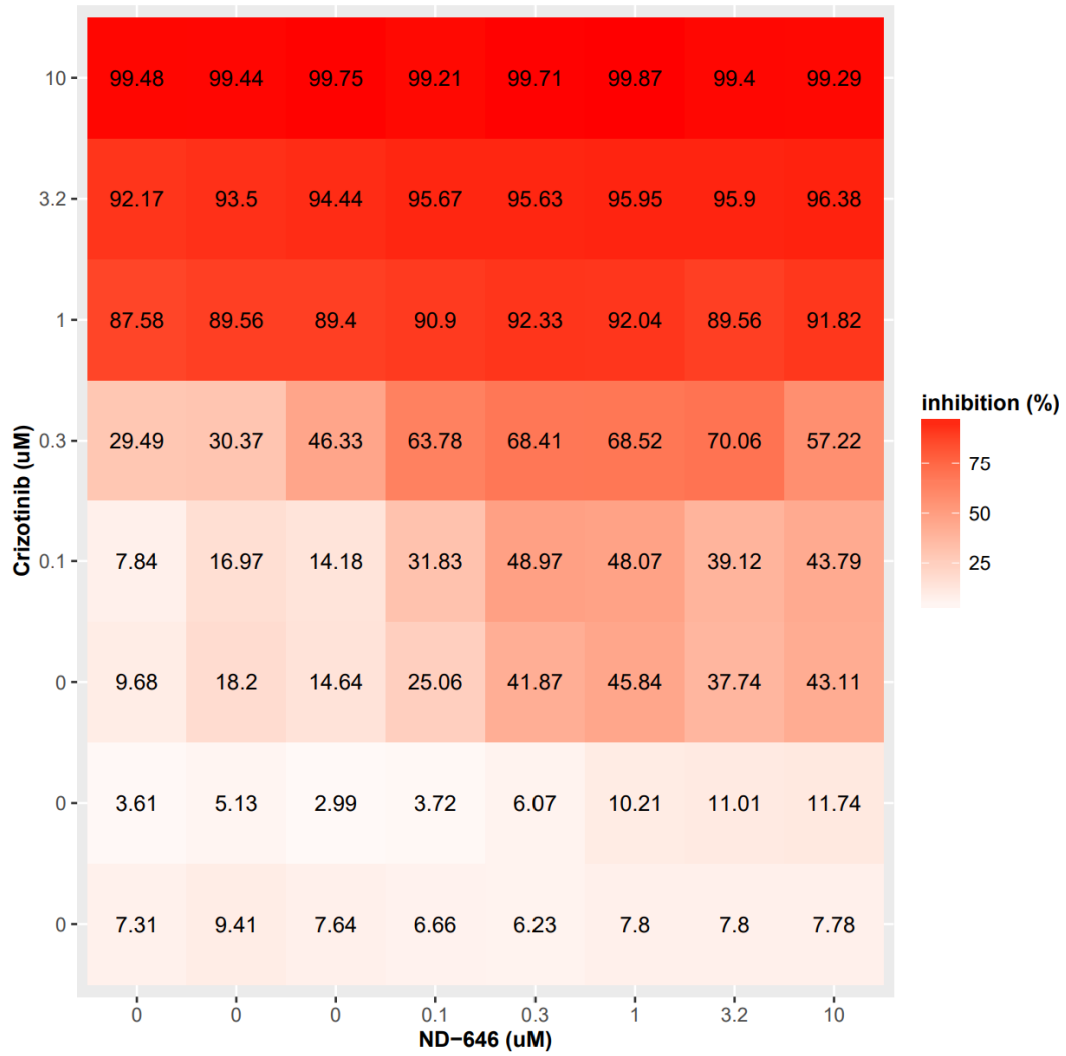
Telaglenestat & Crizotinib



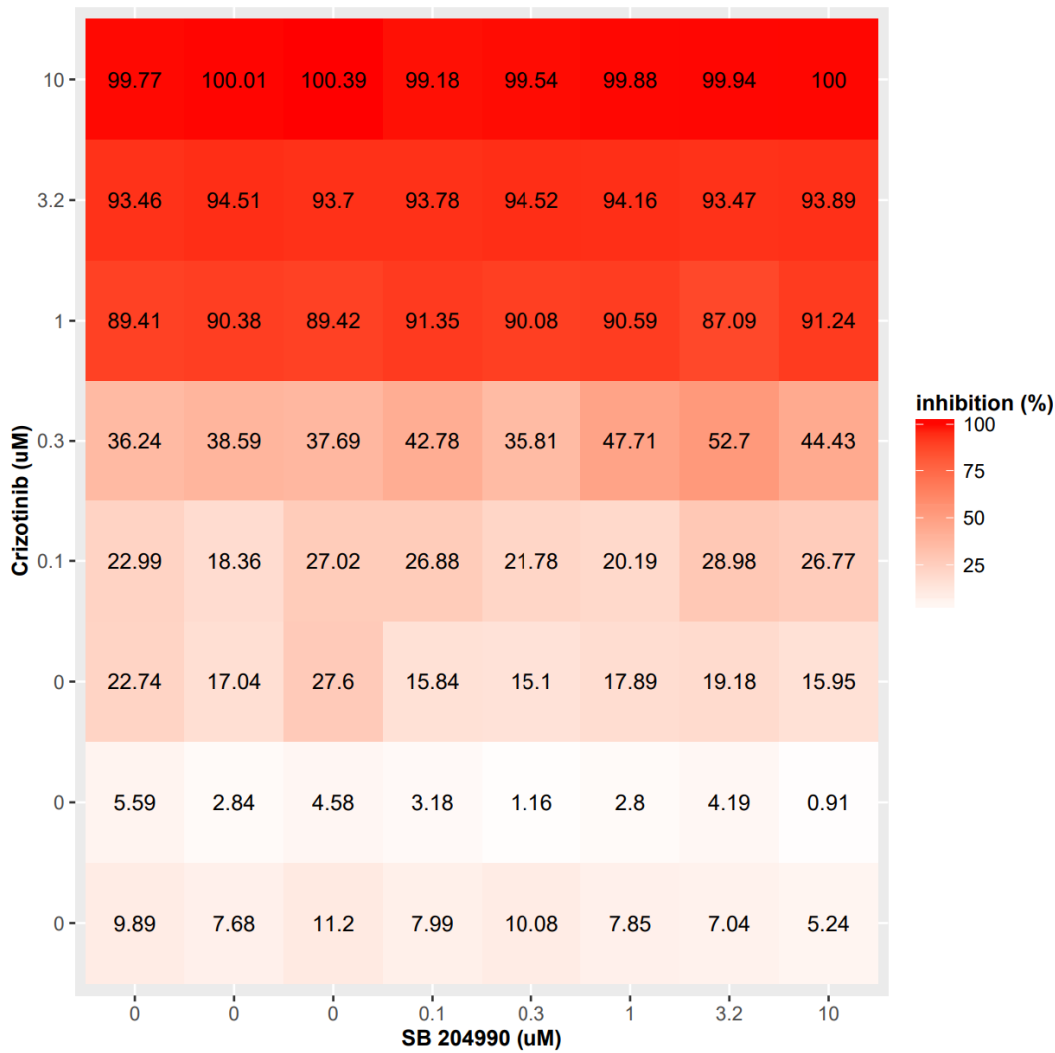
Telaglenestat No Glut & Crizotinib No Glut



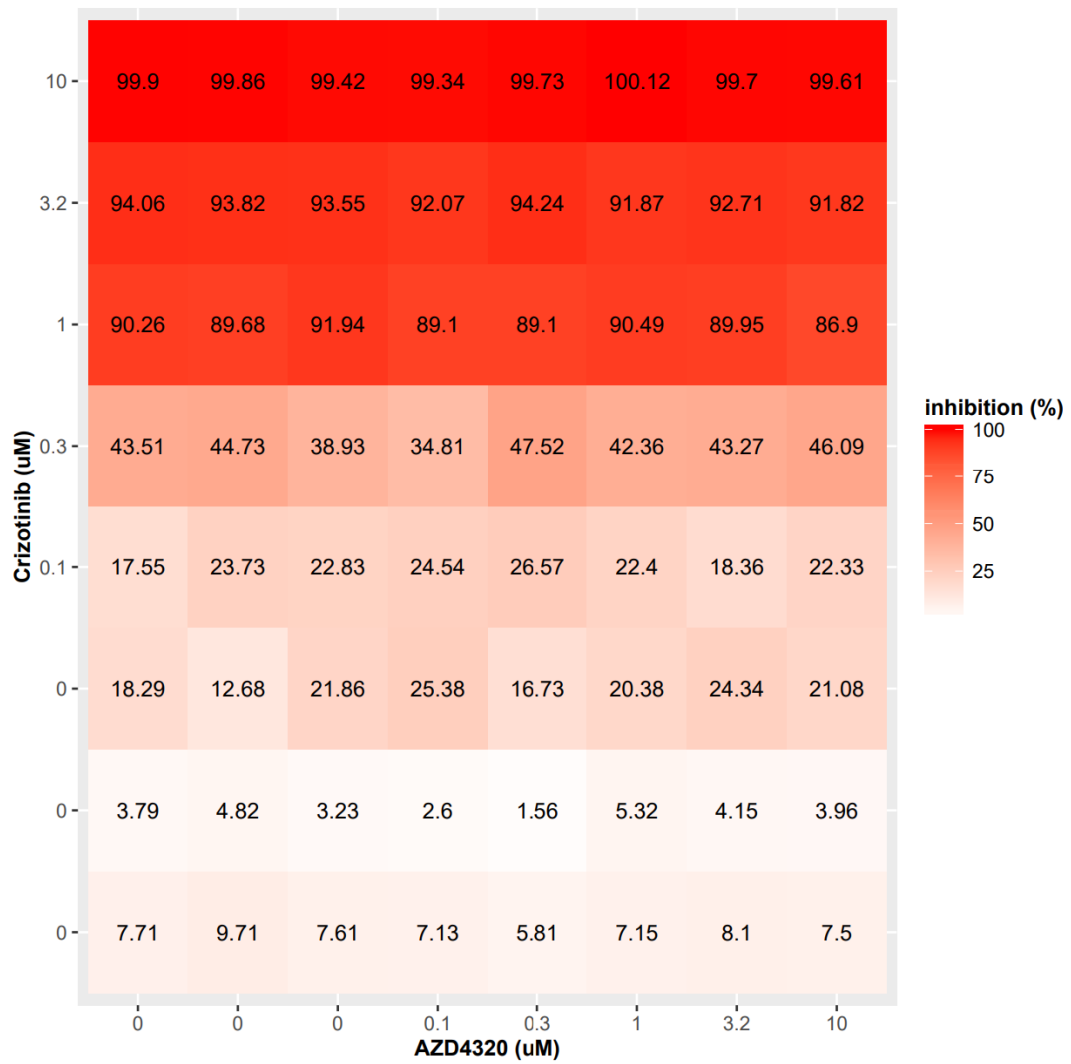
ND-646 & Crizotinib



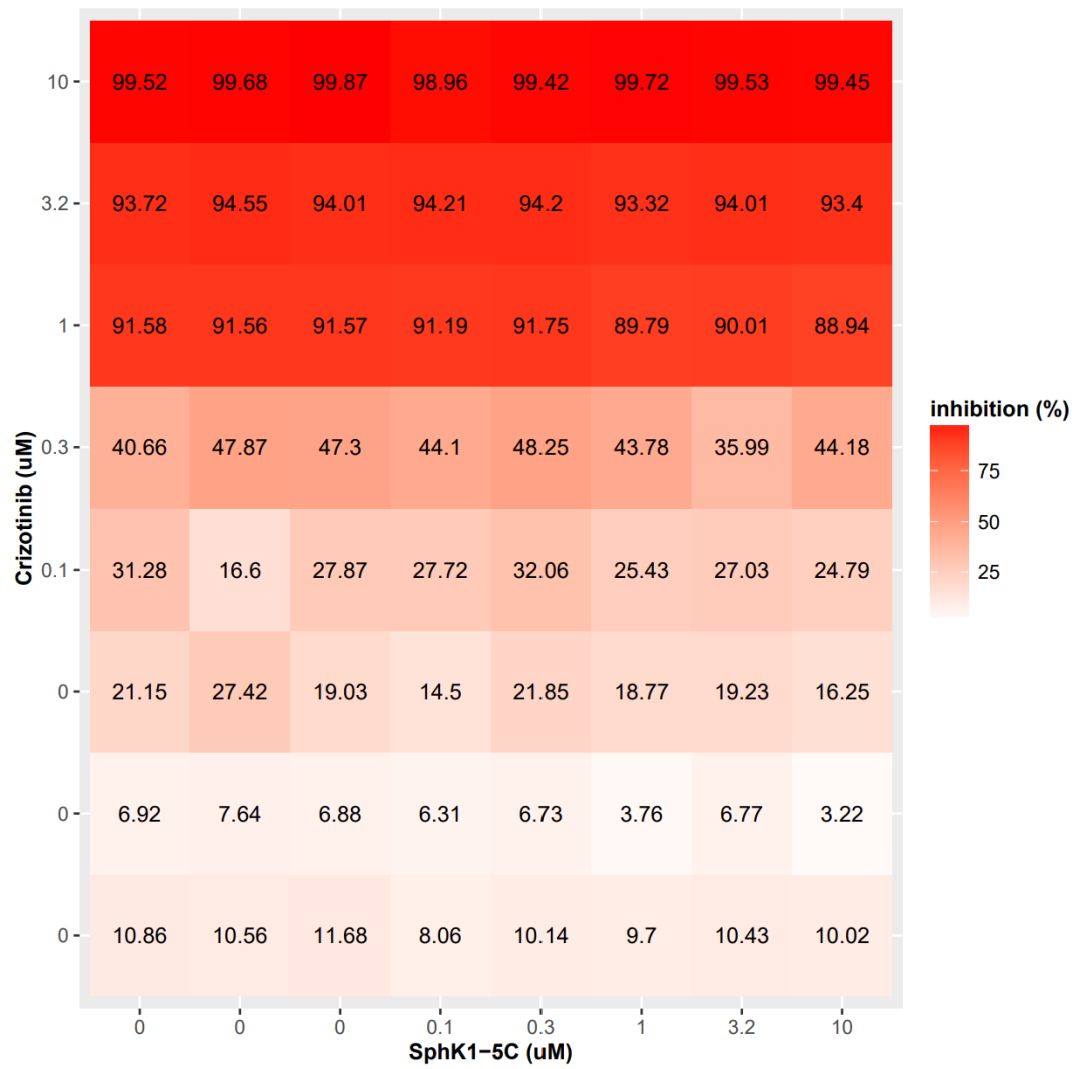
SB 204990 & Crizotinib



AZD4320 & Crizotinib

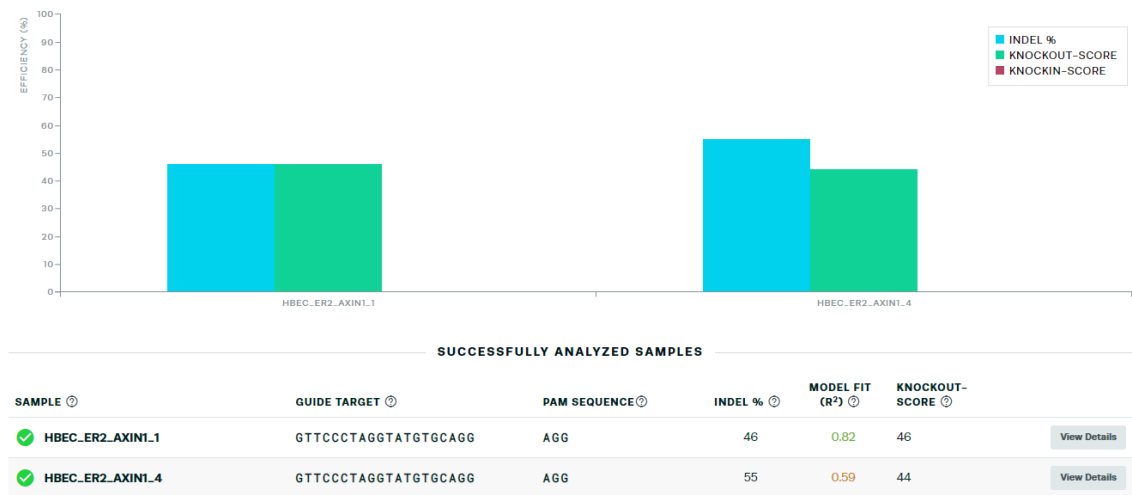


SphK1-5C & Crizotinib

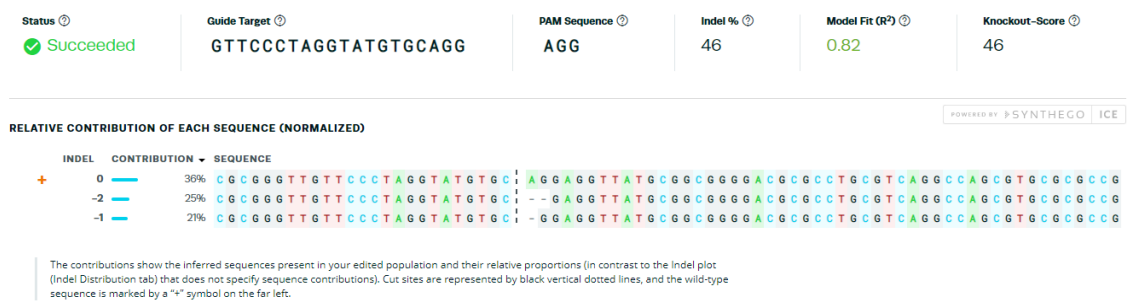


TIDE analysis of HBEC ER cells engineered with CRISPR-Cas9 to express the AXIN1 heterozygous variant found in LUAD-0006 cells

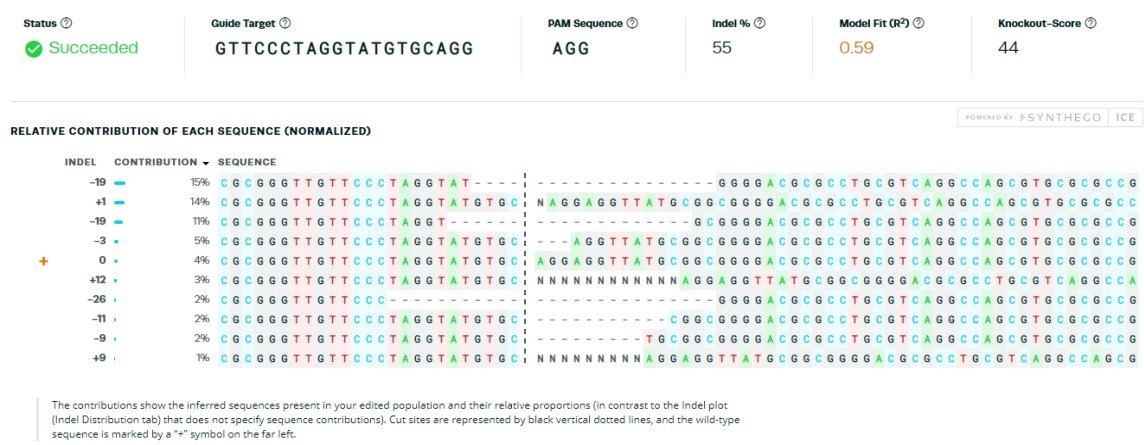
Summary chart



Alleles expressed in HBEC-ER2 AXIN1 clone #1



Alleles expressed in HBEC-ER2 AXIN1 clone #2



Chapter 3: Activating point mutations in the receptor tyrosine kinase, ROS1, drive oncogenesis

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Abstract

Targeted therapy of *ROS1*-positive cancers by tyrosine kinase inhibitors (TKIs) such as crizotinib has achieved clinical success in several cancer types, notably in lung cancer. *ROS1* TKI-sensitive cancers are driven by chimeric *ROS1* fusion proteins with constitutive tyrosine kinase (TK) activity that are generated from chromosomal rearrangements involving the *ROS1* gene. Unlike other receptor tyrosine kinases (RTKs) such as EGFR and ALK, it is currently unknown whether missense mutations in the context of the full length *ROS1* RTK can constitutively activate the TK domain (TKD) and if this is sufficient to drive tumor formation.

We queried the AACR Genie database for cancer associated somatic *ROS1* aberrations and found that 12% of the 5148 *ROS1* aberrations consisted of nonsynonymous mutations residing within the *ROS1* TKD. We prioritized a subset of *ROS1* mutations based on the following criteria: 1) predicted functional impact with the ‘Sorting Intolerant From Tolerant’ (SIFT) algorithm, 2) either confirmed as somatic or with a mean allele frequency < 0.01, and 3) in tumor samples that contain ≤ 15 total mutations. With these criteria, we identified 34 *ROS1* missense mutations for further functional interrogation using *in vitro* and *in vivo* methods. Transient transfection of engineered *ROS1* mutant plasmids in HEK-293A cells and immunoblotting with *ROS1* phospho-specific antibodies revealed distinct functional impact of these substitutions, ranging for loss of kinase activity to gain of kinase function. We selected five candidate activating mutations for further validation in two established *in vitro* cell transformation models (NIH3T3 and MCF10A). One mutant, *ROS1*^{D2113N}, correspondingly induced anchorage-independent colony formation that was abrogated with the *ROS1*-TKIs crizotinib and lorlatinib. Using MCF10A cells, we performed cell proliferation assays and saw increased proliferation of cells expressing the *ROS1*^{D2113N} relative to *ROS1*^{WT}; importantly, this

cell proliferation was ROS1-TKI sensitive. Phosphoproteomics studies further revealed that ROS1^{D2113N} promotes increased STAT and mTORC2 signaling To evaluate *in vivo* tumorigenicity, we subcutaneously injected NIH3T3 cells that expressed empty vector, ROS1^{D2113N}, or SLC-ROS1 fusion oncoprotein into nude mice and observed tumor formation with both the SLC-ROS1 positive control and the ROS1^{D2113N}. ROS1^{D2113N}-driven tumor formation was attenuated after oral treatment with the ROS1-TKIs, crizotinib and lorlatinib, in mice.

Taken together, these data demonstrate for the first time that point mutations within the TKD of the full length ROS1 receptor can drive oncogenic transformation and tumor growth that can be successfully blocked with existing ROS1-TKI targeted therapy. Our data suggest cancer patients with tumors that harbor activating ROS1 missense mutations may benefit from ROS1-TKI treatment.

Respective Contributions

SI wrote the manuscript and created the figure panels while data collection was performed by numerous co-authors.

Figure 3.1: SI generated panel A from a publicly available dataset. MD generated panel B. EA and KJ cloned the plasmids that were screened. Panel C data generation was done by MD, KJ, and SI while panel was created by SI. SI generated the data and panels for E and F.

Figure 3.2: KN generated the data and panels for A and B. SI generated the data and panels for C-E.

Figure 3.3: SI generated the samples and analyzed the data collected by the Fred Hutch Proteomics Core. SI created the panels.

Figure 3.4: KJ generated the data and panel for A. SI generated the data and panel for B.

Figure 3.5: KN generated the data. SI created the panels with help from MD.

Figure 3.S1: SI generated the samples and analyzed the data collected by the Fred Hutch Proteomics Core. SI created the panels.

Introduction

Targeted therapy of cancers driven by aberrant receptor tyrosine kinase (RTK) signaling has achieved remarkable clinical outcomes over the past two decades (125,258). RTKs normally serve to link extracellular signals to intracellular signaling pathways to influence various cell-based phenotypes such as, but not limited to, cell proliferation, differentiation, and changes in metabolism (6). In the case of cancer, these proteins become aberrantly activated, which leads to upregulation of several downstream signaling pathways where the net result becomes uncontrolled cell proliferation and survival (115). To combat such molecular aberrations, tyrosine kinase inhibitors (TKIs) have been developed to abrogate kinase activity and thus shut off constitutive upregulation of pro-cell proliferation signaling programs. TKIs have shown robust efficacy in reducing cancer burden in patients as detailed in many clinical trials (125,157,259,260).

Among other mechanisms, gain-of-function, nonsynonymous point mutations such as EGFR^{L858R} have been shown to cause oncogenic RTK signaling (120,261). The gold standard approach to detect gain-of-function RTK mutations involves characterizing mutations discovered in patient tumor samples that respond to TKI treatment in absence of other RTK driver mutations; however, this approach does not provide a streamlined, high-throughput way to anticipate new gain-of-function mutations. While *in silico* algorithms have been developed to predict the effect of mutations on protein structure and function (262-266), they are not accurate enough to replace functional validation in a lab setting. With the overwhelming number of “variants of unknown significance” detected by clinical sequencing, a formidable challenge exists to distinguish “driver” from “passenger” mutations. An approach that involves an initial *in silico* prediction step

that generates a smaller list of potential variants that is then functionally tested may facilitate efficient discovery of new oncogenic, gain-of-function mutations.

Like other RTKs, the orphan receptor tyrosine kinase, *ROS1*, has shown an ability to promote oncogenic transformation when aberrantly activated (125). Up to this point in time, *ROS1* has only been shown to play this role via fusion oncogenes that have been shown to serve as oncogenic drivers in a diverse set of cancers, most notably in NSCLC (83,199). Previous preclinical studies establish that oncogenic *ROS1* fusion proteins can be targeted effectively with tyrosine kinase inhibitors (TKIs) (140,200). Notably, robust clinical responses were achieved with the TKIs crizotinib and entrectinib in *ROS1*-fusion-positive NSCLC, which led to their FDA-approval for the treatment of *ROS1* fusion-driven NSCLC (164,201). With this said, it has not been demonstrated if either gene amplification of or gain-of-function point mutations in *ROS1* can also drive tumor formation (125). Such knowledge could potentially expand the cohort of cancer patients that may benefit from efficacious FDA-approved *ROS1*-TKI therapy.

In this study, we aimed to identify gain-of-function nonsynonymous mutations in *ROS1* that can be targeted with TKI treatment. We queried clinical sequencing data from the AACR Genie dataset in a tumor-agnostic fashion and utilized the “Sorting Intolerant From Tolerant” (SIFT) algorithm along with other filters to identify several *ROS1* TKD somatic mutations that warranted further wet-lab characterization (114,262,263). We then performed a series of cell-based *in vitro* experiments in multiple cell models to determine that two mutations centered around amino acid 2113, *ROS1*^{D2113N} and *ROS1*^{D2113G}, promoted increased cell proliferation and oncogenic growth that could be attenuated with *ROS1*-TKI. Finally, we performed *in vivo* studies to validate not only the tumor-forming potential of these *ROS1* mutants but also TKI efficacy in attenuating tumor growth driven by these mutants. These results establish that gain-of-function

mutations in *ROS1* can drive cancer formation and that FDA-approved targeted therapy can potentially be leveraged in an expanded cohort of cancer patients.

Materials and Methods

Cell lines, compounds, and plasmids

HEK-293T/17 (ATCC Cat# CRL-11268, RRID:CVCL_1926), MCF10A cells (ATCC Cat# CRL-10317, RRID:CVCL_0598), and NIH-3T3 cells (ATCC Cat# CRL-1658, RRID:CVCL_0594) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). HEK-293A cells (Cat# R70507; RRID:CVCL_6910) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Platinum-A and Platinum-E cells were purchased from Cell BioLabs, Inc. (San Diego, CA, USA). Primary antibodies used in the study are as follows: phospho-ROS1 (Cell Signaling Technology, Inc. (CST; Danvers, MA, USA); Y2274; #3078; RRID:AB_2180473), ROS1 (CST; #3287; RRID:AB_2797603), phospho-STAT3 (CST; Y705; #9145; RRID:AB_2491009), STAT3 (CST; #9139; RRID:AB_331757), phospho-ERK (CST; T202/Y204; #9101; RRID:AB_331646), ERK (CST; #4696; RRID:AB_390780), anti-FLAG (Thermo Fisher Scientific Cat# 701629, RRID:AB_2532497), α -TUBULIN (Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA, USA); 12G10; RRID:AB_1157911), ACTIN (DSHB; JLA20; RRID:AB_528068), GAPDH (Santa Cruz Biotechnology, Inc.; Dallas, TX, USA; sc-47724; RRID:AB_627678), phospho-S6 (CST; S235/236, #4858; RRID:AB_916156), and S6 (CST; #2317; RRID:AB_2238583). DMEM, DME-F/12, L-glutamine, and antibiotics were purchased from Genesee Scientific (San Diego, CA, USA). Fetal bovine serum (FBS) was procured from Neuromics (CA3 Biosciences, Inc., Edina, MN, USA). Bovine growth serum (BGS) was procured from VWR International (Radnor, PA, USA). Horse serum (HS) and calf serum (CS) were purchased from Thermo Fisher Scientific. Bovine pancreas insulin solution was purchased from Millipore Sigma (Cat# I0516; Burlington, MA, USA). Recombinant human EGF

was purchased from Peprtech (Cranbury, MA, USA), hydrocortisone powder from Millipore Sigma (Cat# H0888), and cholera toxin from Cayman Chemical (Ann Arbor, MI, USA). 0.05% Trypsin/0.53 mM EDTA was procured from Corning Inc. (Corning, NY, USA) while TrypLE™ was purchased from Thermo Fisher Scientific. Crizotinib, cabozantinib, and staurosporine were purchased from LC Laboratories® (Woburn, MA, USA), and lorlatinib, entrectinib, repotrectinib, and cycloheximide from Selleck Chemicals Inc. (Houston, TX, USA). TransIT-LT1 transfection reagent was purchased from Mirus Bio LLC (Madison, WI, USA). Gateway LR clonase was purchased from ThermoFisher. Protease and phosphatase inhibitors were purchased from Bimake (Houston, TX, USA). Pre-cast Bolt™ and NuPAGE™ 4-12% Bis-Tris protein gels and 4X LDS sample buffer were purchased from ThermoFisher. Pierce™ BCA Protein Assay Kit was purchased from ThermoFisher. CCK-8 reagent was purchased from Bimake. (Milpitas, CA, USA). Infusion Cloning HD Kit was procured from Takara Bio USA Inc. (San Jose, CA, USA). pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454; <http://n2t.net/addgene:8454>; RRID:Addgene_8454) (224), pENTR4-FLAG (w210-2) was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid # 17423 ; <http://n2t.net/addgene:17423> ; RRID:Addgene_17423), pGP (retroviral Pol and Rev gene plasmid) was a gift from Romel Somwar, and pCX4-puro was a gift from Tsuyoshi Akagi. Wild-type full-length ROS1 human cDNA was purchased from DNASU (Tempe, AZ, USA). Oligonucleotides were ordered from Integrated DNA Technologies Corp. (Newark, NJ, USA) and Eurofins Genomics (Eurofins Scientific, Luxembourg).

Growth and propagation of cell lines

All cell lines were grown in a tissue culture incubator with 5% CO₂ at 37°C. Cells were maintained in 75-cm² flasks and subcultured when approaching 75% confluence. NIH-3T3 cells were maintained in DMEM medium supplemented with 10% (vol/vol) CS and 1% (vol/vol) L-glutamine solution and detached using 0.05% Trypsin/0.53 mM EDTA. HEK-293T/17, HEK-293A, Platinum-A, and Platinum-E cells were maintained in DMEM medium supplemented with 10% (vol/vol) BGS and 1% (vol/vol) L-glutamine solution and detached using 0.05% Trypsin/0.53 mM EDTA. MCF10A cells were maintained in DMEM/F12 medium supplemented with 5% (vol/vol) HS, 1% (vol/vol) L-glutamine solution, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 µg/mL insulin, and 20 ng/mL EGF (MCF10A growth medium). MCF10A cells were detached using 2X TrypLE™ in 1X Versene and neutralized with MCF10A resuspension medium (DMEM/F12 supplemented with 20% (vol/vol) HS and 1% (vol/vol) L-glutamine solution). All cell lines were routinely tested for mycoplasma contamination (Lonza MycoAlert™ PLUS Mycoplasma Detection Kit) and verified to be free of contamination. Antibiotics were included in all cell culture media.

Cloning

The retroviral construct pCX4-ROS1 was cloned as described previously (140). Briefly, full-length cDNA was PCR amplified and inserted via infusion cloning into pENTR4-FLAG to append a C-terminal FLAG tag to ROS1 cDNA (pENTR4-ROS1-FLAG). Simultaneously, pCX4-puro was converted into a Gateway destination vector (pCX4-DEST-Puro) by inserting necessary att recombination sites and ccdB gene into the multiple cloning site. ROS1-FLAG was then placed into pCX4-DEST-Puro via an LR clonase reaction. ROS1 mutants were generated by

performing site-directed mutagenesis (QuikChange Mutagenesis Protocol, Agilent Technologies Inc., Santa Clara, CA, USA) of the pENTR4-ROS1-FLAG construct and then performing LR clonase reactions to place these mutants into pCX4-DEST-Puro.

Generation of stable isogenic cell lines

Replication incompetent, infectious ecotropic and VSV-G pseudotyped retroviral particles were generated using Platinum-E and HEK293T/17 cells, respectively. Platinum-E cells were transfected in 6-well plates with 2 µg pCX4 ROS1 transfer plasmid complexed with 8 µL TransIT-LT1 reagent while HEK293T/17 cells were transfected in 10 cm dishes with 6 µg transfer plasmid, 5 µg pGP, and 4 µg pCMV-VSV-G complexed with 45 µL TransIT-LT1 reagent. Supernatant was collected at 48- and 72-hours post transfection, filtered with 0.45 µm syringe filter, and then either applied directly to cells (ecotropic) or concentrated via ultracentrifugation (VSV-G pseudotyped) and then resuspended in 200 µL DMEM (MCF10A, HEK-293A). 2 µg/mL polybrene was then added along with virus to cells and incubated for 72 hours. Cells were then selected with 1 µg/mL puromycin for 3 days.

Immunoblotting

Lysates were prepared from cells using a standard cell lysis buffer as described before (220). Protein quantitation of cleared cell lysates was performed using BCA assay, and 15 µg of total protein with LDS sample buffer and reducing agent (beta-mercaptoethanol) was loaded on pre-cast 4-12% Bolt™/NuPAGE™ Bis-Tris Protein Gels. Proteins were transferred to nitrocellulose membranes and probed with relevant antibodies. All antibodies were used at

dilutions recommended by manufacturer. Blots were imaged using a Bio-Rad Laboratories (Hercules, CA, USA) ChemiDoc™ imaging station according to the manufacturer's protocol for immunoblot detection with use of horseradish peroxidase-conjugated secondary antibodies. Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific) was used to determine relative molecular weights of protein bands after imaging. Phospho-ROS1 detection required the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Densitometry was performed using Image Lab™ Software (RRID:SCR_014210, Bio-Rad Laboratories, Hercules, CA, USA).

Transient transfection assays to screen ROS1 TKD mutants

HEK-293T/17 cells were seeded in 12 well dishes and then grown to 70% confluence. Cells were then transfected with 2 µg pCX4 ROS1 DNA (wild-type and mutant variants in separate wells) complexed with 8 µL TransIT-LT1 reagent for 48 hours. Cells were then lysed and prepared for immunoblotting as prepared above. Lysates were loaded at equal volume (10 µL) onto 4-12% NuPAGE™ Bis-Tris protein gels and processed as described above. ROS1 catalytic activity was assessed by comparing phospho-ROS1 (pROS1) signal to total ROS1 (tROS1) signal via densitometry as a surrogate measure, where a higher pROS1/tROS1 ratio relative to wild-type was indicative of potentially higher catalytic activity.

Cell proliferation and oncogenic transformation assays

MCF10A proliferation assay: MCF10A cells transduced with different ROS1 variants were cultured (n=5) in 96 well plates and treated with DMSO or different ROS1-TKIs, dispensed with

multi-channel pipettes. For this assay, a modified growth medium was used where EGF and HS concentration was reduced (DMEM/F12, 2% (vol/vol) HS, 0.5 ng/mL EGF, other component concentrations kept the same as MCF10A growth medium described above). The final DMSO % was kept at $\leq 0.1\%$. 5000 cells were seeded and final working volume was 200 μ L. After addition of cells and treatment, cells were allowed to grow for 7 days. Cell proliferation/viability was assessed using a CCK-8 (WST-8, tetrazolium)-based assay and 490 nm absorbance was read at 4 hours post addition of CCK-8 reagent using a BioTek Synergy™ H1 plate reader (BioTek Instruments, Inc., Winooski, VT, USA). CCK-8 absorbance values of inhibitor-treated wells were normalized to vehicle-treated wells. Resulting data analysis was performed and plotted using GraphPad Prism v9.3 (GraphPad Software, RRID:SCR_002798, San Diego, CA, USA).

NIH-3T3 anchorage-independent soft agar growth assay: Anchorage-independent soft agar growth experiments were performed as described in detail previously (69). Briefly, 8000 cells (NIH-3T3 cells transduced with various ROS1 variants) were seeded in a lower % upper agarose layer, on top of a denser lower agarose layer, and treatment was added after cells were suspended in the agarose for 24 hours. 50 nM lorlatinib was used. % DMSO kept at $< 0.01\%$. Colonies were counted using the Gelcount™ colony counting platform (Oxford Optronix Ltd., Milton Park, Abingdon, UK). Resulting data was analyzed with Microsoft Excel (Microsoft Excel, RRID:SCR_016137) and GraphPad Prism.

Cycloheximide chase assay to assess ROS1 protein degradation half-life

HEK-293A cells transduced with pCX4 ROS1^{WT} or ROS1^{D2113N} were seeded on 6 well plates and grown to 70% confluence. Cells were then treated with 50 μ g/mL cycloheximide for

varying time points between 0 and 24 hours. Lysates were then collected, quantified, and loaded onto protein gels as described above. Prior to membrane blocking, Revert Total Protein Stain (LICOR Biosciences, Lincoln, NE, USA) was applied to quantify protein loading. Stain was then removed, and membranes were blocked and probed with anti-ROS1 antibody to assess total ROS1 expression. Densitometry was then performed as described above to quantify decrease in ROS1 expression with cycloheximide treatment over time.

Analysis of AACR Genie clinical sequencing data to find somatic ROS1 TKD mutations

AACR Genie data was queried for the *ROS1* gene and then downloaded from genie.cbioportal.org (112,113). All datasets on the website, including data from GENIE Cohort v11.0-public, DFCI-Profile Glioma Cohort 2013-2018, AACR Project GENIE AKT1 Cohort, and Metastatic Breast Cancer 2013-2016 datasets were included (114,267,268). The data was then annotated using the ‘Sorting Intolerant From Tolerant’ (SIFT) algorithm following the published protocol and then filtered with Microsoft Excel (263).

***In vivo* efficacy studies.**

Four-to eight-week-old female and male athymic nude (Nu/j) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed and handled under specific pathogen-free conditions in the University's Animal Care Facilities, which meet international standards; mice were regularly checked by a certified veterinarian responsible for health monitoring, animal welfare supervision, experimental protocols and procedures revision. All protocols involving animals and their care were conducted in accordance with the following laws, regulations, and policies governing the care and use of laboratory animals: Animal Welfare Act (AWA), Public

Health Service (PHS), the United States Department of Agriculture (USDA), and the Institutional Animal Care and Use Committee (IACUC), which self-regulates experiments at our institution. Experimental protocols have been reviewed and approved by the OHSU IACUC including members “ad hoc” for ethical issues. All animals and animal protocols used in research are covered by our IACUC protocol, overseeing all aspects of animal care and use to assure compliance with AWA regulations and PHS Policy. In compliance with federal regulation and OHSU institutional policy, all research staff completed Responsible Conduct of Research (RCR) and Animal Care and Use (ACU) education.

After an initial two-week environmental adjustment period, mice were placed under anesthesia using 2% isoflurane/oxygen, weighed, and ear punched for identification purposes. Tumor cells ($1-5 \times 10^6/50\mu\text{l}$) were mixed with matrigel (50ul on ice) to ensure tumor cells would remain in place and injected into the left or right flank. Animals were allowed to recover under supervision before being returned to animal facilities. Post-injection, animals were checked daily until tumor formation was detected at which time animal weight and tumor size were measured thrice weekly using balance and a digital caliper (cat 14-648-17, Fisher Scientific, Federal Way, WA). Tumors were allowed to grow until they reached 1500-2000 cc at which time animals were sacrificed and tumors were collected. At collection, tumors were washed in PBS and dissected with sterile scalpel blades with approximately Half of the tumor was fixed in 10% normal formalin (24 hours then placed in 70% EtOH) for immunohistochemistry, fresh froze in liquid nitrogen for DNA/RNA analysis, placed in bambanker and frozen for future cell line culture or placed in cell lysis buffer for future western blotting.

Initial studies of tumor formation were carried out in 4 female Nu/j mice (JAX lab) 4 to 6 weeks of age, mice were injected with ROS1-D2113N mutated NIH 3T3 cells (1×10^6) in the right flank after animals had been anesthetized with 2.5% isoflurane/2.5% oxygen. Mice were

monitored for tumor growth by caliper measurements twice weekly until tumor formation reached 2cc at which time mice were anesthetized with 2.5% isoflurane/2.5% oxygen and tumors were recovered at necropsy. Tumors were sliced and placed in the following conditions for future use; 1) fast freeze in liquid nitrogen and then stored in -80, 2) DNA/RNA SHIELD and stored at -80 3) BAM BANKER and stored in -80 4) CELL LYSIS BUFFER and stored at -80 and 5) FIXATION in 10% buffered formalin overnight and then stored at room temperature in 70% ethanol. Cell lines from tumors stored in BAM BANKER were grown out in DMEM/10% FBS and stored at -80 for further study.

Once tumor formation was documented, a follow-up study to test effect of TKI inhibitors on tumor growth was performed. ROS1-D2113N mutated NIH 3T3 cells were transduced with a lentivirus containing luciferase according to the manufacturer protocol (Cellomics Technology, Rockville, MD). Cells were grown in DMEM containing 10% FBS, penicillin/streptomycin, glutamate and fungicide for 24 hrs before transduction. Prior to transduction, polybrene was added to medium at 10 ug/ml while the lentiviral stock was thawed. Lentiviral stock was added to cells at 1:100. Cells were centrifuged at 1,200 x g for 60 minutes at room temperature and then incubated at 37°C overnight in a CO2 incubator at which time virus-containing transduction medium was removed and replaced with fresh growth medium. Cells were cultured for 48 hours and then selected for lentiviral transduction using blasticidin at 10ug/ml.

Tumor growth in vivo from luciferase producing cells was monitored using an in vivo imaging system (IVIS Lumina II, Caliper LifeSciences, Hopkinton, MA). For this, luciferin dissolved in PBS was administered to mice by IP injection at a final dose of 150mg/kg. After 6 minutes anesthesia was induced by 2.5% isoflurane/2.5% oxygen and mice were imaged with data being recorded.

For the TKI inhibitor study, 26 Nu/j mice (13 females/13 males) were injected with luciferase expressing ROS1-D2113N mutated NIH 3T3 cells (1×10^6) in the right flank after animals had been anesthetized with 2.5% isoflurane/2.5% oxygen. In addition, of these mice, 6 (3 female/3 male) were also injected on the left flank with ROS1-D2113N mutated NIH 3T3 cells (1×10^6) that had been plated and grown from the preliminary study to determine the feasibility of ROS1-D2113N mutated NIH 3T3 cell tumor growth from mouse passed cells. Cells cultured from the original tumor study are called PM14 (post-mouse ID#14) ROS1-D2113N mutated NIH 3T3 cells. Control animals (5 males and 5 females) were treated with vehicle by gavage (ethanol/PEG200/Water 10%/40%/50%). The lorlatinib animals (5 males and 5 females) were treated with lorlatinib (3mg/kg daily by gavage in ethanol/PEG200/Water 10/40/50). Lastly, the crizotinib animals (3 males and 3 females with PM14 and ROS1-D2113N mutated NIH 3T3 cells) were daily treated by gavage with crizotinib (100mg/kg in 0.5% methyl cellulose/0.5% tween 80).

In vivo tumor measurement began 3 days after injection. Tumor development was tracked and photon emission increased every measurement. TKI treatment began as soon as caliper measurement was possible (29 days after initial injection). At this time, animals were randomly assigned to TKI treatment after photon measurements were ranked from high to low to ensure higher expressing tumors based on photon emission were randomly dispersed amongst lower expressing tumors.

Mice were monitored for tumor growth by caliper measurements twice weekly until tumor formation reached 2cc at which time mice were anesthetized with 2.5% isoflurane/2.5% oxygen and tumors were recovered at necropsy. Tumors were sliced and placed in the following conditions for future use; 1) fast freeze in liquid nitrogen and then stored in -80, 2) DNA/RNA SHIELD and stored at -80 3) BAM BANKER and stored in -80 4) CELL LYSIS BUFFER and

stored at -80 and 5) FIXATION in 10% buffered formalin overnight and then stored at room temperature in 70% ethanol.

Statistical analysis

Mean $\pm$ SEM are shown unless otherwise stated. Student's t-test, one-way, or two-way ANOVA with either Dunnett's T3 (one-way) or Tukey's (two-way) multiple comparisons tests were used. P values < 0.05 were deemed statistically significant. Asterisks used in figures correspond with descriptions in figure legends that denote level of statistical significance. All data were plotted and analyzed using GraphPad Prism v9.3 (RRID:SCR_002798).

Results

Functional screening of clinical tumor sequencing data identifies several *ROS1* variants that potentially exhibit increased catalytic activity relative to wild-type *ROS1*

Our primary goal with this study was to identify potential gain-of-function mutations in the receptor tyrosine kinase, *ROS1*, that drive oncogenic transformation. To obtain a list of somatic mutations to test, we queried all samples in the AACR Genie database (genie.cbioportal.org), a database that contains clinical tumor sequencing data from multiple cancer centers and multiple cancer types (112-114). We found that 4,871 of 138,915 samples (4,545 of 124,014 patients) in AACR Genie contained an alteration in *ROS1* (112-114). We then applied a three-step filter to generate a final list for wet-lab analysis: 1) included only *ROS1* tyrosine kinase domain (TKD; amino acids 1945-2222 of *ROS1*) missense mutations that were either confirmed as somatic or that had a mean allele frequency (MAF) < 0.01, 2) removed any mutations detected in samples with >15 total alterations (>15 total mutation burden for samples with this information), 3) annotated remaining mutations with the ‘Sorting Intolerant From Tolerant’ (SIFT) algorithm and kept mutations that were predicted to have a deleterious impact. This filtering process yielded 34 missense somatic mutations in the *ROS1* TKD that we functionally characterized. As shown in **Figure 3.1A**, these mutations do not cluster in one area of the TKD, although some can notably be found in key regions of the TKD such as in and around the DFG motif and the kinase activation loop.

A

samples in AACR Genie

1900 2000 2100 2200 2300

Nucleotide binding

Activation Loop

B

GFP Wildtype L164G L164I G180E A188G S200N E202K G204E L208M R209C R210K R211N R212M R213N R214E R215G R216A R217V R218L R219K R220N R221E R222G R223E R224E R225E R226E R227E R228E R229E R230E R231E R232E R233E R234E R235E R236E R237E R238E R239E R240E R241E R242E R243E R244E R245E R246E R247E R248E R249E R250E R251E R252E R253E R254E R255E R256E R257E R258E R259E R260E R261E R262E R263E R264E R265E R266E R267E R268E R269E R270E R271E R272E R273E R274E R275E R276E R277E R278E R279E R280E R281E R282E R283E R284E R285E R286E R287E R288E R289E R290E R291E R292E R293E R294E R295E R296E R297E R298E R299E R300E R301E R302E R303E R304E R305E R306E R307E R308E R309E R310E R311E R312E R313E R314E R315E R316E R317E R318E R319E R320E R321E R322E R323E R324E R325E R326E R327E R328E R329E R330E R331E R332E R333E R334E R335E R336E R337E R338E R339E R340E R341E R342E R343E R344E R345E R346E R347E R348E R349E R350E R351E R352E R353E R354E R355E R356E R357E R358E R359E R360E R361E R362E R363E R364E R365E R366E R367E R368E R369E R370E R371E R372E R373E R374E R375E R376E R377E R378E R379E R380E R381E R382E R383E R384E R385E R386E R387E R388E R389E R390E R391E R392E R393E R394E R395E R396E R397E R398E R399E R400E R401E R402E R403E R404E R405E R406E R407E R408E R409E R410E R411E R412E R413E R414E R415E R416E R417E R418E R419E R420E R421E R422E R423E R424E R425E R426E R427E R428E R429E R430E R431E R432E R433E R434E R435E R436E R437E R438E R439E R440E R441E R442E R443E R444E R445E R446E R447E R448E R449E R450E R451E R452E R453E R454E R455E R456E R457E R458E R459E R460E R461E R462E R463E R464E R465E R466E R467E R468E R469E R470E R471E R472E R473E R474E R475E R476E R477E R478E R479E R480E R481E R482E R483E R484E R485E R486E R487E R488E R489E R490E R491E R492E R493E R494E R495E R496E R497E R498E R499E R500E R501E R502E R503E R504E R505E R506E R507E R508E R509E R510E R511E R512E R513E R514E R515E R516E R517E R518E R519E R520E R521E R522E R523E R524E R525E R526E R527E R528E R529E R530E R531E R532E R533E R534E R535E R536E R537E R538E R539E R540E R541E R542E R543E R544E R545E R546E R547E R548E R549E R550E R551E R552E R553E R554E R555E R556E R557E R558E R559E R560E R561E R562E R563E R564E R565E R566E R567E R568E R569E R570E R571E R572E R573E R574E R575E R576E R577E R578E R579E R580E R581E R582E R583E R584E R585E R586E R587E R588E R589E R590E R591E R592E R593E R594E R595E R596E R597E R598E R599E R600E R601E R602E R603E R604E R605E R606E R607E R608E R609E R610E R611E R612E R613E R614E R615E R616E R617E R618E R619E R620E R621E R622E R623E R624E R625E R626E R627E R628E R629E R630E R631E R632E R633E R634E R635E R636E R637E R638E R639E R640E R641E R642E R643E R644E R645E R646E R647E R648E R649E R650E R651E R652E R653E R654E R655E R656E R657E R658E R659E R660E R661E R662E R663E R664E R665E R666E R667E R668E R669E R670E R671E R672E R673E R674E R675E R676E R677E R678E R679E R680E R681E R682E R683E R684E R685E R686E R687E R688E R689E R690E R691E R692E R693E R694E R695E R696E R697E R698E R699E R700E R701E R702E R703E R704E R705E R706E R707E R708E R709E R710E R711E R712E R713E R714E R715E R716E R717E R718E R719E R720E R721E R722E R723E R724E R725E R726E R727E R728E R729E R730E R731E R732E R733E R734E R735E R736E R737E R738E R739E R740E R741E R742E R743E R744E R745E R746E R747E R748E R749E R750E R751E R752E R753E R754E R755E R756E R757E R758E R759E R760E R761E R762E R763E R764E R765E R766E R767E R768E R769E R770E R771E R772E R773E R774E R775E R776E R777E R778E R779E R780E R781E R782E R783E R784E R785E R786E R787E R788E R789E R790E R791E R792E R793E R794E R795E R796E R797E R798E R799E R800E R801E R802E R803E R804E R805E R806E R807E R808E R809E R810E R811E R812E R813E R814E R815E R816E R817E R818E R819E R820E R821E R822E R823E R824E R825E R826E R827E R828E R829E R830E R831E R832E R833E R834E R835E R836E R837E R838E R839E R840E R841E R842E R843E R844E R845E R846E R847E R848E R849E R850E R851E R852E R853E R854E R855E R856E R857E R858E R859E R860E R861E R862E R863E R864E R865E R866E R867E R868E R869E R870E R871E R872E R873E R874E R875E R876E R877E R878E R879E R880E R881E R882E R883E R884E R885E R886E R887E R888E R889E R890E R891E R892E R893E R894E R895E R896E R897E R898E R899E R900E R901E R902E R903E R904E R905E R906E R907E R908E R909E R910E R911E R912E R913E R914E R915E R916E R917E R918E R919E R920E R921E R922E R923E R924E R925E R926E R927E R928E R929E R930E R931E R932E R933E R934E R935E R936E R937E R938E R939E R940E R941E R942E R943E R944E R945E R946E R947E R948E R949E R950E R951E R952E R953E R954E R955E R956E R957E R958E R959E R960E R961E R962E R963E R964E R965E R966E R967E R968E R969E R970E R971E R972E R973E R974E R975E R976E R977E R978E R979E R980E R981E R982

To functionally screen for gain-of-function mutations, we cloned the 34 *ROS1* variants and wild-type ROS1 (ROS1^{WT}) into a retroviral backbone (pCX4-puro) and transfected these into HEK-293T/17 cells. To assess gain-of-function, we immunoblotted the resulting lysates and probed for C-terminal ROS1 autophosphorylation (Y2274), which can be used as a surrogate

measure for kinase catalytic activity (**Fig. 3.1B**). We then calculated the ratio of phosphorylated ROS1 to total ROS1 protein via densitometry for each variant and observed three categories of activity: variants that exhibited no discernable change in activity, variants that potentially exhibited a loss of catalytic activity such as ROS1^{K1980E}, and 5 variants that potentially exhibit a gain of catalytic activity relative to ROS1^{WT} (ROS1^{L1949F}, ROS1^{S1986F}, ROS1^{E2071K}, ROS1^{D2113N}, and ROS1^{I2151F}) (**Fig. 3.1C**). Of the variants that exhibit increased autophosphorylation, we observed ROS1^{D2113N} to have a marked increase relative to ROS1^{WT}. To further investigate the importance of amino acid 2113, we cloned a series of potential missense mutations at the 2113 site (ROS1^{D2113E}, ROS1^{D2113G}, ROS1^{D2113Q}, and ROS1^{D2113Y}) and a compound mutation variant ROS1^{D2113N + K1980E} (combines the gain-of-function and loss-of-function variants into one allele) and performed a similar transient transfection immunoblot analysis (**Fig. 3.1D**). Along with ROS1^{D2113N}, it was found that ROS1^{D2113G} also exhibited increased autophosphorylation, while the combined variant ROS1^{D2113N+K1980E} expectedly did not phenocopy ROS1^{D2113N} (**Fig. 3.1E**). Thus, our functional screening approach yielded several potential gain-of-function variants that warranted further functional validation.

Cell-based transformation and proliferation assays reveal that ROS1^{D2113N} is a gain-of-function missense variant that promotes oncogenic growth that can be attenuated with ROS1-TKI

Next, we stably transduced the five ROS1 variants of interest (L1949F, S1986F, E2071K, D2113N, I2151F), ROS1^{WT}, the potential loss of function variant ROS1^{K1980E}, and a positive control ROS1 fusion oncogene, SLC-ROS1, into NIH-3T3 cells (**Fig. 3.2A**). This cell model has been used extensively in the past to test the oncogenicity of a diversity of alterations, including those related

to *ROS1* (69,83,140). We performed an anchorage-independent soft agar assay, leveraging the fact that cancerous cells are likelier to proliferate and form robust colonies of cells in anchorage-independent conditions (immobilized in an agarose matrix) relative to pre-malignant cells, where little colony formation is normally observed. We seeded NIH-3T3 cells in agarose, measured colony formation after 3 weeks, and discovered that cells that expressed *ROS1*^{D2113N} promoted robust colony formation relative to NIH-3T3 empty vector and *ROS1*^{WT} and to a similar degree as SLC-*ROS1* (**Fig. 3.2B**). Importantly, this colony formation was successfully attenuated by concurrent *ROS1*-TKI treatment with lorlatinib, reducing colony growth back to empty vector and *ROS1*^{WT} levels (**Fig. 3.2B**). *ROS1*^{K1980E} indeed did not promote colony formation and exhibited decreased *ROS1* autophosphorylation relative to WT (**Fig. 3.2A-B**), increasing confidence that this confers loss-of-function to *ROS1*. Finally, the remaining five *ROS1* variants exhibited a statistically non-significant degree of colony formation relative to empty vector control and did not feature the same degree of growth as *ROS1*^{D2113N}.

Figure 2

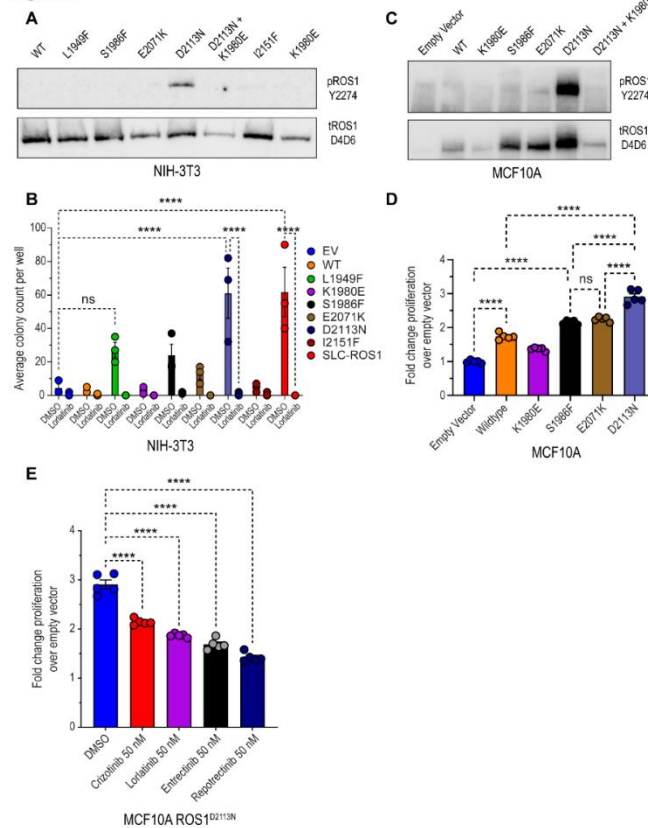


Figure 3.2: *In vitro* functional validation of screened ROS1 TKD variants. **A.** Immunoblot analysis of NIH-3T3 cells stably transduced with ROS1 variants. **B.** Soft-agar assay of NIH-3T3 ROS1 cell lines (n=3) treated with DMSO or 50 nM lorlatinib for 3 weeks. **C.** Immunoblot analysis of MCF10A cells stably transduced with ROS1 variants. **D.** MCF10A proliferation assay of ROS1 variants with reduced EGF (0.5 ng/mL) as assessed via CCK-8 assay after one week. Absorbance measured at 490 nm. Values represent fold change relative to cells transduced with empty vector. **E.** MCF10A proliferation assay of ROS1^{D2113N} with reduced EGF (0.5 ng/mL) treated with DMSO (< 0.01%) or various ROS1-TKIs as assessed via CCK-8 assay after one week. Absorbance measured at 490 nm. Values represent fold change relative to cells transduced with empty vector. Asterisks indicate statistical significance (**** P < 0.001; ns- P > 0.05).

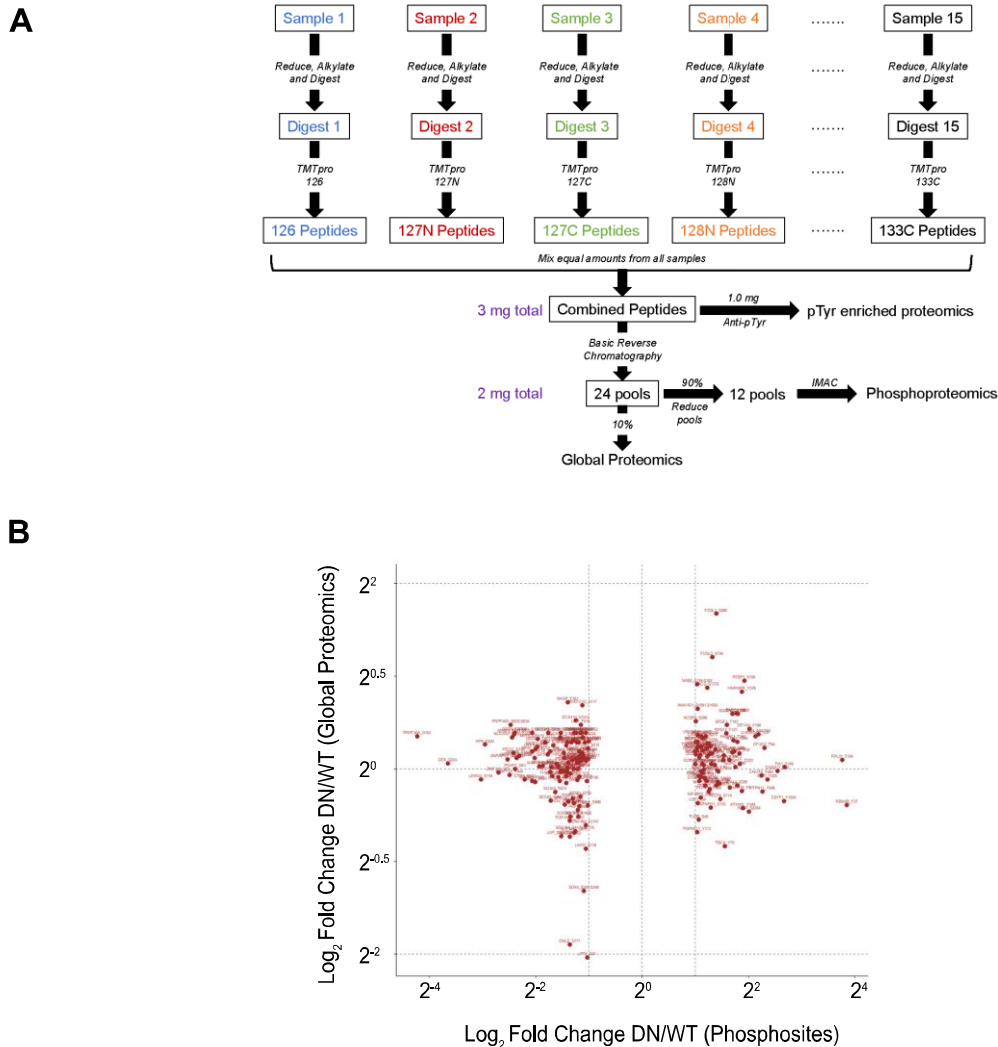
To verify that the phenotype seen with ROS1^{D2113N} was not unique to one cell line, we also transduced some of the ROS1 variants of interest into MCF10A cells, a pre-malignant breast epithelial cell line also utilized for assessment of oncogenic variants (269,270) (**Fig. 3.2C**). We performed a one-week proliferation assay where we reduced the concentration of EGF (20 ng/mL to 0.5 ng/mL) used to promote proliferation of MCF10A cells as normal MCF10A cells proliferate

less robustly at lower EGF concentrations. Similar to what we observed in the NIH-3T3 cells, ROS1^{D2113N} drove increased MCF10A cell proliferation at a lower EGF concentration relative to all other tested conditions (**Fig. 3.2D**). ROS1^{WT}, ROS1^{S1986F}, and ROS1^{E2071K} also promoted a slight increase in cell proliferation, while ROS1^{K1980E} predictably had minimal impact on proliferation (**Fig. 3.2D**). Finally, we tested a series of ROS1-TKIs (crizotinib, lorlatinib, entrectinib, repotrectinib, and cabozantinib) and found that each significantly attenuated MCF10A proliferation mediated by ROS1^{D2113N} like what was seen in the NIH-3T3 soft agar assay (**Fig. 3.2E**). As a result, our studies with both NIH-3T3 and MCF10A cells point to ROS1^{D2113N} as a gain-of-function mutation that promotes increased oncogenic transformation and cell proliferation in different cell types.

Phosphoproteomics reveal that ROS1^{D2113N} promotes increased PTPN11, GAB1, STAT, and mTOR activity relative to ROS1^{WT}

With the discovery that ROS1^{D2113N} can promote oncogenic transformation in NIH-3T3 cells and increased cell proliferation in MCF10A cells, we wanted to study how ROS1^{D2113N} affects downstream signaling. To achieve this, we performed global proteomics and phosphoproteomics (IMAC and phosphotyrosine enrichment) on HEK-293A cells that were stably transduced with either ROS1^{WT} or ROS1^{D2113N} as described in Supplementary Fig. 3.1A. Interestingly, there were relatively few differentially expressed proteins discovered via global proteomics, suggesting that bulk protein expression is not vastly affected by the presence of the

Figure S1



Supplementary Figure 3.1: Setup of global proteomics and phosphoproteomics plus analysis that ROS1^{D2113N} impacts post-translational modifications of downstream signaling mediators more so than total protein expression in comparison to ROS1^{WT} **A.** Setup of global proteomics and phospho-proteomics enrichment after lysate generation and quantification. **B.** Comparison of log₂ fold change values (ROS1^{D2113N}/ROS1^{WT}) obtained from phosphoproteomics (x-axis) vs. global proteomics (y-axis).

D2113N activating mutation (Supplementary Fig. 3.1B). In contrast, IMAC and pTyr phosphoproteomics revealed numerous statistically significant differences in a host of key downstream signaling proteins (**Fig. 3.3A**). Increased phosphorylation and activation were

Figure 3

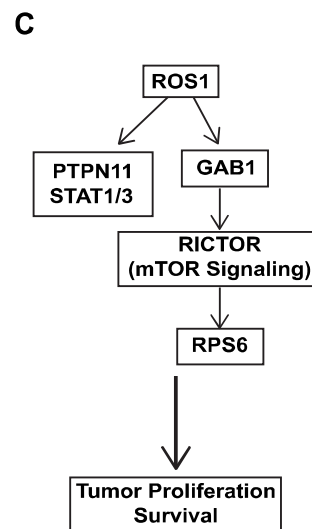
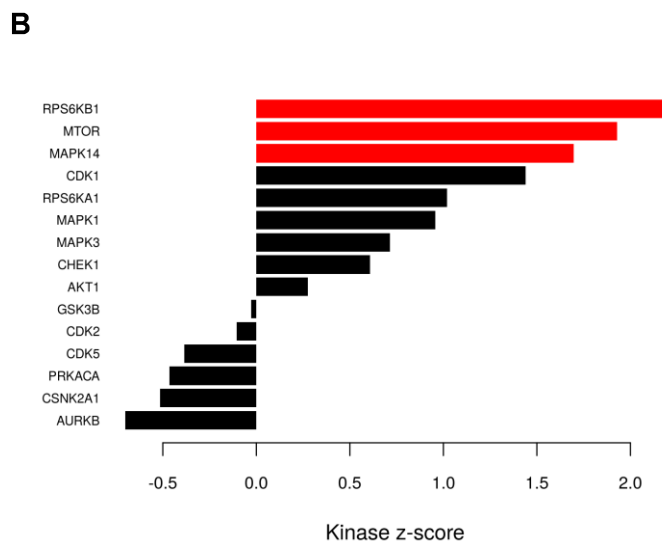
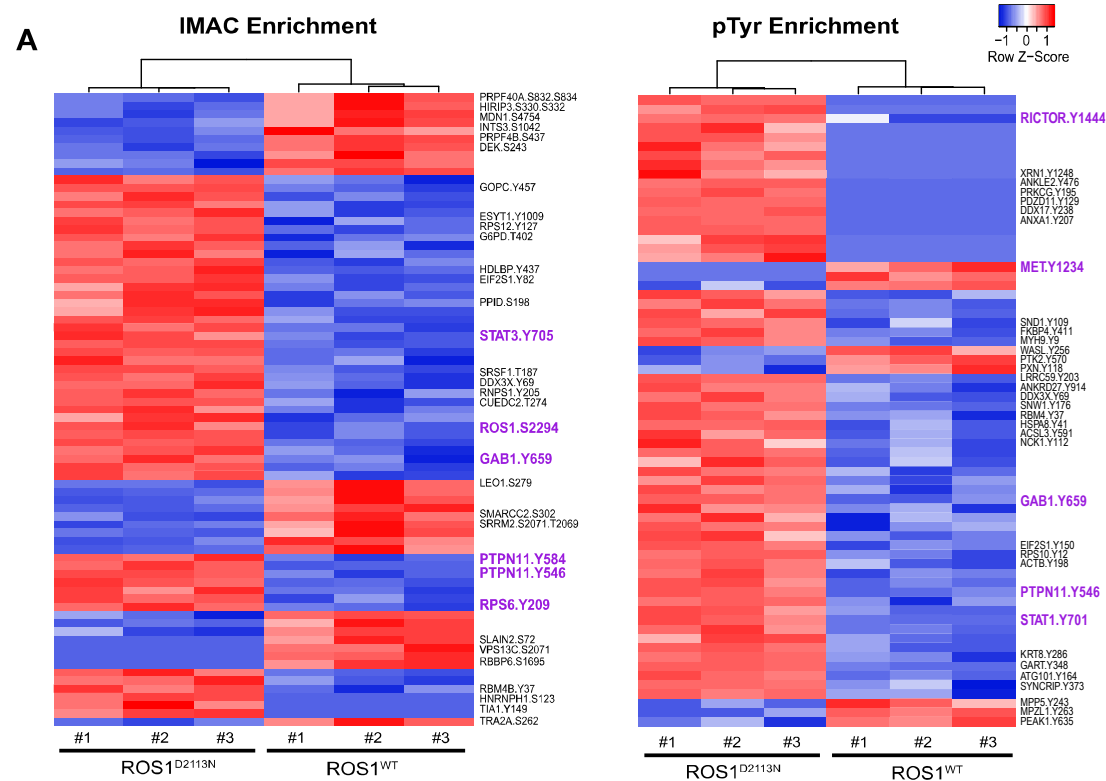


Figure 3.3: Phosphoproteomics identifies PTPN11 and STAT as major contributors to ROS1^{D2113N}-mediated downstream signaling. **A.** Heatmaps of IMAC enriched phosphoproteome (left) and phospho-tyrosine (pTyr) enriched phosphoproteome (right) of HEK-293A cells stably transduced with ROS1^{WT} or ROS1^{D2113N}. Red color indicates upregulation while blue color indicates downregulation. All phosphosites displayed are statistically significant ($q < 0.1$ for IMAC; $q < 0.05$ for pTyr). Rows and columns were clustered with the Average Linking method while the distance measurement method was Euclidean. **B.** Kinase substrate enrichment analysis (KSEA) of IMAC phosphoproteome from **(A)** comparing ROS1^{D2113N} to ROS1^{WT}. Red color indicates $q < 0.05$ while positive z-score indicates that kinase is predicted to be upregulated in ROS1^{D2113N} relative to ROS1^{WT}. **C.** General model of likely signaling paradigm driven by ROS1^{D2113N}.

observed in the RTK adapter proteins PTPN11 (SHP2) and GAB1, the transcription factors STAT1 and STAT3, RICTOR (contributor to the mTORC2 signaling complex), and ribosomal protein s6 (downstream effector of mTOR signaling). To better understand the effects on downstream signaling, we also performed Kinase Substrate Enrichment Analysis (KSEA) (271,272) on the phosphoproteomics data and found that substrates known to be phosphorylated by ribosomal S6 kinase and mTOR were increased in expression in ROS1^{D2113N} relative to ROS1^{WT} (**Fig. 3.3B**). Importantly, minimal evidence was observed of any increased activity of either the RAS-RAF-MEK-ERK or PI3K-AKT signaling pathways, which contrasts with the current thinking of signaling effects of ROS1 overactivation as studied with ROS1 fusion oncogenes. Hence, our current working model of ROS1^{D2113N}-mediated downstream signaling is summarized in **Figure 3.3C**, where PTPN11 and GAB1 likely interact with the ROS1 kinase domain and promote signaling through STAT activation and mTOR signaling (likely mTORC2 with the presence of RICTOR phosphorylation).

ROS1^{D2113N} responds to MEK inhibition but not JAK or mTOR inhibition

We next wanted to apply the findings from our phosphoproteomics study to our *in vitro* cell models from **Figure 3.2**. Immunoblotting of MCF10A cells revealed that ROS1^{D2113N} and SLC-ROS1 similarly activated SHP2 and STAT3, while ERK1/2 was relatively unaffected in both,

validating what was observed in the phosphoproteomics study (**Fig. 3.4A**). We then a separate NIH-3T3 soft agar assay with ROS1^{D2113N} and SLC-ROS1 and treated with vehicle, trametinib (MEKi), LY-294002 (PI3K/MTORi), or ruxolitinib (JAKi) and found that both cell lines responded similarly to the same drugs, with trametinib being the most effective inhibitor (**Fig. 3.4B**). This suggests that MEK signaling plays an important role in mediating the effects conferred by ROS1^{D2113N}; however, neither proteomics nor immunoblotting demonstrated any remarkable increase in MEK phosphorylation relative to other ROS1 variants tested. Importantly, neither inhibition of JAK (ruxolitinib) nor inhibition of mTOR (rapamycin) affected ROS1^{D2113N}-mediated colony formation, which somewhat conflicts with what was observed in the phosphoproteomics and immunoblotting experiments (**Fig. 3.4B**). It is possible that STAT activation may be independent of JAK activity in the case of ROS1^{D2113N}. Furthermore, the relative insensitivity of NIH-3T3 ROS1^{D2113N} to rapamycin may be due to the fact that mTOR signaling is facilitated via mTORC2 (RICTOR, rapamycin-insensitive) rather than mTORC1 (RAPTOR, rapamycin-sensitive). Thus, alternative small-molecule compounds that directly target STAT and mTORC2 signaling may need to be tested

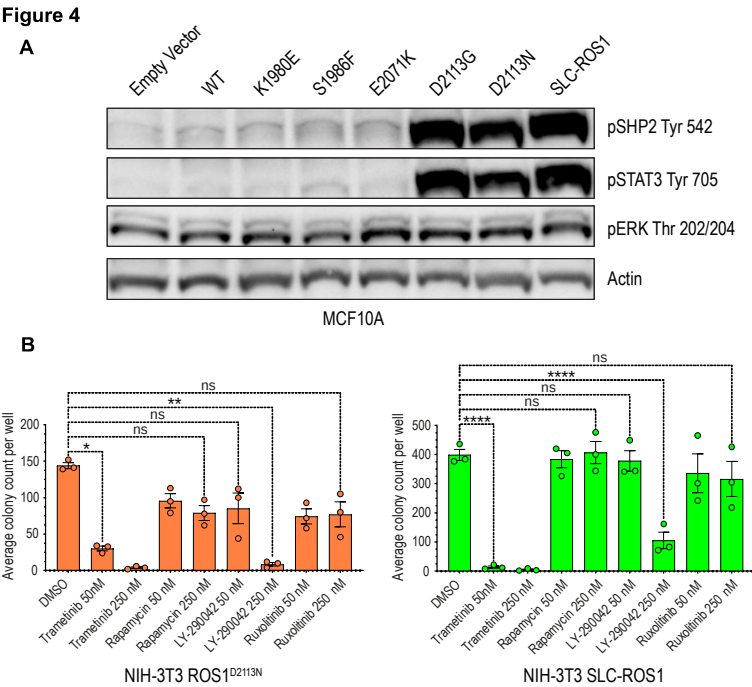


Figure 3.4: MEK inhibition attenuates ROS1^{D2113N} driven oncogenesis. **A.** Immunoblot analysis of MCF10A ROS1 cell lines probed for various downstream signaling proteins. **B.** Soft-agar assay of NIH-3T3 ROS1^{D2113N} and SLC-ROS1 cell lines (n=3) treated with DMSO (< 0.01%) or indicated ROS1-TKIs for 4 weeks. Asterisks indicate statistical significance (**** P < 0.001; ** P < 0.01; * P < 0.05; ns- P > 0.05).

to better elucidate how downstream signaling contributes to the oncogenic transformation mediated by ROS1^{D2113N}.

In vivo studies validate that ROS1^{D2113N} can promote tumor formation that can be reduced with ROS1-TKI treatment

Finally, we wanted to assess the tumor-forming potential conferred by the gain-of-function mutation ROS1^{D2113N} and in-vivo efficacy of ROS1-TKI against this mutation. First, we implanted NIH-3T3 empty vector, ROS1^{WT}, ROS1^{E2071K}, ROS1^{D2113N}, ROS1^{K1980E}, and SLC-ROS1 cells subcutaneously into Nu/J mice. As expected, the mice injected with NIH-3T3 cells that expressed the SLC-ROS1 fusion oncoprotein had the fastest growing tumors, with all mice (n=4) being sacrificed at 26 days post injection, while mice injected with either empty vector or ROS1^{K1980E} cells did not form tumors throughout the duration of the study (74 days) (**Fig. 3.5A-B**). While ROS1^{D2113N} did not drive tumor formation as quickly, tumor formation was next fastest in this group, where all replicates had noticeable tumor formation by day 32 and all mice were sacrificed by day 52 (**Fig. 3.5A-B**). Intriguingly, mice injected with ROS1^{WT} or ROS1^{E2071K} cells did grow tumors, but the growth rate was slower than ROS1^{D2113N}; ROS1^{E2071K} on averaged lagged in tumor growth by 1 week relative to ROS1^{D2113N} while ROS1^{WT} lagged by two weeks (**Fig. 3.5A-B**). Hence, our study suggests that ROS1^{D2113N} can promote oncogenic growth both *in vitro* and *in vivo*.

We also wanted to examine if ROS1-TKI treatment could attenuate the tumors driven by ROS1^{D2113N}. We subcutaneously injected NIH-3T3 ROS1^{D2113N} cells that were modified to express luciferase into Nu/J mice and waited for tumor growth to be detectable via caliper measurement

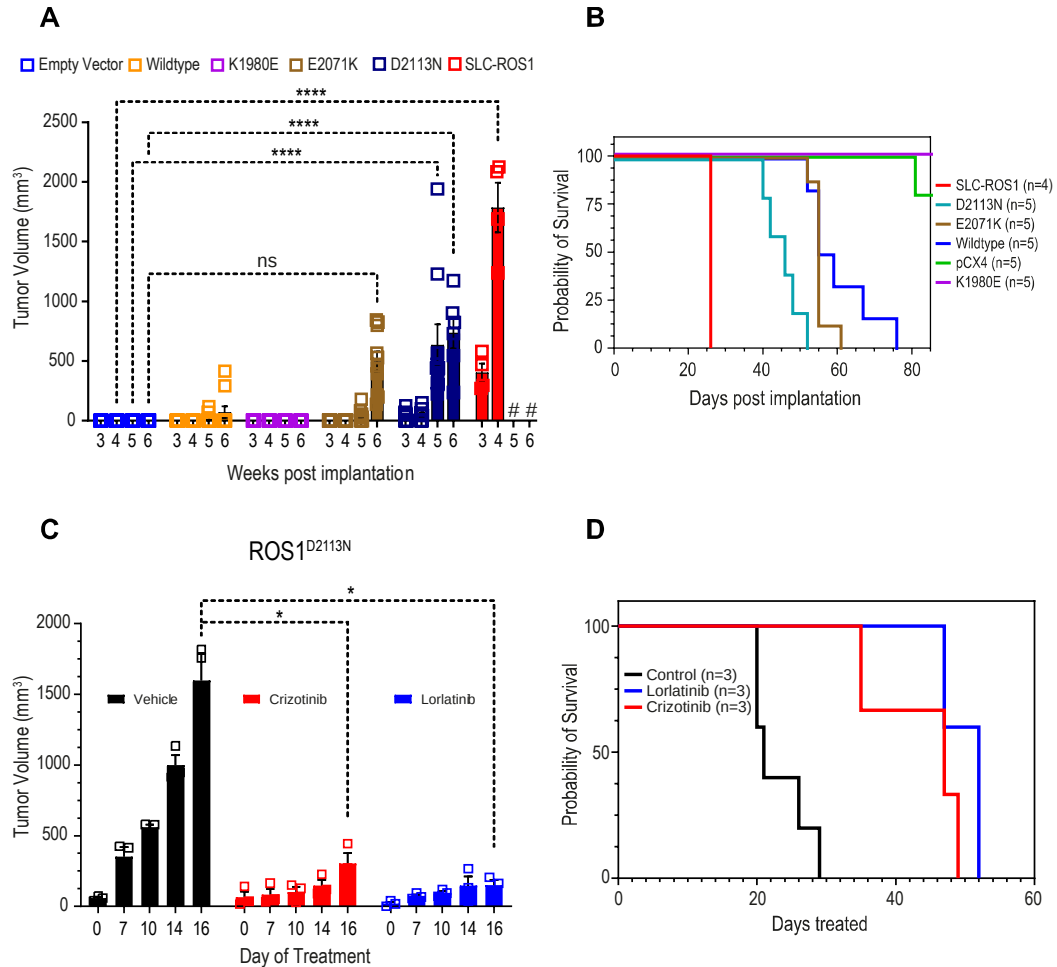
Figure 5

Figure 3.5: *In vivo* functional validation of ROS1^{D2113N} as a gain-of-function missense mutation. **A.** Tumor growth of NIH-3T3 empty vector, ROS1^{WT}, ROS1^{K1980E}, ROS1^{E2071K}, ROS1^{D2113N}, and SLC-ROS1 cells subcutaneously injected into female Nu/J mice over 6 weeks. n=5 for all except SLC-ROS1 (n=4). **B.** Kaplan-Meier survival curve of experiment described in (A). p < 0.0001. **C.** Tumor growth of NIH-3T3 ROS1^{D2113N} cells subcutaneously injected into female Nu/J mice and treated for 16 days with vehicle, crizotinib (100 mg/kg), or lorlatinib (3 mg/kg). n = 3. **D.** Kaplan-Meier survival curve of experiment described in (C). p < 0.0001. Asterisks indicate statistical significance (**** P < 0.001; * P < 0.05; ns- P > 0.05).

(29 days). We then randomized mice into vehicle, crizotinib, and lorlatinib treatment groups (n=3) based on photon emissions derived from an IVIS imaging platform. Over two weeks of treatment, both crizotinib and lorlatinib significantly attenuated ROS1^{D2113N}-driven tumor growth in mice

relative to vehicle as expected (**Fig. 3.5C**). Furthermore, mice treated with crizotinib or lorlatinib survived several weeks longer than vehicle-treated mice (**Fig. 3.5D**). Therefore, our data suggest that not only can ROS1^{D2113N} drive tumor formation but also can be targeted with ROS1-TKI *in vivo*, supporting the data collected earlier in our cell-based *in vitro* models.

Discussion

Receptor tyrosine kinases (RTKs), which consist of an extracellular ligand-binding region (ECD), a single pass transmembrane helix, and an intracellular region that features a juxtamembrane domain (JM), tyrosine kinase domain (TKD) and a C-terminal domain (CTD), normally serve to link extracellular signals to intracellular signaling pathways that affect cell proliferation, differentiation, and changes in metabolism (3). However, it has been well-documented in a diverse set of cancers that aberrant, constitutively active RTKs can upregulate downstream signaling pathways to promote uncontrolled cell proliferation and survival (115,273). To combat such molecular aberrations, small-molecule compounds (tyrosine kinase inhibitors; TKIs) have been developed that competitively or allosterically bind the TKD to shut down aberrant kinase activity (258). These TKIs have demonstrated immense success in a number of clinical trials and many have achieved FDA approval (123,125,155). Discovering novel alterations that can be targeted with TKIs will expand the number of patients that can benefit this efficacious form of anti-cancer therapy.

Multiple mechanisms exist by which RTKs become overactivated. First, many RTK genes have been shown to participate in chromosome rearrangements that are transcribed and translated into chimeric fusion oncoproteins, which feature constitutively active TKDs that can drive tumor formation in a wide variety of liquid and solid tumors (274). Various groups have found a number of 5' partner genes that can fuse via translocation or intrachromosomal deletion to the intracellular regions of RTK genes; in particular, studies have identified that *ROS1* can form fusion oncogenes with more than 55 partner genes (125). A second mechanism of RTK overactivation involves either complete or partial RTK gene amplification that leads to increased RTK expression and downstream cell signaling. Notable examples include *HER2* amplification in

breast cancer (275), *EGFR* amplification in gliomas (276,277), and *MET* amplification in non-small-cell lung cancer (NSCLC) (278). Finally, gain-of-function, nonsynonymous point mutations have also been shown to cause RTK overactivation that leads to cancer. While gain-of-function RTK mutations have been functionally characterized in both the ECD and intracellular regions of several RTKs, many mutations detected via clinical sequencing often group in the TKD, specifically in areas adjacent to the ATP-binding pocket and activation loop (118,279,280). A major issue is that a bottleneck currently exists to functionally validate gain-of-function mutations. The gold standard approach to detect gain-of-function RTK mutations involves characterizing mutations discovered in patient tumor samples that respond to TKI treatment in absence of other RTK driver mutations; however, this approach does not provide a streamlined, high-throughput way to anticipate new gain-of-function mutations. To better utilize clinical sequencing data to identify gain-of-function mutations, multiple *in silico* algorithms have been developed to predict the effect of mutations on protein structure and function (262-266); however, these algorithms are not accurate enough to entirely replace wet-lab functional validation. Hence, one potential solution to discover novel gain-of-function RTK mutations may be to first screen clinical sequencing data with *in silico* algorithms to generate a concise list of mutations and then apply functional validation approaches to distinguish “driver” from “passenger” mutations.

In this study, we discovered a gain-of-function missense mutation, ROS1^{D2113N}, in the orphan RTK, *ROS1*; no activating mutations have previously been described for this RTK (125). To identify ROS1^{D2113N}, we employed a two-step approach: first, we used an *in silico* algorithm, “Sorting Intolerant From Tolerant” (SIFT) (262,263), to identify a subset of missense mutations detected by clinical tumor sequencing that were predicted to have a deleterious impact on protein function (**Figure 4.1**). We then functionally tested this refined list of variants in multiple *in vitro* and *in vivo* models to demonstrate that ROS1^{D2113N} can drive oncogenic growth (**Figure 3.2 and**

Figure 3.5). Importantly, we also observed that treatment with the ROS1-TKIs crizotinib (FDA-approved) and lorlatinib (phase III clinical trials) can attenuate this oncogenic growth both *in vitro* and *in vivo* (**Figure 3.2 and Figure 3.5**). We also discovered that the MEK inhibitor, trametinib, was able to attenuate oncogenic growth mediated by ROS1^{D2113N} (273,281) (**Fig. 3.4B**). A future goal would be to better elucidate the similarities and differences of the downstream signaling effects between ROS1^{D2113N} and ROS1 fusion oncoproteins. We also found ROS1^{D2113N} to have oncogenic effects in multiple cell types (NIH-3T3- murine fibroblast, MCF10A- human breast epithelial, HEK-293A: human embryonic kidney). This potentially suggests that ROS1^{D2113N} can promote oncogenic transformation in a variety of cancer types and would concord with what has been observed with ROS1 fusion oncoproteins. This observation has support from clinical sequencing: ROS1^{D2113N} was detected in two melanoma cases and one oligodendroglioma in the AACR Genie database (112-114). More studies of ROS1^{D2113N} in a number of cell types would be beneficial to validate that TKI treatment against this variant can be beneficial in a diverse set of cancers.

One key takeaway from our study is that functional validation is necessary to distinguish “driver” and “passenger” mutations. While the use of *in silico* modeling allowed us to functionally characterize a smaller list of potential gain-of-function mutations, many of the mutations that were predicted to have a deleterious impact had minimal impact on ROS1 kinase activity. Several mutations were also found to exhibit decreased catalytic activity, which technically represents a deleterious impact on protein function but also highlights the limitations of using *in silico* algorithms to only detect gain-of-function alterations. Finally, we utilized another *in silico* algorithm, “Mutation Assessor”, to check the SIFT-generated mutation list and found widely differing predictions (266). Most notably, Mutation Assessor predicted ROS1^{D2113N}

to have minimal impact on protein function. Thus, *in silico* algorithms will need to be optimized and improved to use them exclusively for gain-of-function mutation screening.

In addition to the effect of ROS1^{D2113N} on promoting oncogenic growth, our group has previously shown that D2113N can also function as a resistant mutation to type II ROS1-TKI inhibitors such as cabozantinib in the context of ROS1 fusion oncoproteins. The amino acid position 2113 resides in the activation loop of the ROS1 tyrosine kinase domain (TKD). Previous modeling by our group has shown that the aspartic acid (D) to asparagine (N) mutation at 2113 decreases the flexibility of the activation loop relative to ROS1^{WT} as a repelling interaction between the negatively charged aspartic acid and glutamic acid at position 2120 becomes converted to a hydrogen bond between an asparagine and glutamic acid, which alters the stabilization of the activation loop conformation (124). As the D2113N mutation affects the allosteric binding site of type II ROS1-TKIs but not the ATP-binding site occupied by type I TKIs, future studies should investigate whether other type II ROS1-TKI resistant mutations confer a similar gain-of-function to full-length ROS1. Furthermore, it would also be important to establish if ROS1^{D2113N} does not respond to type II ROS1-TKI treatment like in the fusion oncoprotein setting (124). Interestingly, a study that characterized gain-of-function mutations in the full-length version of the RTK, *ALK*, also discovered that some of these mutations had previously been associated with resistance in an *ALK* fusion oncoprotein setting (118). A key difference is that the mutations in question, *ALK*^{L1196M} and *ALK*^{F1174L}, confer resistance to type I (crizotinib) rather than type II TKIs and this study did not test the ability of type I or II TKIs to attenuate oncogenic growth (118). Thus, more work in this area could greatly benefit the ability to better optimize TKI treatment for patients.

Finally, a key limitation of our study is that we focused only on potential gain-of-function mutations in the ROS1 TKD. While the majority of functionally characterized gain-of-function

RTK mutations have resided in the TKD (115,118,120,121), a recent study by Joshi et al. found several gain-of-function mutations that resided in the extracellular and juxtamembrane regions of NTRK1, NTRK2, and NTRK3 (119). The authors of this study discovered that these mutations promoted increased kinase activity by increasing the probability of receptor dimerization that normally plays a key role in promoting activation of the kinase-domain via trans-autophosphorylation (119). These findings demonstrate that multiple types of mutations in different regions of RTKs can therefore promote aberrant kinase activation and thus, future studies should expand the search area for potential gain-of-function variants. One way to potentially simplify such a search would be to leverage any structural information that may lead to key functional insights. As no crystal structure currently exists for the full-length ROS1 receptor (125), the discovery of such a structure would ease the discovery of novel gain-of-function mutations in locations outside the ROS1 TKD.

Acknowledgements

This project was supported with funding from the National Institutes of Health (NRSA: F30CA247253, R01CA233495), OHSU Knight Cancer Institute, and OHSU Doernbecher Children's Hospital Foundation. We also would like to acknowledge the American Association for Cancer Research and its financial and material support in the development of the AACR Project GENIE registry, as well as members of the consortium for their commitment to data sharing. Interpretations are the responsibility of study authors.

Summary and Conclusions

The role of MYC overexpression in promoting resistance to ROS1-TKI

The objective of my dissertation was to expand the scope and increase the durability of tyrosine kinase inhibitor treatment targeted against the receptor tyrosine kinase (RTK), ROS1. In chapter 2, we demonstrate that overexpression of MYC functions as a mechanism of therapeutic resistance to tyrosine kinase inhibitors (TKIs) in ROS1-positive NSCLC. We show this by performing MYC knockdown experiments in a patient-derived cell model and MYC overexpression experiments in multiple ROS1-fusion-positive cell models. Furthermore, our work indicates that this mode of ROS1-independent resistance will affect all generations of ROS1-TKIs, suggesting that treatment with a second, MYC targeted therapeutic option rather than development of a newer TKI will be necessary to overcome it. Future studies to understand the role of metabolic changes in ROS1-TKI resistance may also provide further options for combination therapy in addition to the CDK4/6 inhibitors and BET bromodomain inhibitors proposed in the study. Ultimately, mapping ROS1-independent mechanisms of resistance to ROS1-TKIs and designing potential therapeutic combination strategies to mitigate them will refine future clinical practice and help improve long-term outcomes of patients with ROS1-driven cancer. With this said, several questions are raised by the work in chapter 2 that require further exploration and I will discuss each question to a greater extent over the next several pages.

How might MYC promote therapeutic resistance to ROS1-TKIs?

MYC has been established as an oncogene in four contexts: as a key component of certain oncogenic retroviruses where wildtype MYC was fused to the viral element *gag*, as a participant in chromosomal translocations (t8:14) that yielded higher expression of wildtype

Figure 1

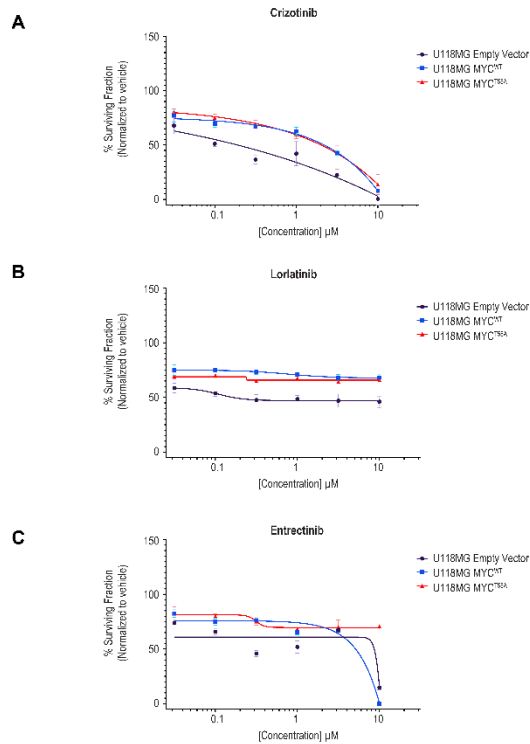


Figure 4.1: Overexpression of MYC in the ROS1-positive U118MG cell line does not confer the same degree of TKI resistance as observed in HBEC-ER and HCC78.

MYC in various lymphomas, upregulation of MYC via indirect mechanisms like growth factor stimulation (via RTK signaling for example) or loss-of-function mutations in tumor-suppressors that promote increased MYC expression, and copy-number amplification of MYC and associated MYC family members (such as MYCN or MYCL) seen in both liquid and solid tumors (282-286). In addition to driving tumor formation, my work detailed in chapter 2 of this dissertation also shows that MYC overexpression can act as a mechanism of therapeutic resistance to ROS1-TKIs. I demonstrated this with both knockdown studies in a patient-derived ROS1-positive cell line with MYC copy-number amplification (LUAD-0006) that featured ultra-high MYC protein levels and overexpression studies in ROS1-TKI sensitive cell lines (HBEC-ER and HCC78) with a degradation-resistant MYC mutant (MYC^{T58A}). From a translational perspective, one immediate

question becomes if MYC overexpression can serve as a resistance mechanism towards ROS1-positive cancers outside of non-small-cell lung cancer (NSCLC)? Work from my lab and others have demonstrated that ROS1 fusion oncogenes can drive formation of various tumor types such as Spitzoid neoplasms and both adult and pediatric gliomas (125). One limitation of my work is that I performed all my studies with cells of a broncho-epithelial lineage and as a result cannot definitively assess if the effects of MYC overexpression on ROS1-TKI efficacy will be as significant in cancers derived from other cell lineages. ROS1-TKI efficacy in the central nervous system (CNS) is particularly important due to both the appearance of ROS1-driven primary CNS tumors and a notable percentage of ROS1-driven NSCLC that metastasize to the CNS (125). Interestingly, I performed a pilot experiment where I overexpressed MYC in the ROS1-driven glioma cell line, U118MG, and did not see the same effect on ROS1-TKI efficacy as seen in the NSCLC cell lines (**Figure 4.1**). While I did not observe decreased sensitivity with ROS1-TKI when MYC was overexpressed in U118MG, this experiment was inherently flawed as I did not observe the same degree of ROS1-TKI sensitivity in unmodified U118MG cells as has been previously published, likely due to differences in cell culture conditions or acquired mutations in the cell line (69). Furthermore, MYC overexpression rendered ROS1-TKIs that can cross the blood brain barrier like entrectinib and lorlatinib just as ineffective as other ROS1-TKIs (**Fig. 2.1 and 2.3**) (164,287). Hence, more experiments will need to be performed on multiple ROS1-positive cell lines from different cell lineages to better elucidate the role that MYC overexpression may play in ROS1-TKI resistance across various cancer types.

A second translational question related to my work in chapter 2 involves if MYC overexpression can promote therapeutic resistance in non-small-cell lung cancers driven by other RTKs such as EGFR or ALK? In my view, this is likely to be the case for a few reasons. First, several mechanisms of resistance are shared between ROS1 and both EGFR and ALK, including

activating alterations in signaling proteins like BRAF that are downstream of RTK activation and alternative RTKs like KIT and MET (258,288,289). Second, other mechanisms of resistance not tied to a particular mutation, like histological transformation from NSCLC to small-cell lung cancer or epithelial-to-mesenchymal transition, have been shown in both EGFR and ALK-driven NSCLC (288). Finally, a case report published recently also identified MYC copy-number amplification as a likely mechanism of resistance to ALK-TKI in an NSCLC patient (251). Ultimately, the definitive approach to determine the role of MYC overexpression in promoting resistance to all TKIs in NSCLC will be to perform a similar series of experiments as performed in chapter 2 in the relevant patient samples and cell models for each RTK in question.

In addition to the research questions with direct clinical impact that stem from my work in chapter 2, more studies can be performed to better elucidate how supraphysiological levels of MYC are achieved and how MYC modifies signaling inside the cell to overcome RTK inhibition.

One challenge I faced in elucidating MYC overexpression as a mechanism of resistance to ROS1-TKIs was to recreate the level of MYC overexpression observed in the patient-derived cell line, LUAD-0006, in alternative human broncho-epithelial cell lines. Two observations from my experiments lead me to speculate that copy-number amplification of MYC may not be sufficient to cause MYC overexpression at the protein level, which contrasts with the oft-held assumption that amplification of a gene automatically leads to overexpression at the protein level. The first observation was that I could not overexpress MYC^{WT} to the same degree observed in LUAD-0006 cells in either the HBEC-ER or HCC78 cell lines (**Fig. 2.3A and 2.S6A**). This was despite expressing MYC^{WT} cDNA from a CMV promoter, a common element in plasmids to promote high intracellular expression of recombinant proteins and one that has been used successfully to promote robust gene expression in human airway epithelium (290). Furthermore, targeted sequencing of LUAD-0006 cells revealed that there were no other alterations of MYC

apart from the copy-number amplification, suggesting that wild-type MYC (MYC^{WT}) is expressed at a high level in these cells. I was only able to achieve similar MYC overexpression as seen in LUAD-0006 by utilizing a variant of MYC that contains a threonine to alanine substitution at position 58 (MYC^{T58A}) (291,292). MYC^{T58A} has been used in several studies to promote overexpression (232,293) and is based on research that identified phosphorylation of the threonine at position 58 (T58) as necessary for ubiquitination and subsequent proteasomal degradation of MYC (230,294). As a result, mutation to an alanine at position 58 prevents phosphorylation and attenuates MYC protein turnover, thus elevating MYC protein expression. The second observation was that I could not reduce LUAD-0006 MYC protein expression with the recently developed, small-molecule PP2A activator, DT-061, which was developed based on prior research that demonstrated how MYC is first activated and then degraded (295,296). First, various kinases like ERK or CDK proteins phosphorylate MYC at a serine at position 62 (S62) to stabilize the protein and increase target gene transcription (230,246). Second, phosphorylation at S62 allows for the kinase GSK-3 β to phosphorylate T58 (297). With T58 phosphorylation and subsequent proline isomerization with Pin1, PP2A can then dephosphorylate S62 and prime MYC for proteasomal degradation (246). While DT-061 has been shown to increase PP2A activity and promote MYC degradation, it was unable to do so in LUAD-0006, which suggests that other alterations in the LUAD-0006 genome may decouple the ability of PP2A to interact with MYC and dephosphorylate S62.

The combination of the inability to overexpress MYC^{WT} in other human broncho-epithelial cell lines and the ineffectiveness of DT-061 to reduce MYC protein expression therefore have led me to hypothesize that other mutations in LUAD-0006 may be present in addition to MYC CNA to allow for elevated MYC protein expression. One research question then becomes if MYC CNA is sufficient on its own to cause MYC protein overexpression? There have

been several studies that have tried to establish a correlation between copy-number amplification and gene overexpression in cancer but only a few that specifically assessed overexpression at the protein level (298-300). One study did indeed find a correlation between CNA and protein overexpression of the RTK, EGFR, in human glioblastoma samples, a cancer type with a high incidence of EGFR CNA, where the authors combined fluorescence *in situ* hybridization (FISH) and immunohistochemical analyses to link copy number variation to protein expression (298). However, subsequent studies in both aneuploid breast and cervical cancer cell lines and genetic loci found little to no correlation between copy number variation (CNV) and respective protein levels (CNV measured with microarray and protein expression with proteomics) (299,300). It is possible that the level of regulation that a protein is subject to may also influence the extent to which CNA can promote protein overexpression. MYC is tightly regulated as previously described, with both MYC mRNA and protein having a short half-life of 30 minutes (244-246,286).

Another beneficial research question to pursue would be if loss-of-function alterations in negative regulators or gain-of-function alterations in positive regulators of MYC can also increase MYC protein levels above a certain threshold that allow for MYC to promote therapeutic resistance? One study found that while MYC may be overexpressed in as high as 70% of all human cancers, only 20% of those cases had concurrent MYC CNA (301). Many proteins have now been discovered to regulate post-translational modification and degradation of MYC, including a number that are commonly mutated in cancer such as the E3 ubiquitin ligase, FBW7 (246). Future studies should recreate alterations in regulators of MYC that increase MYC protein expression to elucidate if these can also induce TKI resistance similar to what I demonstrated in chapter 2.

Yet another future question relevant to my work in chapter 2 concerns how MYC functions to allow cancer cells to continuously survive and proliferate in the absence of constitutive ROS1 signaling? MYC can be thought of as a master transcription factor; studies have shown that it can regulate 10-15% of the human genome (302,303). Normally, MYC functions by dimerizing with another protein, MAX, and then binding to DNA at E-box motifs (CACGTG) via its positively charged helix-loop-helix leucine zipper region (304). However, MYC has been shown to bind non-E-box DNA sites at higher, aberrant levels of expression (305,306). The increased engagement of MYC with non-canonical DNA binding sites presumably activates new signaling paradigms that can then contribute to oncogenic transformation and TKI resistance. Studies have described many oncogenic phenotypes that MYC can promote, which include cell proliferation, stem cell maintenance, genetic instability, and changes in metabolism (302,307-311). Hence, there are multiple downstream effects by which MYC can potentially mediate TKI resistance and as a result it would likely be impossible to narrow down the contribution of MYC to one target gene or pathway. Nonetheless, one could conduct experiments in ROS1-positive, TKI-sensitive cells (like HBEC-ER as shown in chapter 2) to compare how MYC overexpression affects cell signaling in the process of rendering cells resistant to TKI. A direct approach with regards to MYC function would involve performing CHIP-seq to assess how promoter and enhancer occupancy of MYC changes with overexpression in TKI-sensitive cells and subsequent resistance. Two other approaches that could lend insight into the specific target genes or pathways that MYC upregulates to promote resistance include RNA-sequencing or functional screening via CRISPR-Cas9, although both approaches require further validation of candidate targets.

MYC is a tough therapeutic target

Figure 2

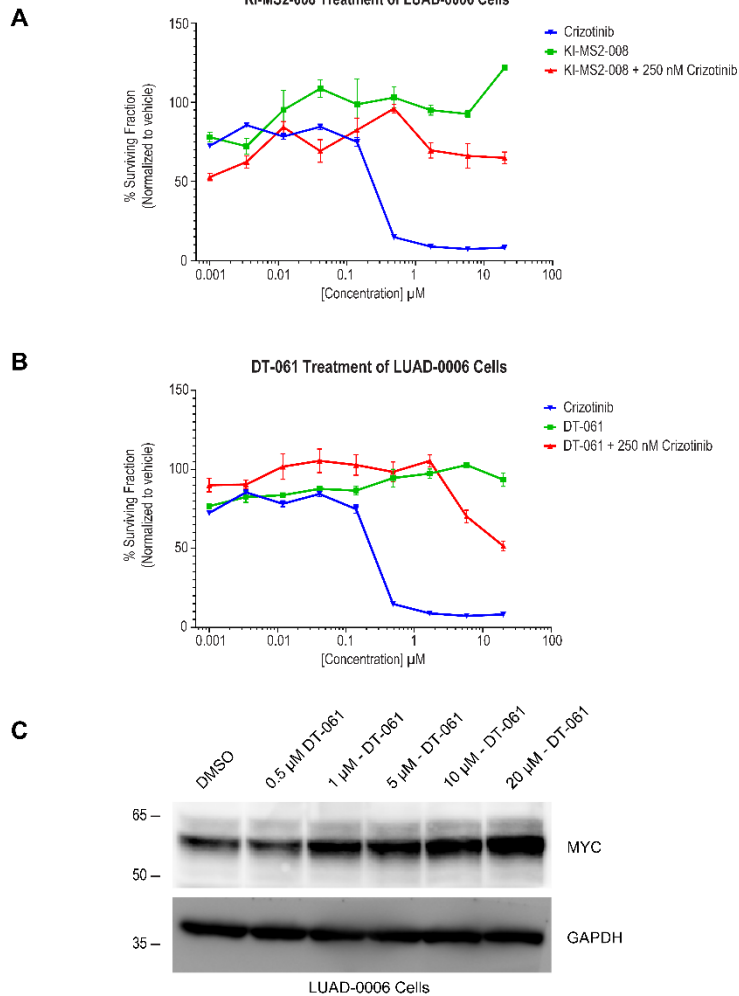


Figure 4.2. The indirect MYC inhibitors KI-MS2-008 and DT-061 do not re-sensitize LUAD-0006 cells to crizotinib and DT-061 does not affect MYC protein expression.

Finally, the key challenge with regards to overcoming MYC-mediated TKI resistance remains the fact that MYC has proven to be a difficult therapeutic target. To date, there has yet to be a reliable direct inhibitor of MYC approved by the FDA that can effectively attenuate its ability to bind DNA and upregulate target gene transcription. Most approaches to target MYC have so far entailed targeting proteins either transcriptionally upregulated by MYC or involved in

regulating MYC at either the transcriptional or post-translational stage (308). In chapter 2, I tested compounds from both categories: CDK4/6 inhibitors ribociclib and palbociclib to inhibit direct transcriptional targets of MYC (238,252,312,313) and the BET bromodomain inhibitors OTX-015 and ARV-825 to inhibit BRD4 (242,243), a protein that binds to acetylated histones and provides a scaffold to recruit transcription factors like MYC to facilitate gene expression (257,314). I demonstrated that both classes of inhibitors were able to synergistically resensitize LUAD-0006 cells to crizotinib (**Fig. 2.5A-D**). Furthermore, a subsequent *in vivo* experiment with subcutaneously implanted LUAD-0006 tumors conducted by our collaborators at Memorial Sloan Kettering Cancer Center (MSKCC) found that ribociclib indeed was more effective at attenuating tumor growth compared to crizotinib alone (**Fig. 2.5E-F**). The downside with these compounds is that they may not have the same efficacy in different ROS1-TKI resistant patients, whether it is due to a limited therapeutic window as has been observed with BET bromodomain inhibitors (315) or lack of efficacy that may be caused by several factors. Hence, there have been recent efforts to develop direct inhibitors of MYC, inhibitors that prevent MYC-MAX dimerization (316), and compounds that directly activate mediators of MYC protein degradation (296), all with promising pre-clinical data. In work that I did not publish, I tested KI-MS2-008, a compound that promotes MAX homodimerization and disrupts MYC-MAX heterodimerization (316), and DT-061, which I described earlier in this chapter, and found that neither compound achieved similar outcomes to CDK4/6 or BET bromodomain inhibition in LUAD-0006 cells (**Figure 4.2A-B**). For DT-061, I also assessed if the compound decreased MYC protein expression and found that MYC protein expression instead increased with increasing concentration of DT-061 (**Figure 4.2C**). This likely suggests that PP2A-mediated degradation of MYC is compromised, possibly due to other concurrent alterations in the LUAD-0006 cell line. Hence, more work will need to be done to establish reliable inhibitors of MYC that can successfully attenuate MYC activity regardless of

other mutations that are likely to be present in a cancer cell genome. One promising compound I didn't get a chance to study is MYCi975, a recently published compound that directly binds to MYC in the N-terminal MBII domain and was found to disrupt MYC/MAX homodimerization and MYC activity *in vitro* and *in vivo* (317). Future studies that aim to develop therapeutic strategies to overcome MYC-mediated TKI-resistance should test this and other similar direct MYC inhibitor compounds to find the most reliable solution to attenuate the many downstream effects of MYC-induced transcription.

Metabolic inhibitors as a strategy to combat ROS1-TKI resistance

An unexpected class of therapeutics that re-sensitized LUAD-0006 cells to ROS1-TKI treatment in chapter 2 were inhibitors of metabolic enzymes located in the mitochondria. The impetus to test these inhibitors initially came from a reverse-phase protein array (RPPA; multiplex antibody detection of cancer-relevant proteins) experiment where I discovered that certain mitochondrial proteins were upregulated when LUAD-0006 cells were maintained in media with ROS1-TKI (crizotinib) relative to vehicle, including proteins involved with cellular respiration and mitochondrial biogenesis (**Fig. 2.4A-B**). I then performed a flow cytometry experiment with the MitoTracker™ Green marker where I observed that mitochondrial mass increased when LUAD-0006 cells were treated long-term with crizotinib, in line with the observations from the RPPA experiment. Finally, I tested a panel of inhibitors that inhibited various metabolic enzymes (**Supplementary Data 2.2**) and found that the glutaminase inhibitor, telaglenastat (318,319), and the acetyl-CoA carboxylase inhibitor, ND-646 (237), synergistically re-sensitized LUAD-0006 cells to the ROS1-TKI, crizotinib (**Figure 2.4C**). These findings, along with other recent publications showing the utility of metabolic inhibition to combat therapeutic

resistance in cancer, may point to an orthogonal avenue by which resistance to TKI might be overcome in the scenario that an undruggable target is identified as a direct cause of resistance.

While the role of metabolism in cancer has been extensively studied for more than 100 years, famously with the discovery of the “Warburg Effect” in the 1920s (320), recent literature has expanded our understanding to the point where any metabolic pathway can fuel oncogenic cell proliferation and that a variety of factors internal and external to the cell may dictate which metabolic fuel is preferred (321). With that said, my work primarily focused on the intracellular signaling mediated by constitutive RTK signaling and MYC overexpression, and both alterations have been shown to be significant contributors to the metabolic landscape in cancer cells (303,307,322-324). As discussed previously in chapter 1, ROS1 fusion oncogenes are thought to traditionally upregulate canonical downstream signaling pathways like RAS-RAF-MEK-ERK and PI3K-AKT-MTOR (125), and both pathways have been studied extensively in the context of metabolism. Research has shown that RAS-RAF-MEK-ERK plays a role in upregulating glycolysis and lactic acid fermentation via the activation of various transcription factors like MYC and HIF-1 α , which increase expression of enzymes like hexokinase 2, phosphofructokinase, and pyruvate dehydrogenase kinase (enzyme that inhibits conversion of pyruvate to acetyl-CoA to reduce TCA cycle flux), while downregulating transcription factors like PGC-1 α that promote mitochondrial biogenesis and oxidative phosphorylation (322). The PI3K-AKT-MTOR pathway complements the effects of ERK via upregulation of glycolysis via post-translational phosphorylation of glycolytic intermediates and of synthesis of cellular building blocks such as nucleotides, proteins, and fatty acids. PI3K-AKT-MTOR has been shown to promote fatty acid synthesis both via activation of sterol regulatory-element binding protein (SREBP) transcription factors and by direct phosphorylation of ATP citrate lyase by AKT, likely to promote the increased membrane generation required by proliferating cells (325,326). As

mentioned earlier, MYC is activated downstream of RAS-RAF-MEK-ERK and PI3K-AKT-MTOR and can upregulate glycolytic enzymes and nucleotide synthesis (307). Interestingly though, MYC also promotes increased mitochondrial biogenesis and increased processing of glutamine (309,327). Studies in multiple cancer models have now shown that glutamine metabolism increases with overexpression of MYC, knockdown of MYC reduces glutamine usage, and that pharmacological attenuation of the enzyme, glutaminase, reduces MYC-driven tumor growth *in vivo* (324,328). Like PI3K-AKT-MTOR, MYC also activates numerous enzymes

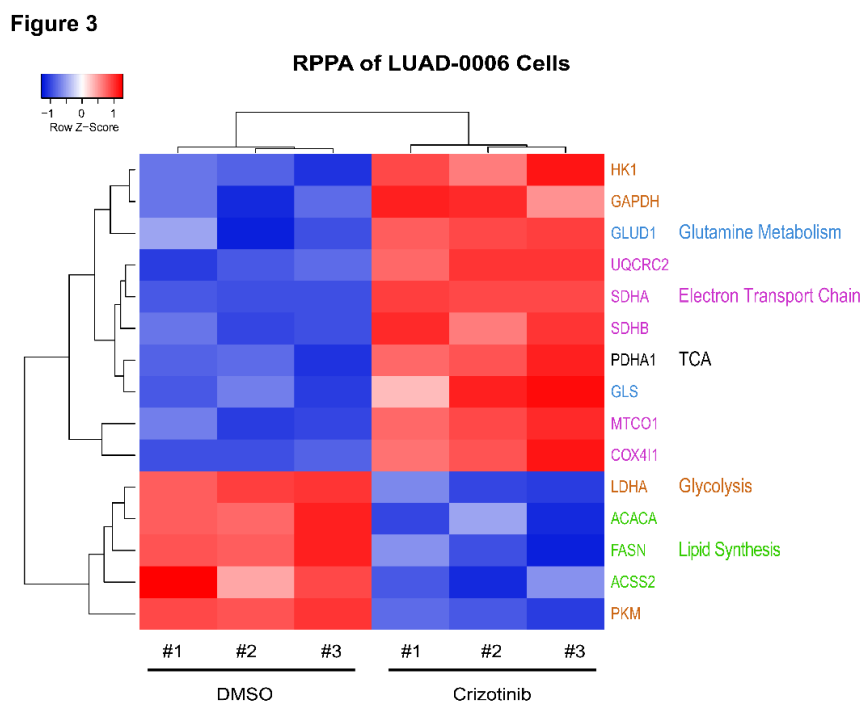


Figure 4.3. Protein expression (as measured by RPPA) of various metabolic mediators in LUAD-0006 changes with ROS1-TKI treatment.

involved in fatty acid synthesis, including acetyl-CoA carboxylase (ACC) (303). With the ever-increasing knowledge of the dynamic nature of metabolism in cancer, numerous small-molecule compounds have been developed to capitalize on the energy-intensive needs of cancer cells,

allowing for interrogation of metabolic dependencies in otherwise hard to treat cancers or cases of therapeutic resistance, as shown in my work.

As discussed in the previous paragraph, both overactive MYC and overactive RTK signaling can drive significant changes in metabolism. Thus, it is unsurprising that maintenance of LUAD-0006 ROS1-positive cells with TKI treatment led to stark changes in mitochondrial protein expression (seen in the LUAD-0006 RPPA experiment in **Figure 2.4A**) and an increased reliance on glutamine metabolism and fatty acid synthesis (**Figure 2.4C**). Further interrogation of the RPPA data also demonstrates that TKI treatment of LUAD-0006 significantly affects many of the metabolic enzymes regulated by ERK, AKT, and MYC signaling (**Figure 4.3**). It also is logical that glutaminase inhibition synergistically with TKI reduces LUAD-0006 cell proliferation per the numerous studies that show an increased reliance on glutamine as a fuel source in MYC-overexpressed cancers and in non-small-cell lung cancer (NSCLC) (324,329). The role of glutamine metabolism has also been explored in the setting of liquid tumors, particularly in the case of RTK-driven AML (FLT3+ AML). Multiple studies from Gregory et al. have shown that glutaminase inhibition can reduce proliferation of FLT3+ AML cells (318,319). Interestingly though, in the context of TKI resistance for liquid tumors, metabolic reprogramming has been shown as an emerging phenomenon. Specifically, in the context of FLT3-TKI resistance, Joshi et al. have shown in the transition from sensitivity to resistance that AML cells switch from glucose to lipid metabolism (CPT1A, sphingolipid metabolism) (330). Hence, it is possible that metabolic reprogramming may occur in the setting of TKI resistance in solid tumors too. In contrast to the efficacy of glutaminase inhibition, the sensitivity of LUAD-0006 cells to combined TKI and acetyl-CoA carboxylase (ACC) inhibition (ND-646) is harder to fully appreciate as although TKI treatment reduces ACC activity via increased phosphorylation of serine 79 and decreased protein expression, LUAD-0006 cells remain sensitive to ACC

inhibition. It is possible that the combination of TKI and extra pharmacological inhibition counteracts an ultra-high elevation of fatty acid synthesis mediated by AKT and MYC, respectively. A recent study also demonstrated the sensitivity of multiple KRAS-driven NSCLC mouse models to ACC inhibition, which may reveal a particular need for ACC signaling in NSCLC cells. Nonetheless, questions remain as to the role of metabolism in ROS1-TKI resistance. For example, were the changes in glutamine metabolism and fatty acid synthesis made possible by MYC overexpression or do these metabolic adaptations occur generally in ROS1-driven cancer cells that can compensate for loss of ROS1 signaling? In addition, are the TKI-driven metabolic adaptations unique to ROS1-positive NSCLC or are they conserved across multiple RTK-driven cancers and types? The use of metabolic analyzers like the Seahorse platform or detailed metabolomics and lipidomics studies may provide further insight into the similarities and differences in metabolic adaptations observed across multiple ROS1-independent mechanisms of resistance.

Pre-clinical screening strategies to pre-emptively predict ROS1-independent mechanisms of TKI resistance

Finally, a key challenge that I have pondered throughout my training to become a physician-scientist consists of the significant bottleneck researchers and clinicians face to validate the number of potential mechanisms of ROS1-independent resistance (and for that matter, RTK-independent resistance) observed in clinical sequencing of tumors. To recap, ROS1- or RTK-independent resistance involves situations where tumors no longer respond to TKI treatment despite having no secondary alterations in the primary RTK oncogene that may reduce TKI binding efficacy. The advent of next-generation sequencing (NGS) and targeted genomic sequencing has allowed clinicians and researchers to sequence patient tumors identify alterations

in numerous known oncogenes at various stages of treatment; this allows one to detect novel alterations that appear once a tumor stops responding to therapy (**Figure 2.S2A**). The common way to accurately identify the causal resistance mechanism in such a sample would either be to knockdown or restore each gain-of-function or loss-of-function alteration, respectively, in a cell line derived from the TKI-resistant sample or to functionally characterize each new alteration in an alternative TKI-sensitive cell line. In chapter 2, I performed both approaches to elucidate MYC overexpression as a ROS1-independent mechanism of resistance. However, the major caveat is we were able to work with a patient-derived cell line (LUAD-0006) that had only a few alterations to test, with MYC copy-number amplification standing out more than the rest. The relative genomic simplicity of the ROS1-TKI-resistant patient-derived cell line was fortuitous to quickly narrow down prospective resistance alterations. The issue arises when a TKI-resistant tumor acquires many new potential oncogenic alterations relative to the TKI-naïve tumor, it becomes cumbersome to functionally characterize and validate every potential resistant alteration in the event a previously known one is not identified.

To address this research and clinical bottleneck, a sophisticated pre-clinical screening approach to pre-emptively identify mechanisms of ROS1-TKI resistance may better inform clinicians to be proactive in prescribing alternative therapeutics when TKI therapy no longer elicits a favorable response. Any such screening approach would need to find both loss-of-function mutations in tumor-suppressors and gain-of-function mutations in oncogenes to capture the entire intracellular spectrum of ROS1-independent resistance. The important caveat would be that such a pre-clinical screening approach would not be able to capture mechanisms of resistance outside of intracellular genetic alterations, such as growth factors from the microenvironment that enable continued cancer cell proliferation or cell lineage switching or plasticity (331,332).

Nonetheless, an intracellular screening study might identify many clinically relevant resistant alterations, which could help combat therapeutic resistance.

The current standard for loss-of-function screening is to utilize genome-wide CRISPR-Cas9 libraries to knockout many genes and assess what knockouts contribute to TKI resistance. Many studies have utilized this approach to identify tumor-suppressors and pathways involved with drug resistance in cancer (333,334). For example, a 2019 study by Nechiporuk et al. identified that knockout of the tumor suppressors TP53, BAX, and PMAIP1 confer resistance to the BCL2 inhibitor, venetoclax (335). Genome-wide CRISPR knockout screens to detect resistant alterations involve a multistep approach that generally involves stable expression of the Cas9 endonuclease in the drug-sensitive cell line of interest, generation and transduction of a single-guide RNA (sgRNA) library with multiple sgRNAs targeted towards each gene of interest, antibiotic or fluorophore selection of cells that express both Cas9 and an sgRNA, and then subsequent treatment of the knockout pool with the drug of interest (335). Once resistant clones grow out after 1-2 weeks, genomic DNA can be collected and then sgRNA regions can be PCR amplified for subsequent amplicon-based NGS. Analysis is then performed to determine which sgRNAs corresponding to knocked-out genes are enriched in the resistant relative to vehicle treated cell pools to determine which genes and pathways can mediate drug-resistance. Such an approach can also be performed in a targeted manner by adjusting the number of sgRNAs in the CRISPR library to focus only on genes and pathways that have a known association with cancer and can be pharmacologically inhibited. The greatest limitation of the CRISPR knockout approach is that essential genes that may contribute to resistance at a lower level of expression will not be detected because a full knockout of such genes will prevent cell proliferation under any circumstance (336). In fact, the best example of this from my work came when I performed loss-of-function studies in LUAD-0006 cells. I found that MYC acted as an essential gene in

LUAD-0006 cells in line with other studies of MYC; this meant I was unable to use CRISPR-Cas9 to study if loss of MYC expression re-sensitized LUAD-0006 cells to crizotinib.

For gain-of-function mutation screening, the simplest approach would be to use a potent mutagen such as the chemical N-ethyl-N-nitrosourea (ENU) or expression of a hyperactive variant of the activation-induced deaminase (AID) enzyme (337-341). Both mutagens primarily promote G/C to A/T transitions, with other point mutations occurring at lower frequencies (337-341). Direct treatment of TKI-sensitive cells with ENU has been used previously to pre-emptively predict TKI-resistant point mutations in the ROS1 kinase domain (124,126). In these experiments, a sizeable number of wild-type colonies (clonal populations that grow out after ENU treatment in the presence of TKI but contain no alterations in ROS1 to explain resistance) are observed to grow out; these colonies likely contain mutations in alternative genes that may cause ROS1-independent resistance (124). The problem with this approach is that whole-genome sequencing (WGS) with sufficient depth would need to be performed on the cells both pre-mutagen treatment and post-mutagen plus TKI treatment to detect all potential alleles that were acquired prior to TKI resistance. Complex analysis would then need to be performed to sort variants based on frequency and genetic locus to generate a list for further validation. To overcome these challenges, one could instead use a CRISPR-based approach like CRISPR-X or TAM to target specific oncogenes to simplify sequencing efforts (341,342). Both CRISPR-X and TAM use a catalytically inactive Cas9 variant (dCas9) fused to hyperactive AID to limit mutagenesis to genes of interest (as specified by the single guide RNAs in the plasmid library) rather than adopting a whole-genome approach. These systems are more economical from a sequencing capacity as one can perform amplicon-based NGS to enrich for the sgRNAs present in the populations that survive TKI treatment. Enriched sgRNAs point to the genes that are modified in a clonal population to confer resistance to TKI and these genes can then be sequenced further

to identify specific resistant mutations. Both CRISPR-X and TAM have been used previously to identify resistant mutations to targeted therapy. Furthermore, CRISPR-based experiments can be fine-tuned to the research question one wants to answer. From a translational perspective, one can use a targeted sgRNA library to focus on known proto-oncogenes to efficiently identify targetable resistant alterations. If a researcher wanted to discover resistant alterations in unstudied genes in the context of cancer, a genome-wide CRISPR library or a CRISPR library with known proto-oncogenes subtracted out could be used instead. Hence, the use of newer CRISPR approaches may represent the best way to pre-emptively identify mechanisms of ROS1-independent resistance. Challenges of implementing CRISPR-based approaches would center around the on-target efficiency of the sgRNAs utilized and the ability to saturate all genes of interest, where gene sgRNA coverage is limited by the presence of protospacer-adjacent motifs (PAMs) in the relevant loci. Certain activating mutations may not be successfully generated with these approaches if PAMs that allow for sgRNA binding to DNA are lacking in hotspot regions of proto-oncogenes.

To overcome limitations of CRISPR-Cas9 knockout screens and CRISPR-AID systems, combined CRISPRa and CRISPRi screens could alternatively be used (336,343-345). CRISPRa/i technology uses dCas9 like CRISPR-X and TAM but instead of being fused to AID, dCas9 in CRISPRa and CRISPRi is fused to a transcriptional activator or repressor, respectively, to boost or attenuate gene expression when bound near a transcriptional start site (TSS). CRISPRa/i can also be made doxycycline-inducible to provide further control in the experiment. The advantage of this approach is that the genome of each cell remains unaltered apart from the stable expression of the dCas9 and sgRNA elements. A few recent papers have now employed CRISPRa/i genome-wide screens to effectively find contributors to drug sensitivity or resistance in multiple areas of study including cancer (336,344,346). The greatest obstacle to effectively applying this technique

to different cancer cell models would be to ensure that a significant magnitude of increased or decreased expression can be promoted across all genes involved in the screen. While CRISPRa/i screening studies have optimized sgRNA placement relative to the TSS (336), more studies would need to be performed to determine and optimize the extent to which individual genes are activated or repressed with this technology and importantly, how protein expression is altered.

Activating mutations in ROS1 promote oncogenesis

In chapter 3, my lab and I collected data that characterizes a novel gain-of-function missense variant, ROS1^{D2113N}, in the ROS1 tyrosine kinase domain. We queried AACR Genie (114), a publicly available clinical tumor sequencing dataset that combines data from multiple cancer centers around the United States, and found many “variants of unknown significance” in the tyrosine kinase domain of *ROS1*. By applying a 3-step filter that included the use of an *in silico* pathogenic mutation prediction algorithm, we generated a list of 34 variants for further functional testing. From this set of 34 variants, we then performed a series of *in vitro* and *in vivo* experiments to demonstrate that ROS1^{D2113N} represents an oncogenic gain-of-function mutation. Importantly, we also showed that ROS1-TKI attenuated the oncogenic proliferation mediated by ROS1^{D2113N}. Thus, our work reveals that gain-of-function mutations exist in ROS1, like other RTKs, which may increase the number of patients that may benefit from FDA-approved targeted therapy. One of the other key takeaways from our work is that *in silico* algorithms are insufficient to accurately predict novel gain-of-function variants. As a result, more functional wet-lab work will need to be performed to discover other mutations both inside and outside the tyrosine kinase domain that confer gain-of-function to ROS1. While such validation work remains inefficient, our discovery of ROS1^{D2113N} illustrates that detection of more gain-of-function mutations will expand the scope of ROS1-TKI anti-cancer therapy.

Like with our work with MYC-ROS1 TKI resistance, several questions arise from our study that may inform future studies; I will discuss these below:

ROS1^{WT} overexpression as a driver of oncogenic growth

Our work in chapter 3 describes how an activating mutation, ROS1^{D2113N}, promotes oncogenic cell proliferation in *in vitro* and *in vivo* models. One interesting finding that also came out of these studies is that wild-type ROS1 (ROS1^{WT}) also seems to confer a weaker but noticeable growth advantage to cells. In MCF10A cells *in vitro*, there was an increase in proliferation observed with MCF10A ROS1^{WT} relative to empty vector cells (**Figure 3.2D**), while in NIH-3T3 tumors *in vivo*, ROS1^{WT} drives tumor formation, albeit at a slower rate than seen with either SLC-ROS1 or ROS1^{D2113N} (**Figure 3.4B**). Notably, mice injected with the kinase-dead ROS1^{K1980E} variant did not grow any tumors, suggesting that overexpression of ROS1^{WT} may confer a growth advantage.

If ROS1^{WT} overexpression is sufficient to drive tumor formation and is targetable with ROS1-TKIs, this would further expand the cohort of patients who can benefit from this efficacious form of targeted therapy. In the AACR Genie database, ROS1 copy-number amplification (CNA) represents 6% of all cancer cases with a ROS1 alteration (114). Other studies would need to be pursued to determine if ROS1 CNA translates to ROS1 protein overexpression, similar to what was discussed previously in this chapter with MYC. Further *in vitro* and *in vivo* studies will also need to be performed to better elucidate if overexpression of ROS1^{WT} is either sufficient on its own or if it needs to cooperate with other oncogenic alterations to drive tumor formation. With this said, amplification of other receptor tyrosine kinases (RTKs) in various cancer types have been shown to drive tumor formation and respond to TKI or monoclonal antibody therapy directed against the RTK. Hence, a clear translational benefit may arise from such studies and as such, are worth pursuing.

How do downstream effects promoted by ROS1^{D2113N} differ to the SLC-ROS1 fusion oncogene?

Another question that came out of our work pertains to how ROS1^{D2113N} affects downstream signaling relative to ROS1 fusion oncogenes to promote oncogenic transformation. While the data presented in chapter 3 demonstrates that ROS1^{D2113N} can drive oncogenic transformation, it was clear throughout multiple experiments that this variant may not be as potent as the SLC-ROS1 fusion oncogene that we used as a positive control. The best example of this was the initial *in vivo* experiment described in **Figure 3.4**. While all mice injected with NIH-3T3 SLC-ROS1 cells grew tumors larger than 1500 mm³ prior to 30 days post implantation, ROS1^{D2113N}-driven tumors passed this threshold only after day 50 (**Fig. 3.4A-B**).

To better understand differences between ROS1^{D2113N} and SLC-ROS1 in terms of downstream signaling, we also performed global proteomics and phosphoproteomics on HEK-293A cells stably transduced with the SLC-ROS1 fusion oncogene and compared this to data garnered from HEK-293A ROS1^{D2113N} cells. In support of the phenotypic observations, the results showed that the SLC-ROS1 fusion oncoprotein exerts a more significant effect on downstream signaling than ROS1^{D2113N} (**Figure 4.4A-B**). In addition to a greater increase in STAT activation relative to the increase seen in the ROS1^{D2113N} to ROS1^{WT} comparison (**Fig. 3.3**), SLC-ROS1 also activated both the RAS-RAF-MEK-ERK and PI3K-AKT-MTOR pathways to a greater extent than was observed with ROS1^{D2113N} (**Figure 4.4C**). Interestingly, the IMAC and pTyr

Figure 4

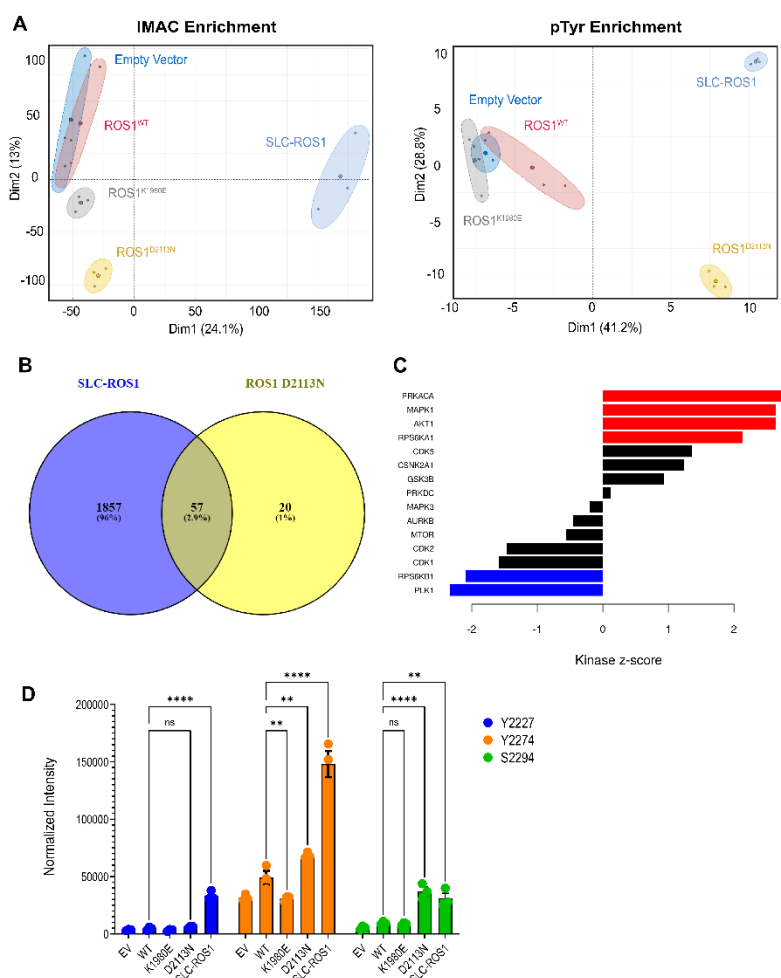


Figure 4.4. Phosphoproteomics of HEK-293A cells stably transduced with either empty vector, ROS1^{WT}, ROS1^{K1980E}, ROS1^{D2113N}, or SLC-ROS1. A. Principal component analysis of both IMAC enriched phosphosites (left) or phospho-tyrosine enriched phosphosites (right). B. Venn diagram of quantity of differential expressed proteins between cells that express ROS1^{D2113N} and SLC-ROS1. ROS1^{WT} is the control group for both conditions. C. Kinase substrate enrichment analysis of HEK-293A cells that express SLC-ROS1 relative to ROS1^{D2113N}. D. Comparison of different ROS1 phosphosites across all tested conditions (taken from IMAC-enriched phosphoproteomic data).

phosphoproteomics data also showed that while activation of ROS1 pS2294 was comparable between ROS1^{D2113N} and SLC-ROS1, SLC-ROS1 featured significantly higher expression of ROS1 pY2227 and ROS1 pY2274 (**Figure 4.4D**). This suggests that ROS1 pS2294 expression may represent an early step in ROS1 activation and be indicative of the capability to drive

increased cell proliferation but insufficient on its own to drive signaling through all canonical RTK signaling pathways.

Multiple experiments can be performed to interrogate the reasons for the difference in downstream activation between ROS1^{D2113N} and the SLC-ROS1 fusion oncogene. IP/MS of cells that contain ROS1^{WT}, ROS1^{D2113N}, or SLC-ROS1 can be performed to assess protein-protein interactions that may provide insight into the specific adapter proteins that are likely to activate certain downstream signaling pathways. Another objective would be to identify a serine-threonine kinase that catalyzes the phosphorylation of ROS1 at S2294, expression of which is comparable between ROS1^{D2113N} and SLC-ROS1 and likely the minimum determinant of the ability of ROS1 to drive oncogenic transformation. A second experiment would involve characterizing the structural differences between ROS1^{WT}, ROS1^{D2113N}, and SLC-ROS1. Two key challenges with this approach are that only the kinase domain of ROS1^{WT} has been successfully purified up till now and purification of the full-length ROS1 receptor represents a significant obstacle to overcome. A third challenge is that as the SLC-ROS1 fusion oncoprotein contains a wild-type kinase domain, purification of the entire SLC-ROS1 protein is likely required to identify structural differences between this and ROS1^{D2113N} that allow for greater kinase activation in the former. I am currently collaborating with Dr. Steven Mansoor at OHSU to tackle these structural biology questions. I have made important progress towards potentially purifying and crystallizing both the ROS1^{WT} and ROS1^{D2113N} kinase domains; however, this will only help us assess the difference in catalytic activity and kinase domain structure between these two variants. More work will need to be done in the coming months and years to optimize purification of both full-length ROS1 and SLC-ROS1 to enable to interrogation of more complex structural questions.

Pre-emptive screening to identify activating point mutations in ROS1

Finally, three major issues were brought up in chapter 3 that pertain to cumbersome nature of functionally characterizing variants of unknown significance (VUS) in ROS1 and other proto-oncogenes: first, there are hundreds of VUS that have been sequenced in cancer patients for ROS1 alone that require functional validation to distinguish driver from passenger alterations (114), two, current algorithms that rely purely on amino-acid sequence to predict pathogenicity of alterations are unable to accurately narrow down a list of potential activating mutations with any confidence, and three, activating mutations in ROS1 are likely to occur in regions outside of the kinase domain as observed in the NTRK family of RTKs. Hence, more efficient functional or structural screening approaches may increase the ability to predict activating mutations; however, each approach has its benefits and drawbacks.

Two potential functional screening approaches would involve either the use of plasmid libraries that contain every possible amino acid substitution in the ROS1 receptor (saturation mutagenesis) or the use of a targeted mutagenesis system like CRISPR-X to restrict mutagenesis to the ROS1 gene as described previously. The advantage of these approaches is that one immediately transitions to functional characterization without a filtering step and can therefore identify most, if not all, possible activating mutations. There are a couple of disadvantages though. First, while the saturation mutagenesis approach would evaluate all amino acid substitutions across all codons in ROS1, these experiments can be incredibly expensive and inefficient to perform due to the difficulty in constructing libraries that contain all possible amino acid substitutions (347). While recent papers claim to make the cloning process for saturation mutagenesis more economical and efficient (348,349), enough potential obstacles remain to doubt the utility of this approach. Second, a high-throughput assay would need to be developed to select

populations with activating mutations. Unlike resistance screens where one can treat a cell population with a drug of interest to apply selection pressure and enrich for potential resistant alterations, there are no reliable options to setup a similar method to screen for activating mutations. One approach might involve the use of a high-throughput ELISA where a phospho-ROS1 antibody might be used to detect increased ROS1 activity in multiple clonal populations containing different candidate variants, but a new antibody that detects ROS1 pS2294 would likely need to be developed as this phospho-site seems to be more sensitive to detect activating alterations than other phospho-sites.

To counter the economic issues with functional screening approaches, an alternative might be to solve the structure of full-length ROS1. As explained in previous chapters, all regions of a receptor tyrosine kinase are likely involved in regulating the kinase domain and activating mutations can be found in regions outside the kinase domain (KD), including the extracellular domain (ECD). While solving the full-length ROS1 structure is no easy task, doing so would allow one to elucidate how the various domains of ROS1 work to autoinhibit the kinase domain. Regions that are identified to keep the kinase in an inactive conformation can then be subject to molecular dynamics simulations to identify amino acid substitutions that undo the ability of any particular domain to restrict intracellular ROS1 kinase activity. With regards to the ROS1 ECD, recent work by a postdoctoral fellow, Chelsea Jenkins, found that deletion of certain regions of the ECD led to autophosphorylation and activation of the ROS1 kinase domain that drove oncogenic transformation in NIH-3T3 soft agar assays. Hence, a structural model of these and other regions in ROS1 can help elucidate specific interactions between amino acids that promote kinase autoinhibition. This filtered list can then be cross-referenced with clinical sequencing data and functionally characterized using the methods described in chapter 3 to identify activating mutations. A final bonus benefit of a structure-guided approach would be to aid in the discovery

of a ligand for ROS1, a finding that may crack the code as to what role ROS1 really performs in normal human cells.

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