Co-targeting kinases and epigenetic regulators for the treatment of Ras-driven lung cancer

Abstract

Lung cancer is the leading cause of cancer deaths worldwide killing over one million people each year. With the advent of targeted therapies, overall survival rates have improved for a subset of lung cancer patients including those with tumors harboring mutations in EGFR, BRAF, and ALK. While KRAS is the most frequently mutated oncogene in lung adenocarcinoma, there are still no effective treatments for these patients. This dissertation characterizes a promising therapeutic combination that targets kinase signaling and epigenetic regulation in KRAS-mutant tumors. Specifically, we show that dual inhibition of MEK and BRD4 triggers cell death and potent tumor regression. Demonstrable tumor regression in this setting is rare, making our work exciting for clinical development.

Moreover, we report that this therapeutic efficacy is dependent on the aberrant expression of the homeobox gene, HOXC10. HOXC10 is highly expressed in nearly half of all KRAS mutant tumors and is largely caused by unappreciated defects in PRC2 genes. Importantly, BET bromodomain inhibitors potently suppress HOXC10 expression and this suppression is required for cell death. We further show that HOXC10 and the Ras pathway cooperatively regulate pre-replication complex proteins in these tumors. It is this co-repression that triggers stalled replication, DNA damage, and ultimately cell death. Together this study characterizes a new therapeutic combination and identifies a predictive biomarker of efficacy.

We also examine this new subset of lung adenocarcinoma tumors characterized by an aberrant PRC2-HOXC10 axis. Here, we present striking preliminary results that indicate PRC2 defects and high HOXC10 expression may enhance the metastatic capabilities of these tumors.

In summary, this dissertation identifies a novel combination treatment, a predictive therapeutic biomarker, and a distinct subset of lung tumors that exhibit high expression of
HOXC10. These findings have promising clinical applications and inspire future mechanistic study of the role of HOXC10 in lung tumorigenesis. We hope this work sparks additional studies that target cancer at the intersection of signaling and epigenetics.
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Chapter 1: Introduction
This dissertation focuses on the development of a promising therapeutic drug combination for patients with KRAS-mutant lung adenocarcinoma. KRAS is the most commonly mutated proto-oncogene in lung adenocarcinomas. Yet despite decades of research, no successful targeted therapy (or chemotherapy) exists for these tumors. My approach has been to investigate the effects of co-targeting specific oncogenic kinases and epigenetic regulators. Here I will describe our success in combining MEK and BET bromodomain inhibitors for the treatment of lung cancer and its mechanism of action. Importantly, this work not only identifies a new promising therapeutic combination but also implicates a new KRAS-mutant subset of tumors warranting further study.

This introduction sets the stage for my work by defining the key signaling nodes and overlapping epigenetic vulnerabilities within KRAS-mutant lung tumors. First, I will discuss the major characteristics of lung cancer that define this clinical unmet need. Since my work focuses on suppressing the oncogenic RAS pathway, I will start by providing background on normal RAS signaling, describing how it is altered in tumorigenesis, and examining previous unsuccessful approaches for combatting KRAS-mutant tumors. I then expand on the role of the PRC2 complex and the BRD4 bromodomain reader in cancer, two epigenetic regulators central to this therapeutic story. Lastly, in the quest to characterize this epigenetics-based therapy, I identified a distinct subset of KRAS-mutant tumors defined by a deregulated PRC2/HOXC10 gene axis. To provide context for this new subtype of KRAS-mutant disease, I will finish this introduction by detailing the increasingly appreciated role for HOX genes in oncogenesis.

**LUNG CANCER**

*Clinical outcomes*

Lung cancer is the leading cause of cancer deaths worldwide resulting in more than one million deaths annually. The disease kills approximately three times as many men and two times as many women as prostate and breast cancer does, respectively (ACS, 2018). Despite
decades of clinical research, the five-year survival rate for lung cancer stands at a dismal fifteen percent, mostly due to poor outcomes associated with metastatic disease (Ding et al., 2008).

Despite the high lethality of this malignancy, funding for lung cancer research receives a paltry $2,399/life-lost versus breast cancer at $24,061/life-lost (SEER Lung Data). Many attribute this disparity due to the perception of lung cancer as a smoker’s disease that can be prevented. But while smoking represents a strong driver of mutagenesis, approximately ten percent of lung cancer cases in the United States occur in never-smokers (Hee1999). This proportion rises to twenty-five percent worldwide and is largely attributed to high environmental pollution and genetic susceptibility (Sellers et al., 1990; Sellers et al., 1998). Even more, smoking rates are on the rise in developing countries thanks to the shrewd marketing tactics of Big Tobacco companies in these unregulated settings (Saffer et al., 2000). Lung cancer also disproportionately affects people of color and individuals from low-income backgrounds (Alberg and Nonemaker, 2012). Taken together, these details point to the need for increased funding for this deadly cancer.

Classes of lung cancer

Lung cancer is divided into two primary classes: non-small cell lung cancer (NSCLC), which comprises 85% of all cases and small cell lung cancer (SCLC), which comprises the remaining 15% (Bender et al., 2014). There are three primary types of NSCLC including large cell carcinoma, squamous cell carcinoma, and adenocarcinoma (see Figure 1-1). Analysis of tumor histology and more recent molecular profiling projects have revealed that the NSCLC classes differ greatly in terms of cell of origin, biology, prognosis, and molecular drivers. Adenocarcinoma represents the most common NSCLC subtype comprising forty percent of tumors and is often the subject of many genomic and translational research studies.
Figure 1-1. Classes of lung cancer
Non-small cell lung cancer (NSCLC) represents eighty-five percent of all lung cancer tumors. Adenocarcinoma and squamous cell carcinoma are the two largest subtypes within the NSCLC class.

Oncogenic drivers of lung adenocarcinoma

Recently, The Cancer Genome Atlas (TCGA) performed a large-scale study of patient lung adenocarcinoma tumors to analyze their genetic makeup via mutational, expression, epigenetic, and copy number analyses (TCGA, 2014). This rich data source is publically available and is used throughout this thesis to generate hypotheses and examine the clinical relevance of our findings. A comprehensive published report confirmed previous studies finding that KRAS is mutated in approximately one-third of all adenocarcinoma tumors (TCGA, 2014). The vast majority of known oncogenic alterations in lung adenocarcinoma activate the RAS-MEK-ERK pathway via mutation at additional points along the Ras signaling pathway including EGFR, BRAF, and ROS/ALK/RET (see Figure 1-2). These mutations, especially those in KRAS, are mutually exclusive from one another and patients are often stratified for treatment according to their presence (TCGA, 2014). Secondary mutations in tumor suppressors such as TP53, CDKN2A, and STK11 are common across all tumor subtypes. Interestingly, the combination of a specific oncogenic driver and secondary mutations can further influence Ras signaling output and determine therapeutic sensitivities (Skoulidis et al., 2015). A more thorough review of these
heterogeneous characteristics in KRAS-mutant subtypes is presented in the introduction to Chapter 3.

Figure 1-2. Oncogenic driver mutations in lung adenocarcinoma. KRAS mutations represent almost one-third of lung adenocarcinoma tumors. NF1 were recently classified as a putative tumor suppressor because loss-of-function mutations in this gene are found in eight percent of tumors without known oncogene mutations. (Source: TCGA, 2014)

THE RAS SIGNALING PATHWAY

Under homeostatic conditions, the RAS pathway positively regulates cell proliferation, survival, and growth. RAS acts as a central signaling node, integrating and amplifying external stimuli into downstream outputs. Because of its central role in regulating cell growth, the regulation of RAS activation is tightly controlled.

Regulation of RAS activity

RAS is a heterodimeric GTPase protein that toggles between an activated GTP-bound state and an inactivated GDP-bound state (see Figure 1-3). The switch between the ON/OFF state of RAS proteins is regulated by two classes of proteins, RAS GEFs and RAS GAPs (Bourne, Sanders, and McCormick, 1990). RAS GEFs act as activators of RAS by facilitating the exchange of GDP for GTP, allowing RAS to adopt its active GTP-bound conformation.
Ras functions as a molecular switch, toggling between its ON GTP-bound state and its OFF GDP-bound state. RAS GEFs are positive regulators of RAS, promoting the exchange of GDP for GTP. RAS GAPs are negative regulators of RAS, stimulating the intrinsic GTP hydrolysis activity of RAS.

Conversely, RAS GAPs act as negative regulators of RAS activity by catalyzing the hydrolysis of GTP to GDP, thereby suppressing RAS activity. Since the GTP hydrolysis activity of RAS proteins is inherently slow, RAS GAPs are essential for ensuring that RAS activity is turned off. When RAS GAP activity is compromised, due to mutations or other genetic or epigenetic events, RAS proteins are more active, resulting in inappropriate proliferative signaling and often tumor growth. In fact, patients with germline inactivation of the RAS GAP protein NF1 often develop hundreds of benign tumors throughout their lifetime due to the resultant Ras activation following loss of heterozygosity of NF1 in somatic cells. Even worse, the risk for the development of uniformly lethal nervous system tumors (MPNSTs) is 8-13% over a patient’s lifetime (Menon et al., 1990; Legius et al., 1994; Evans et al., 2002). In this context, NF1 loss acts as an oncogenic driver primarily through the hyperactivation of RAS activity and its downstream effector pathways (Hiatt et al., 2001; Johannessen et al., 2008). NF1 mutations, as well as KRAS mutations, are relevant to the work described in this dissertation.
**RAS effector pathways**

Upstream induction of the RAS pathway often begins with the activation of receptor tyrosine kinases by growth factors. Upon stimulation, RAS adopts its activated guanosine triphosphate (GTP)-bound conformation, in part through the effects of GEFs such as SOS1. This activated conformation then induces the activation of several signaling cascades (including but not limited to) A) the RAF/MEK/ERK, B) PI3K/AKT/mTORC1 and C) Ral-GEF/PLCε pathways (see Figure 1-4). While other Ras effector pathways have been identified, these three major signaling cascades have been shown to promote growth, survival, and cell cycle progression and are the best studied.

![Diagram of RAS effector pathways](image_url)

**Figure 1-4. RAS effector pathways regulate cellular growth and proliferation**
Simplified schematic showing the main downstream effector pathways activated by RAS that jointly regulate transcription, cell cycle progression, survival, calcium signaling, and other pathways. Figure adapted from (Downward 2003).

This thesis primarily focuses on suppressing RAS signaling through inhibition of the RAS-MEK-ERK effector pathway. The RAS-MEK-ERK pathway is directly regulated by input from activated RAS, which then binds and recruits RAF proteins (e.g. ARAF, BRAF, and c-
RAF1) to the membrane. Raf subsequently phosphorylates and activates mitogen-activated protein kinases 1 and 2 (MEK1 and MEK2), leading MEK to phosphorylate and activate extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) (Leevers and Marshall, 1992; Wood et al., 1992; Marais et al., 1995; Leevers, Paterson and Marshall, 1994). ERK drives cell cycle progression through phosphorylation of cytoplasmic kinases (i.e. RSK and MNK family kinases) and through its phosphorylation of key transcription factors (e.g. ETS family transcription factors) (Downward, 2003; Roux and Blenis, 2004). The transcriptional RAS-MEK-ERK response is often used as a downstream read-out of RAS activation and can help quantify how strongly the RAS pathway is activated or suppressed under a variety of conditions, a point that is relevant for this dissertation.

**The RAS pathway in cancer**

The RAS pathway plays a fundamental role in regulating pro-proliferative and pro-survival signals in normal cells. Considering this normal function, it is unsurprising that activating mutations occur frequently in either RAS itself, upstream regulators, and/or downstream effectors in a majority of cancers. While there are over thirty characterized RAS family genes, to date, only three have established roles in carcinogenesis. Mutations in these \( H \)-, \( K \)-, and \( NRAS \) isoforms occur in approximately 20-30% of all human cancers. \( KRAS \) is the most frequently mutated isoform in RAS-driven cancers and through alternative splicing, its locus encodes two different protein forms, KRAS4A and KRAS4B. Both isoforms are mutated as a consequence of \( KRAS \) mutations (Tsai et al., 2015). While \( KRAS \) is mutated in 33% of lung adenocarcinomas, \( NRAS \) is only mutated in 1% of these tumors and \( HRAS \) mutations have not been detected (Ding et al., 2008). Conversely, \( HRAS \) and \( NRAS \) mutations abound in other tumor types, most notably skin cancers (TCGA, 2015). The tissue-specificity of RAS mutations is an area of active study. Work so far suggests that specific cancers select for certain mutations due to differential tissue expression patterns and unique functional activities of each RAS family member. These
features include different effectors or subcellular localizations, but more work is needed to fully understand these differences (reviewed in Lau and Haigis, 2009).

The majority of KRAS mutations occur in codons 12 or 13 in which the glycine residue is replaced with a more bulky amino acid. This single amino acid substitution does not affect the active site of RAS, but changes the protein conformation so that it is no longer susceptible to RAS GAP regulation. Since the process of GTP hydrolysis is inherently slow, lack of negative RAS GAP regulation effectively leaves RAS constitutively active. Another recurrent KRAS mutation occurs at Q61. This mutation impairs RAS’ catalytic site by decreasing the intrinsic GTP hydrolysis process through stabilization of the transition state, resulting in a protein with low hydrolysis rates and high signaling output (Der, Finkel, and Cooper 1986; Ahmadian et al., 1999).

To better understand the functional consequences of different mutant alleles, labs have more recently relied on phospho-proteomic and other global approaches to examine downstream differences in signaling among codon site changes and mutations within the same allele (i.e. G12V vs. G12C) (Lau et al., 2013). Not only do different types of KRAS mutations lead to different prognoses and therapeutic sensitivities, but each allele-specific change has also been associated with differential pathway activation (as reviewed in Haigis, 2017). For example, in isogenic colorectal cancer cell lines, codon 12 mutants exhibit stronger activation of the proteome than G13D mutations (Hammond et al., 2015). More studies are needed to determine if allele-specific downstream signaling differences are largely qualitative or quantitative in nature.

While mutations in RAS itself are quite common, occurring in up to 90% of some cancers, mutations in upstream inputs and downstream effector proteins are also frequent.
**RAS-driven lung cancer**

As stated previously, KRAS is the most frequently mutated oncogenic driver in lung adenocarcinoma, occurring in almost one-third of all tumors. But KRAS mutations are only one mechanism by which the RAS pathway is activated in lung cancer. Other common oncogenic drivers include EGFR, ALK, MET, and ROS mutations, all signaling proteins that rely on RAS activation for their oncogenic activity. More recently, the TCGA identified additional putative drivers that stimulate the RAS pathway including a large subtype (8.3%) characterized by mutations in NF1, a RAS GAP protein. Interestingly, loss of NF1 in combination with TP53 mutation has been reported to represent a distinct subset of patient tumors. While these NF1-TP53 mutant tumors share many of the same clinical characteristics as KRAS-mutant lung adenocarcinoma, NF1-TP53 mutant tumors appear to be more histologically diverse with poor differentiation as compared to KRAS-mutant tumors (Redig et al., 2016). This observation suggests that there may be both similarities and differences between KRAS and NF1 mutant lung cancers. Nevertheless KRAS mutation and loss of NF1 represent two types of RAS-driven lung adenocarcinoma and both are studied throughout this thesis with the goal of developing clinically effective treatment regimens (see Figure 1-5).

![Figure 1-5. Different mechanisms of RAS hyperactivation in cancer](image)

At baseline, RAS is regulated by the coordinate efforts of RAS GAPs and RAS GEFs. Two mechanisms are appreciated for RAS hyperactivation in sporadic cancer: RAS mutation that often disrupts the RAS GAP ability to regulate RAS and LOF mutations in RAS GAPs. Figure adapted from (Maertens and Cichowski, 2014)
TARGETING the RAS SIGNALING PATHWAY IN RAS-DRIVEN NSCLC

Molecularly targeted treatments have been developed for lung adenocarcinoma tumors with activated oncogenes such as EGFR, BRAF, ROS1, and ALK. Four FDA-approved EGFR tyrosine kinase inhibitors (TKIs) are currently in clinical use and response rates for these drugs range from 50-80% (reviewed in Rotow and Bivona, 2017). Similarly, half of BRAF-mutant lung adenocarcinomas exhibit the V600E mutation and thus are amenable to V600E-specific targeted therapies such as combined vemurafenib and dabrafenib. Thus, both TKIs and BRAF inhibitors are respectively effective alone in EGFR- and BRAF-mutant NSCLC.

Targeting KRAS

The targeted treatment revolution has thus far failed to deliver clinically effective treatments for patients with KRAS-mutant lung adenocarcinoma (reviewed in Garrido et al., 2017). This is in part because direct RAS targeting has been historically challenging due to its high affinity for GTP and its limited number of accessible active binding sites, which are typically used as targets in small molecule drug development (Spoerner et al., 2001). Because of these limitations, investigators have focused on alternative methods of RAS suppression, such as siRNA approaches and inhibiting its post-translational modifications. Thus far, attempts to limit mutant KRAS expression with siRNA have proven untenable due to technical issues such as efficient delivery, uptake, and off-target effects. However, the development of new tumor specific delivery systems is an active area of clinical investigation (Yuan et al., 2014). Efforts to prevent post-translational RAS modifications using farnesyl transferase inhibitors (FTIs) also showed preclinical promise in their capacity to hinder KRAS’ ability to incorporate into the cell membrane, its site of action (Kohl et al., 1993). However, follow-up studies indicated that an alternative process called geranylgeranylation could compensate for the effect of FTIs, preventing their therapeutic utility (James et al., 1996; Whyte et al., 1997).
More recently, there is renewed interest in covalently targeting a subset of KRAS mutant alleles with small molecule inhibitors. These strategies aim to block GTPase activity in the GTP binding pocket and/or slow the process of nucleotide exchange (Ostrom et al. 2013; Janes et al., 2016). Small molecule inhibitors that specifically target KRAS with a G12C mutation are currently being evaluated in preclinical models. One such inhibitor, ARS-1620, prevents nucleotide exchange of the G12C-mutant KRAS by binding cysteine in the active site, locking KRAS in its inactive state (Lito et al., 2016; Patricelli et al., 2016). ARS-1620 was shown to slow tumor growth and initiated modest tumor regression in human xenograft and patient-derived xenograft models (Janes et al., 2016). This study also demonstrated that the inhibitor was specific to G12C and had no preclinical activity against other KRAS-mutant alleles. While this work is exciting, single targeted treatments are rarely curative in humans and often lead to the quick onset of resistance. Therefore, even if these agents exert some clinical activity in humans, combining this KRAS-specific inhibitor with other targeted therapies could improve efficacy, which is relevant to this dissertation.

**Targeting downstream RAS effectors**

In the absence of direct KRAS inhibitors, investigators have focused on evaluating the effects of the downstream effector, MEK. Two FDA-approved MEK inhibitors, trametinib and selumetinib, have been tested in KRAS-mutant NSCLC. Unfortunately, both preclinical and clinical studies have demonstrated that MEK inhibitor monotherapy is not sufficient to drive durable responses in KRAS-mutant lung adenocarcinoma (Davies et al., 2007; Hainsworth et al., 2010). Thus, the majority of clinical trials have focused on combining MEKi with therapies to improve responses (Table 1-1). Preclinical studies suggested trials of selumetinib in combination with standard chemotherapy (Chen et al., 2012; Holt et al., 2012). Despite initial improved response rate and progression-free survival in a Phase II study, a Phase III trial of selumetinib plus docetaxel (chemotherapy) versus docetaxel alone did not significantly improve
OS, RR, or PFS in patients with \textit{KRAS}-mutant tumors (Janne et al., 2013). A murine co-clinical trial identified secondary mutations in \textit{TP53} and \textit{LKB1} as possible modifiers to response, suggesting that future clinical trials should stratify patients according to this additional criteria (Chen et al., 2012). Specifically, \textit{KRAS-LKB1} mutant mice exhibited primary resistance to the combination therapy.

\textbf{Combination targeted therapies}

Efforts to combine inhibitors of more than one RAS effector pathway are also being evaluated in clinical trials based on pre-clinical reports of efficacy in mouse models. Because mutant \textit{KRAS} activates the RAS-MEK-ERK pathway and the PI3K-AKT-mTORC1 pathway, combined inhibition of pathways have shown promising preclinical responses in RAS driven tumors (Engelman, et al., 2008; Malone et al., 2014; Posch et al., 2013). However, to date no effective clinical combinations have been identified, which may be due to combined toxicity in humans, ineffective target inhibition at tolerable doses, or targeting the wrong signaling node.

Other combination therapies currently in clinical trials for patients with \textit{KRAS}-mutant disease include MEKi plus erlotinib (EGFRi, NCT01859026), MEKi plus lapatinib (HER2i, NCT02230553), MEKi plus palbociclib (CDK4/6i, NCT03170206), and MEKi plus navitoclax (BCL2i, NCT02079740 ) (see Table 1-1 for details). All of these trials have been sparked by preclinical studies with promising therapeutic combinations, but it is still unclear whether this benefit will translate to patients. Thus far, translation of combination treatments to the clinic has been disappointing, possibly due to lack of standardization for tumor regression in preclinical mouse models and/or differences in toxicities between humans and mice. This topic of successfully translating preclinical studies to patients is discussed further in Chapter 4.

Regardless, additional approaches are needed to design rational combination treatments that may produce more robust and durable responses for lung adenocarcinoma patients. One promising strategy that we have successfully explored is to target both oncogenic
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<td>ORR</td>
<td>completed</td>
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<td>Recruiting</td>
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Table 1-1. Combination MEKi+ for the treatment of KRAS-mutant NSLCC
kinases and epigenetic regulatory proteins in cancers (De Raedt, et al., 2014; Malone et al., 2017).

**EPIGENETIC LANDSCAPE OF CANCER**

Like the Greek prefix “epi” implies, epigenetics is a field of research that concerns features that are “on top of” or “in addition to” typical genetic changes. In other words, epigenetic changes within the cell do not necessarily involve discrete changes in DNA sequences. Epigenetics was first studied in the context of development but has since become appreciated as a mechanism of gene regulation in adult cells via genomic imprinting, X chromosome inactivation in females, and carcinogenesis (Li et al., 1993; reviewed in Baylin, 2005). A classic example of epigenetic regulation is DNA methylation in which the DNA backbone itself is altered by covalent attachment of methyl groups to the cytosine nucleotide. This change in DNA leads to compacted chromatin that is not physically accessible by the transcriptional machinery.

Another key epigenetic mechanism of gene regulation is histone modification. Histones are proteins that package and compact DNA into structures called nucleosomes. The level of chromatin compaction affects how physically available a particular gene is for transcription. Histones and their corresponding modifications create a “histone code” that is key for transcriptional regulation of the genome (reviewed in Strahl and Allis, 2000). Each histone consists of four core proteins. Histones 3 and 4 possess a long tail at the N-terminal end of their structure that is amenable to covalent modifications, such as methylation, acetylation, SUMOylation, and phosphorylation. These modifications play a role in chromatin conformation, and thus, transcriptional regulation of the adjacent genetic loci (reviewed in Li et al., 2007). This thesis focuses on epigenetic regulators that generate global transcriptional changes via histone modification. Perturbations in one or more epigenetic regulators can lead to global
transcriptional consequences and those that occur during tumor progression are potential therapeutic targets in cancer.

**Classes of epigenetic regulators**

Epigenetic proteins play a fundamental role in controlling gene expression primarily by regulating the local chromatin conformation. There are three primary classes of epigenetic regulators that affect how genes are expressed: writers, erasers, and readers (see Figure 1-6A). Writers and erasers are responsible for catalyzing the placement and removal of epigenetic marks onto histones and DNA. The balance of writer and eraser activity determines transcriptional output at poised genetic loci and key markers of histones correspond with either active or repressed chromatin states (Figure 1-6B). Epigenetic readers do not have catalytic activity, but instead activate gene expression by recognizing a specific modification and recruiting transcription and elongating factors to its site. To demonstrate how histone regulators work in both normal and oncogenic contexts, I will describe two key epigenetic proteins that are relevant to this thesis: the PRC2 complex (writer) and BRD4 (reader).

**The PRC2 complex**

The polycomb repressive complex 2 (PRC2) is one of two complexes in the polycomb-group (PcG) proteins. First characterized in *Drosophila* as a silencer of *HOX* genes, PRC2 is known primarily as an epigenetic writer because of its primary function placing trimethylation marks onto histone 3 at K27. The H3K27me3 mark silences the transcription of a specific but large number of PRC2 targets. The PRC2 complex consists of four protein subunits: SUZ12, EED, EZH2 or EZH1, and RBAP48. Strikingly, both activation (GOF mutations, amplification, and overexpression of the catalytic subunit) and inactivation (copy number loss, mutation of PRC2 complex components) can be tumor-promoting depending on the tumor type and setting. EZH2 overexpression is a well-known oncogenic insult in solid tumors, including castration-
Figure 1-6. Epigenetic proteins that regulate gene transcription through histone modifications

(A) Histones (represented in yellow) are proteins that organize chromatin. Modifications to histone tails determine the local compaction of the chromatin, influencing gene expression. Three primary classes of epigenetic regulators are erasers, readers, and writers. Readers function by recognizing histone marks and recruiting transcriptional activators. Writers and erasers modify histone tails by placing and removing key marks.

(B) Chart shows a simplified categorization of key epigenetic regulators and histone marks (acetylation and methylation only) and their relative influence on the local chromatin state.
resistant prostate cancer and breast cancers (Varambally et al., 2002; Kleer et al., 2003; Min et al., 2010). In both cases, EZH2 expression correlates with poor clinical prognosis, increases with tumor aggressiveness, and triggers enhanced invasion and metastasis in vivo. As expected, tumors with high EZH2 expression have increased H3K27 trimethylation and subsequent global transcriptional repression. Little is known about the specific genes responsible for the oncogenic consequences of EZH2, although there is evidence suggesting that suppression of cell cycle genes and tumors suppressors such as the DAB2IP RasGAP play a causal role in prostate cancer (Varambally et al., 2002; Min, et al., 2010). In addition to EZH2 overexpression, recurrent activating mutations in EZH2 are common in a subset of lymphomas. This mutation changes EZH2 substrate specificity and results in hypertrimethylation of genomic sites (Morin et al., 2010). But again, little is known about how this change directly promotes tumorigenesis (Huet et al., 2017).

More recently, inactivating mutations and copy number loss of PRC2 components have been shown to promote tumor progression. For example, in NF1-mutant nervous system tumors known as MPNSTs, homozygous loss of SUZ12 or EED frequently co-occurs with NF1 loss. Loss of PRC2 activity in this context potentiates oncogenic RAS signaling programs, in part, to promote tumor growth (De Raedt et al., 2014). Similarly, PRC2 loss cooperates with oncogenic NOTCH signaling to promote tumorigenesis in patients with acute lymphoblastic leukemia (T-ALL). Both heterozygous and homozygous loss of SUZ12 or EZH2 occurs with similar frequencies in T-ALL, suggesting a dose-dependent role for PRC2 loss (Ntziachristos et al., 2012). Moreover, heterozygous loss of PRC2 components promotes tumor development in Myc-driven lymphomas, in part by accelerating the normal process of lymphomagenesis (Lee et al., 2013). In fact, the partial loss of PRC2 function enhances hematopoietic stem cell renewal whereas complete deletion of SUZ12 leads this process to fail (Lee et al., 2015). Together, these examples demonstrate how partial loss of a PRC2 complex protein can promote a pro-tumorigenic phenotype, a theme that may also be reflected in solid tumor environments.
**PRC2 complex in lung adenocarcinoma**

In NSCLC, both activating and inactivating alterations in PRC2 have been implicated in tumorigenesis. The oncogenic role of PRC2 was recently investigated in several murine models of lung adenocarcinoma driven by EZH2 overexpression (Zhang et al., 2016). Intriguingly, these EZH2-high tumors did not exhibit high MAPK or PI3K-AKT activation as compared to KRAS-mutant tumors. In addition, layering EZH2 expression on top of a KRAS mutation did not affect overall survival or increase tumor aggressiveness. The authors concluded that EZH2-high lung adenocarcinomas develop separately from RAS-driven lung adenocarcinomas and have their own oncogenic dependencies including the silencing of key developmental genes (Zhang et al., 2016). Further, EZH2 plays a distinct role in EGFR-mutant lung tumors as a recent study shows that EZH2 inhibition in this setting induces sensitivity to chemotherapies such as topoisomerase inhibitors (Fillmore et al., 2015).

Loss of PRC2 components has also been implicated in the development of lung cancer. A recent study in a KRAS-mutant mouse model of lung adenocarcinoma demonstrated that loss of EED cooperates with mutant KRAS to increase tumor aggressiveness through the activation of metastatic programs and inflammation pathways (Serresi et al., 2016). Similar to the NF1-mutant MPNST context, EED loss can also enhance the oncogenic KRAS transcriptional output in this lung adenocarcinoma setting (De Raedt et al., 2014).

While PRC2 loss has already been implicated in the development of KRAS-mutant NSCLC, analysis of TCGA data in this dissertation shows that these tumors only possess heterozygous defects in PRC2 components (Chapter 2). In fact, homozygous loss of PRC2 components SUZ12, EED, and EZH2 is rarely observed in NSCLC overall (TCGA, 2014). The few cases of homozygous SUZ12 and EED loss in NSCLC co-occur with mutation or copy number loss of NF1. Based on this copy number data in lung and other tumor settings, we hypothesize that weaker RAS-driven insults like NF1 inactivation benefit from stronger potentiation of oncogenic output via homozygous PRC2 loss while stronger alterations like
KRAS mutations do not. Clearly, the functional relevance of heterozygous loss in the NSCLC is an area for active study. Regardless, heterozygous loss of SUZ12, EED, or EZH2 occurs in 35% of KRAS-mutant tumors, representing a large subset of the overall patient population. In chapters 2 and 3, I will explore how decreased PRC2 activity (low expression, heterozygous loss) leads to a number of cancer-promoting phenotypes and sensitizes tumors to combined MEK and BRD4 inhibition.

Bromodomain Reader, BRD4

Like PRC2, BRD4 is a commonly studied epigenetic regulator in cancer. BRD4 falls into the class of epigenetic readers because it recognizes activating H3K27 acetylation marks throughout the genome, recruiting transcription and elongation factors to these loci to activate their transcription. BRD4 is a member of the BET family of proteins characterized by conserved domains, two amino-terminal Bromodomains that bind acetylated chromatin and an ExtraTerminal domain, which interacts with specific binding partners. This family of proteins is conserved from yeast to mammals and includes four proteins in humans, BRDT, BRD2, BRD3, and BRD4. BRD2 and BRD4 has been shown to be embryonic lethal in mice, demonstrating the fundamental role of these epigenetic regulators in normal development (Houselstein et al., 2002; Gyuris et al., 2009).

BRD4 is unique among the BET family members because it has a carboxyl-terminal domain (CTD) domain, which recruits the elongation complex pTEFb, stimulating the transcription of responsive genes including many growth-promoting genes during mitotic progression (Hargreaves et al., 2009). First identified as a Mediator (MED1)-interacting protein, BRD4 co-localizes with MED1 throughout the genome, stabilizing one another’s occupancy at certain loci including thousands of enhancers and promoters (Loven et al., 2013). A subset of particularly active genes is regulated by genomic regions occupied by an exceptionally high level of factors like BRD4-MED1. Known as super enhancers, these regions are essential for
establishing cellular identity and driving the expression of oncogenes during cancer development and progression (Chapuy et al., 2013; Hnisz et al., 2013; Loven et al., 2013). BRD4’s well-defined role as a transcriptional activator at crucial oncogenic loci makes it an attractive target for epigenetic-based therapies.

TARGETING EPIGENETIC REGULATORS

BET inhibitors are small molecules that interact with the acetylated lysine-binding pocket of the BET family proteins, triggering displacement of BRD4 at enhancers and promoters genome wide, concomitant with downregulation of target transcripts. JQ1, a thienodiazepine-based small molecule used throughout this dissertation, exhibits activity against the BET family in low-nanomolar range concentrations, with particular affinity for BRD4 (Filippakopoulous et al., 2010). JQ1 works by preferentially displacing BRD4, Mediator, and p-TEFb from super enhancers of tumor cells. Some of the oncogenes associated with BRD4-driven enhancers include MYC, TMPRSS2-ETS fusion genes, FOSl1, CDK6, and TERT (Dawson et al. 2011; Segura et al., 2013). Genetic signatures of BET inhibitor exposure vary according to cellular context and many studies have shown that BET inhibition affects tissue- and cancer-specific signaling pathways including Hedgehog (Hh) signaling in Hh-driven medulloblastoma and androgen receptor (AR) signaling in AR-driven prostate cancer (Tang et al., 2014; Xu et al., 2012). In other words, BET inhibitors adapt to the signaling environment to deliver maximum therapeutic benefit.

BET proteins were first targeted in NUT midline carcinoma (NMC), a rare squamous cell carcinoma that depends on the BRD4-NUT fusion oncoprotein. Investigators found that inhibition of BRD4-NUT in this setting promotes differentiation and triggers growth arrest in multiple preclinical NMC models (French et al., 2008). This observation sparked the development of multiple small molecules targeting the bromodomain of BET proteins and completed clinical trials have shown clinical efficacy for NMC patients with BETi monotherapy
(Stathis et al., 2017; NCT02259114). Targeting cancers without BRD4 mutations quickly followed after a study demonstrated BETi efficacy in multiple myeloma, characterizing BETi as an indirect inhibitor of the ‘undruggable’ transcription factor MYC (Delmore et al., 2011).

MYC is a master transcription factor that modulates the expression of pro-survival genes, drives proliferation, and regulates cell growth. High expression and amplification of MYC occurs frequently across tumor types and has been tied to poor survival outcomes in breast cancer, NSCLC, leukemia, prostate cancer, among others (Beroukhim et al., 2010; Schaub et al., 2018). Since MYC, like other transcription factors, has largely been considered ‘undruggable’, the ability to target this gene indirectly with BET inhibitors generated much excitement within the cancer therapeutics field. In fact, the initial clinical success of BETi and the discovery of proto-oncogene c-MYC as a key BRD4 target, has paved the way for testing this epigenetic therapy in other solid and hematological tumor settings. For example, BETi monotherapy increased overall survival and decreased tumor burden in models of multiple myeloma and acute myeloid leukemia, both therapies requiring sustained downregulation c-MYC expression (Delmore et al., 2011; Zuber et al., 2011). However, BET inhibitors do not exclusively rely on downregulation of the MYC axis for their therapeutic effects as demonstrated in studies of BET inhibitors in NF1-mutant MPNSTs and castration-resistant prostate cancer where MYC expression is either not downregulated or the observed therapeutic effect does not rely on MYC downregulation (De Raedt et al. 2014; Asangani et al., 2014). In Chapter 2, I present data to show that MYC is not regulated by BETi in my therapeutic combination and I further examine MYC-independent BET combinations in Chapter 4.

Current BET inhibitors are considered pan-BET inhibitors as they affect the activity of all BET proteins at clinically relevant doses. Thus far, at least fifteen different BET inhibitor compounds have entered clinical trials with three reaching Phase II for hematological malignancies including OTX015/MK-8628 from Merck, BMS-9861158 from Bristol-Myers Squib,
### Table 1-2. BET inhibitors in Clinical Development

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and GSK52762 from GlaxoSmithKline. Six other BETi compounds are in ongoing safety trials for solid tumors (Table 1-2). Clinical response to BET inhibitor monotherapy has been limited with only partial responses observed in small trials and inevitable tumor relapse in all responding patients (reviewed in Stathis and Betoni, 2018). These responses are perhaps unsurprising considering the majority of preclinical studies with BET monotherapy exhibit tumor stasis and decreased cellular proliferation rather than pronounced tumor regression (Shimamura et al., 2013; Shu et al., 2016; Asangani et al., 2014).

There are ongoing efforts to more deeply suppress BET protein activity with next-generation compounds. One active field of study is the development of degrader drugs in which a BET inhibitor is merged to a small molecule that recruits an E3 ubiquitin ligase to the BET protein, resulting in its proteasomal degradation. This strategy has produced more potent cytotoxic results in preclinical studies likely due to its deeper suppression of BET protein activity and the strategy’s additional suppression of bromodomain-independent activities. Some of the more promising BET degraders include dBET1 which showed superior responses in T-ALL preclinical models and a VHL E3 ligase-based drug called BET PROTAC, which achieves greater responses in CRPC as compared to first generation BET inhibitors (Winter et al., 2015; Raina et al., 2016). Another drug, BETd-246, uses cereblon as an E3 ubiquitin ligase and show potent antitumor activity in triple negative breast cancer models as a monotherapy (Bai et al., 2017). While the results of this thesis focus on JQ1 as a tool compound, it seems likely that usage of this degrader class of inhibitors would amplify clinical responses.

In Chapters 2 and 3, I show that BET inhibitors induced downregulation of HOXC10 that triggered therapeutic effects at the intersection of signaling and epigenetics. The final section of this introduction provides context for the emerging paradigm of HOX genes in cancer.
HOX GENES

In the course of my thesis work I identified an important subset of NSCLC that aberrantly express HOXC10. Here I provide background on HOX genes and their role in development and cancer.

Hox genes during development

HOX genes are master regulatory transcription factors expressed throughout early development of multicellular organisms. First functionally described during the embryogenesis of Drosophila, these highly conserved members of the homeobox superfamily were later discovered throughout the human genome. Humans express 39 different HOX genes, which are organized into four distinct clusters characterized by the letters A-D. Each cluster lies on a different chromosome and contains HOXA-D genes numbered 1-13, though no HOX cluster contains a full set of thirteen (see Figure 1-7A; reviewed in Shah and Sukumar, 2010). Interestingly, each HOX paralog, which refers to genes located in the same cluster position (i.e. HOXA9 and HOXB9) have more similar developmental function to one another than to the adjacent HOX genes within their cluster (i.e. HOXA9 vs. HOXA10). This has been borne out in a number of studies where genetic knockouts of all existing HOX paralogs are necessary to observe a phenotypic effect, suggesting that compensation is possible for loss of one, but not all HOX paralogs (reviewed in Maconochie et al., 1996). In the developing lung, all three HOX paralogs (HOXA5, B5, C5) play redundant roles in the developing lung such that only triple mutants are sufficient to trigger severe hypoplastic lungs with deficient proximal-distal patterning (Hrycaj et al., 2015). Typical invertebrate genomes possess only one HOX cluster, suggesting that HOX clusters have been copied and pasted across the genome throughout vertebrate evolution. Thus, the functional redundancy of HOX paralogs likely has an evolutionary basis (Pascual-Anaya et al., 2013).
HOX transcription factors share a 61 amino acid long functional protein domain called the homeobox, which selectively binds DNA and is largely responsible for its transcription factor activity. HOX proteins interact with a common DNA consensus sequence that contains the motif (C/G)TAATTG. Due to the promiscuous nature of the homeobox domain, *bona fide* target genes for each HOX transcription factor remain elusive. The functional specificities of each HOX gene remain elusive. The functional specificities of each HOX gene remain elusive.

Figure 1-7. HOX gene regulation in humans
(A) The chromosomal organization of the four HOX gene clusters. Each cluster is organized from 3' to 5' with 3' genes expressed early and in proximal regions of the embryo and 5' genes expressed progressively later and in anterior regions. HOX10 has two paralogous genes, HOX10 and HOX11.
(B) Epigenetic regulation of the HOX gene locus depends on the opposing functions of the PRC2 complex and TrXG proteins, which act to repress and activate transcription, respectively. Figure adapted from (Abate-Shen, 2002).
depends on a number of factors including post-transcriptional control, subcellular localization, and binding partner specificities (reviewed in Svingen and Tonissen, 2006). But again, few of these factors are exclusive to one HOX gene over another, making identification of specific targets difficult.

During development, the transcription of HOX genes may be triggered by retinoic acid, fibroblast growth factors (FGFs), Wnts or CDX transcription factors. More study is needed to clearly define how and when HOX gene expression is initiated, but our current understanding indicates that HOX genes are largely regulated both temporally and spatially within their respective cluster (reviewed in Montavon and Shoshnikova, 2014). For example, collinearity is when HOX genes in the same cluster (A-D) are expressed in the 3' to 5' direction with 3' HOX genes (HOX1-5) activated first followed by progressively later activation of 5' HOX genes (HOX9-13). It is still unclear how this tightly controlled regulatory process is mediated in vertebrates though there has been suggestion that HOX genes can transcriptionally cross-regulate expression of one another in Drosophila (Miller et al., 2001). Overall, these observations show that coordinate regulation of HOX expression is a developmental paradigm that could be re-deployed in adult cancer cells.

**Regulation of HOX genes post-development**

Like other developmental genes, HOX genes are primarily regulated by epigenetic mechanisms during embryogenesis. CpG island methylation is a common feature within the promoters of HOX genes when they are silenced following embryonic development. HOX gene regulation in adult cells depends on the antagonistic functions of polycomb group proteins and trithorax complexes, which direct histone trimethylation and acetylation at HOX promoters. Specifically, the polycomb group complex (i.e. PRC2) writes H3K27 trimethylation, which ultimately represses genetic loci, whereas the trithorax complex (i.e. MLL) directs H3K4
trimethylation, which activates transcription. These two epigenetic regulators balance genetic activation and repression and are thought to be the major mechanism for HOX regulation post-development (see Figure 1-7B, reviewed in Mallo and Alonso, 2013).

**HOX genes in oncogenesis**

Traditionally known for their function in body patterning during embryogenesis, HOX genes are established regulators of cellular proliferation, differentiation, and apoptosis. HOX gene expression is generally restricted to undifferentiated and proliferative cells, with expression typically silenced in adult tissues (reviewed in Abate-Shen, 2002). However, aberrant expression of HOX genes is common in both hematological and solid tumor environments with three primary categories defining HOX deregulation in cancer. First, HOX genes can be temporally misregulated when a HOX gene that is normally expressed in a developing tissue is re-expressed post-development. Examples of re-deployment of HOX developmental programs abound in brain, kidney, and prostate tissues in which developmental HOX genes are re-expressed during cancer progression (reviewed in Abate-Shen, 2002).

The second mode of deregulation is aberrant spatial expression in which a HOX gene is ectopically expressed in a tissue that does not normally express that gene during its development. For example, HOXA7 and HOXB7 are aberrantly expressed in ovarian epithelial tumors but these genes are not normally expressed during ovarian development (Naora et al., 2001). These first two mechanisms of deregulation occur when the HOX gene actively promotes cellular growth and proliferation.

The final deregulated homeobox paradigm is aberrant silencing of these genes in an adult tissue that normally expresses them. This paradigm is rare within the HOX gene family as most HOX genes correspond to a proliferative state. But other genes within the homeobox superfamily, such as NKX3.1 in prostate cancer, showcase this pattern of expression more
readily and are considered *bona fide* tumor suppressors (Kim et al., 2002; Abdulkadir et al., 2002).

HOXA9 and HOXB13 are two well-characterized HOX genes that promote tumor development via aberrant activity in acute myeloid leukemia (AML) and prostate cancer, respectively. In AML, activating alterations and gene fusions in the trithorax group gene MLL causes increased expression of HOXA9. Data shows HOXA9 is necessary for maintaining leukemic transformation and that high expression of HOXA9 correlates with poor clinical prognosis (Faber et al., 2009). Large-scale studies to identify HOXA9 binding sites have demonstrated that HOXA9 targets include genes with poor prognosis in leukemia such as *BCL-2*, *c-MYB*, *ERG*, and *FGF2* (Huang et al., 2012).

Within prostate cancer, germline mutations in HOXB13 have been identified to increase cancer risk. The mechanism by which this HOXB13-G84E allele exerts its tumor-promoting effects is still unknown but likely relies on a unique ability to change its downstream transcriptional targets or its binding co-factors (reviewed in Brechka, et al., 2017). Overexpression of HOXB13 is also being explored for its oncogenic potential in this setting (Ewing, et al. 2012).

Beyond aberrant expression patterns, *HOX* genes can also acquire new functions when they are altered via gene fusion. One rare example occurs in leukemia in which HOXA9 is translocated to create a fusion protein with the nucleoporin protein NUP98 (Nakamura et al., 1996). The fusion protein has the DNA binding capability of HOXA9 and the amino terminal region of NUP98. Together, the fusion protein promotes transformation in hematopoietic progenitor cells largely by inducing long-term proliferation of hematopoietic stem cells and upregulating a larger set of genes than HOXA9 alone (Takeda et al., 2006; Ghannam et al. 2004).

Consensus mechanisms of oncogenesis and key target genes have yet to be attributed to even these most well studied HOX genes. Functional insights thus far have attributed
oncogenic activity to general promotion of proliferative and survival pathways. In my study of HOXC10 in lung cancer, I find that downregulation of HOXC10 is necessary but not sufficient to drive response to combined MEK and BRD4 inhibition. This fits with other studies of HOX genes in cancer where activities have been classified as tumor promoting rather than as bona fide oncogenes (reviewed in Abate-Shen, 2002).

**HOXC10 in Cancer**

HOXC10 is a member of the HOX family of transcription factors and its normal and oncogenic roles in KRAS-mutant lung cancer are explored throughout this thesis. HOXC10 and the related HOX10 genes are expressed throughout the neural tube and developing vertebrae and their normal developmental function is to establish lumbar skeletal identity. These HOXC10 expression patterns and developmental functions have been explored in depth in a number of model organisms including *Mus musculus* (mouse), *Xenopus laevis* (frog), and *Ambystoma mexicanum* (axolotl) (Hostikka et al. 2009; Christen et al., 2003; Carlson et al., 2001). Interestingly, there is currently no evidence that HOXC10 or its paralogs are expressed during lung development in these models or in humans. This suggests that the presence of HOXC10 in this context is not aberrant re-deployment of a developmental program, but rather ectopic expression.

HOXC10’s role as a pro-proliferative gene and putative promoter of tumorigenesis was first suggested by its characterization as a regeneration-specific gene. In a study of limb and tail regeneration in axolotls, HOXC10 was specifically expressed in regenerating limbs following experimental resection (Carlson et al., 2001). Interestingly, HOXC10 is not expressed in the forelimbs during development, but is deployed during forelimb regeneration where it recruits and attracts mesenchymal cells. This observation not only supports its role as a pro-proliferative gene, but also showcases its ability to be newly expressed in a tissue without its developmental footprint. Studies within *Xenopus* also support HOXC10’s regenerative capacity as strong
HOXC10 expression is deployed in the stump of regenerating embryonic limbs (Christen et al., 2003). Again, this observation demonstrates HOXC10’s specific regenerative function because HOXC10 expression was not observed during normal development of these limb buds.

The limited nature of HOXC10 developmental studies is only matched by its limited characterization in oncogenesis. Few studies exist and most are small-scale, relying on correlative observations. These studies have shown that high HOXC10 expression correlates with poor survival outcomes and increased invasiveness in gastric, thyroid, breast, and cervical cancers (Guo et al., 2017; Feng et al., 2015; Ansari et al., 2012; Zhai et al., 2007). The significance of HOXC10 expression within lung tumors is explored in depth in Chapters 2 and 3 of this thesis.

While there is little insight into how HOXC10 may function in cancer, studies in gastric cancer have suggested that HOXC10 can activate the expression of pro-proliferative and pro-survival genes such as BCL-2 and c-MYC (Guo et al., 2017). In Chapter 2, I explore the function of HOXC10 in NSCLC and find that it coordinately regulates E2F and downstream replication proteins in conjunction with the RAS pathway. And in Chapter 3, I preliminarily examine HOXC10’s function in cell cycle regulation and metastasis, suggesting areas for further exploration within NSCLC.

**Targeting HOX genes in cancer**

As aberrant expression of HOX genes become increasingly appreciated as tumor-promoting, interest in targeting them therapeutically has grown. Since most of these aberrant HOX genes are not normally expressed in adult human cells, direct targeting could have a fairly large therapeutic window. However, direct targeting of transcription factors and especially the structurally redundant HOX genes may not be feasible.

Within MLL-rearranged leukemia, where the oncogenic nature of HOXA9 is well established, attempts have been made to inhibit HOXA9 by targeting the positive regulators of
HOXA9 including MLL, menin, and methyltransferase protein DOT1L. In AML, DOT1L is recruited to MLL-target genes like HOXA9 and subsequently methylates H3K79 to mark these genes for expression (Okada et al., 2005; Bernt et al., 2010). Thus, DOT1L inhibitors are under active clinical development where they have been shown to inhibit H3K79 methylation, decrease the expression of MLL-fusion target genes including HOXA9 and HOX co-factor MEIS1, and decrease tumor burden in leukemia models (Daigle et al., 2013). Thus, DOT1L and other MLL-related inhibitors may be able to induce therapeutic action by indirectly targeting HOXA9.

In this thesis, I find that the BET protein inhibitor JQ1 potently suppresses HOXC10 expression and that this suppression cooperates with MEK inhibitor to trigger tumor regression in KRAS-mutant lung adenocarcinoma (Chapter 2). Interestingly, JQ1 also induces global HOX downregulation, suggesting that BET inhibitors could prove useful in other tumor settings with high HOX activity (Appendix A). I also explore the effects of ectopic HOXC10 expression in KRAS-mutant lung cancer and its potential tumorigenic implications (Chapter 3).

OVERVIEW OF DISSERTATION

KRAS-mutant lung adenocarcinoma is a lethal disease with no clinically effective treatments available to patients. Despite decades of research on the RAS oncogene, current attempts to treat these tumors have failed. This thesis largely describes a new promising therapeutic combination that targets oncogenic RAS signaling and the epigenetic landscape. I show that combined MEK and BET inhibition is able to not only kill cells but also shrink tumors in four independent models of KRAS-mutant lung adenocarcinoma (two human xenografts, one mouse allograft, and one patient-derived xenograft). Tumor shrinkage of established tumors in these models is rare across the literature, making my data both unique and exciting for the field of lung cancer research.
In Chapter 2, I describe the identification of this therapeutic combination. Strikingly, half of the KRAS-mutant human cell lines screened in vitro exhibited potent cell death responses whereas the second half exhibited cell cycle arrest and no death. I use this clear phenotypic distinction as a tool to identify the therapeutic mechanism of action. Interestingly, I find several molecular differences that distinguish the sensitive versus the resistant group of cell lines including PRC2 status and expression of HOXC10. HOX genes have become increasingly appreciated for their role in carcinogenesis, specifically their functions in balancing proliferation and differentiation phenotypes. In fact, we show that HOXC10 cooperates with the RAS-MEK-ERK pathway to regulate expression of key mediators of DNA replication and cellular proliferation. It is this combined regulation that leads to replication stress and ultimately cell death.

In Chapter 3, I explore how PRC2 defects and HOXC10 overexpression function in KRAS-mutant lung adenocarcinoma by looking at their unique effects on tumor aggressiveness, cell cycle regulation, biomarker potential, and differentiation/stemness. By utilizing a combination of in vivo and in vitro studies, I show how that these new subtypes of KRAS-mutant lung adenocarcinoma warrant further study.

Lastly, in Chapter 4, I make conclusions and discuss the future implications of this work. One section of the discussion reviews the literature related to combination treatments that target cancer at the intersection of signaling and epigenetics. There is a wealth of studies that utilize this strategy for the purposes of preventing resistance and enhancing therapeutic response to targeted kinase inhibitors. Overall these combinations take advantage of an epigenetic therapy’s ability to downregulate multiple points along oncogenic pathways- a mechanism that prevents common modes of resistances including kinome reprogramming. Interestingly, epigenetic therapies first became appreciated in hematological malignancies, but their ability to globally affect the signaling landscape has invigorated their usage in solid tumors. Sections of this chapter will serve as the basis for a review article in Annual Reviews in Cancer Biology.
Together, my thesis proposes further study in 1) combining kinase-targeting drugs with epigenetic-based therapies in solid tumor environments and 2) characterizing HOX genes in the oncogenic process.
Chapter 2: A deregulated PRC2/HOX gene axis controls replication and sensitizes KRAS mutant lung cancer to an epigenetic-based therapy
A deregulated PRC2/HOX gene axis controls replication and sensitizes KRAS mutant lung cancer to an epigenetic-based therapy

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**Stephanie Louise Guerra**- Planned and performed experiments and data analysis

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**Shawna Guillmete**- Trained S. Guerra to perform FACS experiments

**Richard Adeyemi**- Performed DNA fiber experiment

**Hong Tiv**- Performed and managed lung PDX mouse experiment

**Pasi Jänne**- Supervised and helped set up lung PDX collaboration

**Steve Elledge**- Instrumental in experimental discussions and replication experiment planning

**Karen Cichowski**- Supervised and helped develop the project

**Note:** Work from this chapter is in preparation for publication.
INTRODUCTION

The development of small molecules that inhibit oncogenic kinases has changed the standard of care for many diseases and has helped establish the paradigm of precision medicine for treating cancer. Accordingly, in NSCLC, specific kinase inhibitors are now routinely used to treat tumors with genetic alterations in EGFR, ALK, ROS1 and MET. However, there are still no effective therapies for NSCLCs harboring mutations in KRAS, the most prevalent oncogenic driver of this disease. One therapeutic approach has been to target downstream Ras effector kinases, such as MEK (Davies et al., 2007; Ji, et al., 2007). Indeed, MEK inhibitors have been evaluated in the clinic, but they exhibit little efficacy as single agents and even when combined with chemotherapy (Jänne, et al., 2016). More recently, covalent KRAS inhibitors have shown promise in preclinical models, although current agents are only effective against a specific mutant (Ostrem, et al., 2013; Lito et al., 2016; Zeng et al., 2017; Janes et al., 2018). In addition, complete regression of this genetic subset of tumors may ultimately require the addition of other agents. Therefore developing effective therapies for KRAS mutant lung cancer remains a critical unmet need.

BET bromodomain proteins are epigenetic readers that bind acetylated histones and serve as scaffolds for transcription factors and other chromatin organizers at active promoters and enhancers (Jang et al., 2005; Hargreaves et al., 2009; Yang et al., 2008). Small molecules that target BET bromodomain proteins have recently emerged as potential therapeutic agents in cancer (reviewed in Stathis and Bertoni, 2018). Clinical trials with these agents are ongoing and have shown preliminary activity in NUT carcinomas, which harbor BRD4-NUT translocations (French et al., 2008). BET bromodomain inhibitors are also being evaluated in various hematopoietic malignancies, in part based on the hypothesis that these agents function by suppressing MYC (Delmore et al., 2011). Preliminary clinical responses have been observed in acute leukemia and some lymphomas, but to date no biomarkers that predict sensitivity have been identified, including MYC expression (Berthon et al, 2016; Amorim et al., 2016; Peirs et al.,
Therefore while clinical observations support the further development and investigation of these and/or other BET bromodomain inhibitors (reviewed in Stathis and Bertoni, 2018), the challenge lies in more precisely understanding how these agents work, identifying biomarkers that predict response, and developing more effective drug combinations, which may be particularly important for solid tumors that lack BET bromodomain translocations.

The development of BET bromodomain-based drug combinations is an active area of research. One solid tumor type in which these agents hold promise are NF1 mutant nervous system tumors, known as MPNSTs. NF1 encodes a Ras GTPase-activating protein (RasGAP) and consequently NF1-mutant cancers are driven by hyperactivated Ras (Hiatt et al., 2001; Johannessen et al., 2008). Interestingly, BET bromodomain inhibitors have been shown to promote robust tumor regression when combined with MEK inhibitors in animal models of these NF1 mutant tumors (De Raedt et al., 2014). Therefore we investigated whether this combination might be more broadly effective in Ras-driven tumors: specifically KRAS mutant NSCLCs. Strikingly, 50% of KRAS mutant NSCLCs were exquisitely sensitive to combined MEK/BET inhibitors, which caused potent cell death in vitro and dramatic tumor regression in vivo in multiple animal models. However, we discovered that these agents were exclusively effective in Ras-driven NSCLCs that aberrantly expressed a specific homeobox gene, HOXC10. Here we show that MEK/BET bromodomain inhibitors function by coordinately suppressing the Ras pathway and HOXC10, which jointly cause a depletion of key replication proteins in cycling cells and consequently cell death. Together these studies have 1) identified an effective therapeutic combination for KRAS mutant NSCLC and established a functional biomarker that both predicts and mediates response; 2) defined an important new subset of vulnerable lung cancers that aberrantly express HOXC10; and 3) have uncovered a unique role for HOXC10 in regulating replication in NSCLC.

RESULTS
**Combined MEK and BET bromodomain inhibitors trigger cell death and tumor regression**

KRAS is mutated in approximately one-third of human NSCLCs and NF1 is mutated or lost in an additional 8.3% (TCGA, 2014). To investigate the therapeutic potential of combined MEK and BET bromodomain inhibitors in these Ras-driven lung cancers, lung adenocarcinoma cell lines harboring either KRAS or NF1 mutations were exposed to a MEK inhibitor (PD901) and/or a BET bromodomain inhibitor (JQ1). Both KRAS mutant cell lines tested were exquisitely sensitive to these agents, which when combined caused a 68-74% decrease in cell number after only 72 hours (Figure 2-1A, left). Surprisingly, this drug combination was cytostatic, at best, in the two NF1 mutant cells examined (Figure 2-1A, right). The observed differences in efficacy were not due to differential effects on ERK phosphorylation, which was effectively suppressed by this combination in all cell lines. Further, we do not observe suppression of MYC protein or mRNA levels upon exposure to BETi or combined treatment in two different sensitive cell lines, suggesting that MYC suppression is not important for treatment-induced cell death (Figure A-1A).

A larger panel of KRAS mutant NSCLCs was used for further analysis. Notably, 50% of the cell lines were extremely sensitive to combined MEK/BET bromodomain inhibitors, which caused a dramatic decrease in viable cells (Figure 2-1B). Variable cytostatic responses were observed in the remaining cells lines, however these cells did not die and were therefore termed ‘resistant’. Live cell analysis revealed a continuous decrease in the number of cells in ‘sensitive’ cultures over time in response to treatment (Figure 2-1C, left), which was accompanied by a concomitant increase in caspase activation (Figure 2-1D, left). Resistant cells remained cytostatic and cell death was not observed in response to either agent alone or together (Figures 2-1C and -D, right).

Before investigating the molecular mechanism of action, the therapeutic effects of these agents were evaluated *in vivo*. Notably, many agents that slow the growth of tumors in animal
Figure 2-1. Combined MEK and BET bromodomain inhibitors trigger cell death and tumor regression in KRAS-mutant lung adenocarcinoma
(A) KRAS-mutant (NCI-H1573, NCI-H23) and NF1-mutant (NCI-H1838, NCI-H1435) lung adenocarcinoma cells were treated with DMSO, PD901 (MEKi, 1000 nM), JQ1 (BRD4i, 1000 nM), or PD901 + JQ1 for 72 hours. The left y-axis indicates the log2 fold change in cell number at 3 days relative to day 0. The right axis indicates percent change in cell number at 3 days relative to day 0. Error bars +/- SD from technical triplicates. Below immunoblot shows levels of phospho ERK after 24 hours of indicated treatment, vinculin serves as control.
(B) Broad panel of KRAS-mutant lung adenocarcinoma cell lines were treated with DMSO, PD901 (500nM) and JQ1 (1000nM) and PD901 + JQ1 for 72 hours. Error bars +/- SD from technical triplicates, each cell line’s data is representative of at least 3+ independent experiments. Below immunoblots show levels of phospho ERK after 24 hours of indicated treatment, tubulin serves as control. Blots are listed for each cell line from left to right, top row indicates sensitive cell lines (red) and bottom row indicates resistant cell lines (black).
(C) Sensitive cell line NCI-H23 and resistant cell line A549 were plated in 96-well plates and analyzed by Incucyte cell counting every two hours for six days. Cell number begins to decrease within twelve hours in sensitive cell line whereas resistant cells arrest over the course of six days.
(D) Cells from experiment in (C) are analyzed for caspase expression by Incucyte. NCI-H23 cells start exhibiting caspase expression within twelve hours of drug treatment whereas NCI-A549 cell lines do not express caspase throughout course of experiment.
(E) Two xenograft experiments were conducted in cohort of Nu/Nu mice with NCI-H23 (left) and NCI-H1573 (right). Cells were injected subcutaneously and grown for 2-3 weeks until tumors reached approximate enrollment size of 300-500 mm³. After enrollment, mice were treated daily with vehicle (black), PD901 (light gray, 1.5 mg/kg), JQ1 (dark gray, 45 mg/kg), or PD901 + JQ1. Waterfall plot depicts log2 fold change in tumor volume (left axis) and percent change in tumor volume (right axis) after 10 days of treatment.
(F) Pharmacodynamic study of NCI-H23 and NCI-H1573 xenograft tumors harvested after three days of daily treatment (six hours after last dose) shows suppression of phospho-ERK occurs in vivo in response to MEKi and combination treatment, vinculin serves as control.
Figure 2-1 (Continued).

A. Percent Change in Cell Number

B. Percent Change in Tumor Volume

C. Log2 Fold Change in Cell Number vs. Day 0

D. Log2 Fold Change in Tumor Volume vs. Day 0

E. Log2 Fold Change in Cell Number vs. Day 0

F. Log2 Fold Change in Cell Number vs. Day 0
models have been proposed to be promising clinical candidates; however, targeted agents that are effective in the clinic, such as BRAF inhibitors in melanoma and EGFR inhibitors in lung cancer, invariably cause frank tumor regression in preclinical studies. To determine whether these agents could shrink established tumors, two human xenograft models were generated. Single agents exerted modest cytostatic effects, however combined MEK and BET bromodomain inhibitors induced potent tumor regression in both models (Figure 2-1E). Pharmacodynamic analysis confirmed that phospho-ERK was effectively suppressed in tumors exposed to MEK or MEK and BET bromodomain inhibitors and no toxicity was observed (Figure 2-1F, Figure A-1B). Taken together these data demonstrate that MEK and BET bromodomain inhibitors are effective in a significant fraction of KRAS-mutant NSCLCs and potently cooperate in vitro and in vivo to induce both cell death and frank tumor regression.

**PRC2 defects confer sensitivity to combined MEK/BET bromodomain inhibitors**

To investigate the molecular basis of drug sensitivity we compared sensitive versus resistant cell lines. Genomic analysis did not reveal any mutations, amplifications, or homozygous deletions that were associated with either group, including alterations in other known oncogenes or tumor suppressors such as TP53, MYC, STK11, CDKN2A/B, or SMARCA4 that have been shown to mediate sensitivity to either MEKi or BETi monotherapies (Figure A-2). Strikingly however, all of the sensitive KRAS-mutant cell lines exhibited copy loss or mutations in at least one obligate component of the PRC2 complex (SUZ12, EED, or EZH2) whereas the resistant lines did not (Figure 2-2A). Interestingly, defects in PRC2 components have been shown to be associated with enhanced sensitivity to MEK/BET bromodomain inhibitors in NF1 mutant nervous system tumors (DeRaedt et al., 2014); however homozygous alterations were common in that setting, whereas only heterozygous alterations were detected in these NSCLCs (TCGA, 2014). Nevertheless, GSEA analysis confirmed that PRC2 targets were significantly suppressed in resistant cell lines as compared to sensitive cells, suggesting
Figure 2-2. PRC2 defects confer sensitivity to combined MEK/BET bromodomain inhibitors in NSCLC and are common in KRAS-mutant NSCLC

(A) Data from the CCLE shows CNV loss and mutation of obligate PRC2 components SUZ12, EED, and EZH2 in our KRAS-mutant lung adenocarcinoma panel (Figure 1B).

(B) CCLE data was analyzed by ssGSEA for expression of PRC2 targets and grouped by sensitivity to combination treatment. Sensitive group was significantly enriched for PRC2 target expression, indicating low PRC2 activity (p< .0005 by two-sided t-test with Welch’s correction).

(C) Two inherently resistant cell lines A549 and NCI-H2030 were treated with indicated drugs for 72 hours. Assay performed simultaneously for cells infected with shSCR, shSUZ12, or shEED. Results are representative of 2-3 independent experiments for each cell line.

(D) Immunoblot showing downregulation of repressive mark H3K27me3 upon knockdown of SUZ12 or EED in the resistant cell lines from Figure 2C. GAPDH is used as a loading control.

(E) CNV and mutational data from published TCGA 2014 dataset showcasing heterozygous loss in obligate PRC2 components in KRAS-mutant lung adenocarcinoma tumors.

(F) Data compiled from publically available TCGA dataset PRC2 het loss was determined by examining CNV data for SUZ12, EED, and EZH1/2. Additional PRC2-defective tumors were identified by ssGSEA analysis of PRC2 targets. Those with PRC2 target expression higher than 2STDEV over the normal tissue were classified as having “defects in PRC2 target suppression”.

Figure 1B

Genetic Alteration
- EZH2
- EED
- SUZ12
- KRAS

Log2 Fold Change in Cell Number
- 10%
- 30%
- 100%

(TCGA 2014, N=79)

Figure 2C

H2030

A549

Figure 2D

H3K27me3

GAPDH

Figure 2F

PRC2 HETLOSS
Defects in PRC2 target suppression
no detected PRC2 defects

(TCGA provisional, N=153)
that these (heterozygous) cells exhibit functional defects in PRC2 activity (Figures 2-2B). Moreover, shRNA-mediated reduction of either SUZ12 or EED expression reduced H3K27 methylation levels and conferred sensitivity to multiple resistant cell lines, demonstrating that PRC2 suppression plays a causal role in conferring sensitivity to MEK/bromodomain inhibitors in NSCLC (Figures 2-2C and -D).

**Loss-of-function defects in the PRC2 complex are common in KRAS mutant NSCLC**

Further genomic analysis of primary human tumors demonstrated that SUZ12 and EED copy number alterations are frequent in KRAS-mutant NSCLC. Approximately 33% of KRAS mutant tumors exhibit heterozygous loss of at least one of the three obligate PRC2 components: SUZ12, EED, or EZH2 (Figure 2-2E; TCGA 2014). Transcriptional profiling further revealed that an additional ten percent of KRAS-mutant tumors harbor demonstrable defects in PRC2 function, as indicated by significantly higher expression of PRC2 targets as compared to normal tissue (Figure 2-2F), suggesting that other genes in this pathway may be altered in those tumors. Unlike T-ALL and nervous system tumors, homozygous loss of PRC2 components was never observed in KRAS-mutant lung adenocarcinoma and was rare in NSCLC overall; however, heterozygous and homozygous alterations both occur with similar frequency in the aforementioned tumor types, underscoring a potential role for haploinsufficiency in tumor development. Indeed, heterozygous mutations and/or partial loss of PRC2 function are thought to be important for the development of myeloid malignancies (reviewed in Margueron and Reinberg, 2011) and heterozygous alterations in PRC2 components promote tumor development in mouse models of Em-myc lymphoma (Lee et al., 2013). During hematopoietic stem cell renewal, partial suppression of PRC2 activity is preferable to complete loss of function, further demonstrating the proliferative advantage of heterozygous PRC2 alterations (Lee et al., 2015).
Regardless, genomic and expression analysis indicates that in total, almost forty-five percent of KRAS-mutant tumors harbor measurable PRC2 defects and thus may be susceptible to combined MEK and BET inhibition (Figure 2-2F).

**Identification of HOXC10 as a biomarker and mediator of sensitivity**

To elucidate the mechanism by which these agents were cooperating, we examined transcriptional profiles of NSCLC cells treated with individual and combined agents. In NF1 mutant nervous system tumors, BET bromodomain inhibitors have been shown to potentiate the suppressive transcriptional effects of MEK inhibitors, as BRD4 binds and regulates a subset of Ras-responsive genes (DeRaedt et al., 2014). Notably, MEK and BET bromodomain inhibitors also cooperatively suppressed Ras transcriptional output in sensitive NSCLCs (Figure 2-3A). However, a cooperative suppression of Ras transcriptional signatures was also observed in resistant cells (Figure A-3A), suggesting that while a more potent suppression of the Ras transcriptional network likely contributes to the enhanced cytostasis observed in insensitive cell lines, it may not be sufficient for cell death. Consistent with this notion, it is well known that genetic ablation of KRAS in many KRAS mutant tumor cells results in cytostasis but not cell death (Singh et al., 2010), as we observed in our sensitive cell lines (Figure 2-3B). While enhancing pathway inhibition with the addition of a MEK inhibitor could further potentiate the effects of KRAS knockdown, a full cytotoxic response could only be achieved with the addition of BET bromodomain inhibitors (Figure 2-3B). Taken together these observations suggest that BET bromodomain inhibitors might function by exerting additional effects beyond suppressing the Ras transcriptional output.

To identify deregulated PRC2 targets in NSCLC that might also provide insight into the therapeutic mechanism of action, a two-class comparison of transcriptional profiles from sensitive versus resistant cell lines was performed. Only 11 genes were found to be differentially expressed between these cell lines, however HOXC10 was by far the most differentially and
Figure 2-3. Identification of HOXC10 as a biomarker and mediator of sensitivity

(A) Heatmap shows suppression of compiled gene list representing the RAS transcriptional output. Data are derived from microarray analysis conducted in triplicate on sensitive cell line NCI-H1573 after 24 hours of treatment with DMSO (black), PD901 (light gray, 500 nM), JQ1 (dark gray, 1000 nM), and PD901 + JQ1 (red).

(B) NCI-H1573 was treated with MEKi, BRD4i, siKRAS, or indicated combinations for 72 hours and results were assayed by cell counting. Cell death between the siKRAS + MEKi and siKRAS +MEKi + BRD4i conditions was significantly enhanced (p<.0005 by two sided t-test with Welch’s correction).

(C) CCLE expression data of KRAS-mutant lung adenocarcinoma cells was analyzed by two-class comparison. All significantly differential genes (corrected p<.001) are graphed according to fold change in expression between the sensitive and resistant group and ordered by rank list. HOXC10 was the top hit and was expressed 13.33 fold higher in sensitive cells.

(D) Expression of HOXC10 was analyzed in the resistant and sensitive groups with data from the CCLE. HOXC10 expression was significantly higher in the sensitive group (p<.05, two-sided t-test with Welch’s correction)

(E) HOXC10 expression was analyzed by qPCR and immunoblot analysis. qPCR data is normalized to GAPDH levels and lowest expression value (H1373) was arbitrarily set at a level of 1.0 as a point of comparison. By qPCR, the median HOXC10 expression of the sensitive group of cell lines is 20-fold higher than the resistant group, comparable to what is found in publically available data.

(F) HOXC10 protein expression was assayed by immunoblot after exposure to indicated treatment for twenty-four hours (PD901: 500nM, JQ1: 1000nM). Alpha-tubulin was used as loading control.

(G) NCI-H23 (top) and NCI-H1573 (bottom) cells expressing LACZ or HOXC10 were treated with indicated drugs for 72 hours. Data is represented as log2 fold change in cell number between day 3 and day 0. Experiments are representative of at least three independent experiments. Immunoblots in Figure A-3D show response of HOXC10 and p-ERK to treatment.

(H) SUZ12 or EED was knocked down in resistant cell lines. SUZ12 knockdown is observed by immunoblot. EED knockdown not observed due to inadequate antibody. HOXC10 levels were assayed in response to PRC2 knockdown. Alpha-tubulin used as loading control.

(I) HOXC10 response to treatment was assayed by immunoblot in sensitized A549 cells (via EED knockdown) treated with indicated drugs. Alpha-tubulin is used as loading control.

(J) Resistant cell line A549 is treated with indicated treatments for 72 hours in proliferation experiment under four conditions: shSCR + LACZ, shSCR + HOXC10, shSUZ12 + LACZ, shSUZ12 + HOXC10. A549 were subjected to double selection; short hairpins under blasticidin selection and expression vectors under puromycin selection.

(K) Three additional KRAS-mutant cell lines were assayed for sensitivity to combined treatment utilizing a 72-hour proliferation assay. Proliferation experiments from Figure 1B for resistant cell line NCI-H1792 and sensitive cell line NCI-H23 are shown for comparison. HOXC10 protein levels were analyzed by immunoblot. GAPDH is used as control.

(L) Original panel of cells from Figure 1A are assayed for HOXC10 protein expression by immunoblot. Sensitive line NCI-H1573 and resistant line NCI-H1792 were run on same blot for comparison. K indicates a KRAS-mutant cell line and N indicates an NF1-deficient cell line.
Figure 2-3 (Continued).
potently upregulated in sensitive cells (Figures 2-3C and D). Upregulation was confirmed by both mRNA analysis and immunoblots, which revealed a dramatic binary difference in expression between sensitive and resistant cell lines (Figure 2-3E). This observation was particularly intriguing because HOX genes are canonical PRC2 targets and the aberrant expression of other HOX genes play a well-established role in cancer, most notably HOXA9 in AML (Milne et al., 2002; Andreef et al., 2008). Interestingly, HOXC10 has been found to be overexpressed in a subset of tumor types, although little is known about its potential function (Sadik et al. 2016; Ansari et al., 2012; Feng et al., 2015). Therefore, we were interested in defining its role in this therapeutic response and its potential utility as a biomarker in human NSCLCs.

**HOXC10 suppression is required for NSCLC cell death**

First, we examined HOXC10 expression in response to MEK and BET bromodomain inhibitors in NSCLCs. Notably, HOXC10 protein and mRNA expressions were potently suppressed by JQ1 alone and this down regulation was sustained in the presence of both agents (Figures 2-3F, Figure A-3B). This observation was consistent with the notion that HOXC10 is a PRC2 target and that BET bromodomain inhibitors can inhibit the expression of many de-repressed PRC2 targets as has been previously demonstrated (Figure A-3C; DeRaedt et al., 2014). Most importantly however, ectopic expression of HOXC10 dramatically suppressed cell death in response to MEK and BET bromodomain inhibitors (Figure 2-3G, Figure A-3D). These effects were not due to enhanced effects on baseline levels of cell proliferation.

To confirm that HOXC10 was indeed a PRC2 target that conferred sensitivity to this drug combination, either SUZ12 or EED were suppressed with shRNA sequences in insensitive cell lines. PRC2 suppression induced the expression of HOXC10 (Figure 2-3H), and as shown in Figure 2-2C these cell lines were rendered sensitive by these events. Similar to innately sensitive cell lines, HOXC10 expression was inhibited by BRD4i and BRD4i/MEKi in this setting.
Importantly, ectopic HOXC10 expression also rescued this acquired sensitivity (Figure 2-3J, Figure A-3E).

Finally, to investigate the potential value of HOXC10 as a predictive marker of sensitivity, KRAS mutant cell lines with either high or low HOXC10 based on CCLE mRNA expression data, were prospectively selected for analysis. Differential HOXC10 protein expression was confirmed and those that expressed HOXC10 were sensitive to MEK/BET bromodomain inhibitors, whereas cells that lacked HOXC10 were not (Figure 2-3K). In addition, retrospective analysis of the two unresponsive NF1 mutant cell lines shown in Figure 2-1A demonstrated that they also did not express HOXC10, as would be predicted (Figure 2-3L). Together these results suggest that HOXC10 is both a predictive and functional biomarker of sensitivity to MEK and BET bromodomain inhibitors.

**HOXC10 marks distinct set of NSCLCs and a PDX model is sensitive MEK/BET inhibition**

Transcriptional analysis of primary human NSCLCs revealed that HOXC10 is overexpressed in 51% of KRAS-mutant tumors (Figure 2-4A; >2SD over expression in normal lung tissue). By analyzing the few KRAS-mutant tumor-normal matched tissue pairs available from TCGA, HOXC10 was found to be specifically overexpressed in tumor versus normal tissue in 8/9 of the HOXC10-expressing samples (Figures 2-4B). To investigate the therapeutic efficacy of this drug combination in primary patient material, a KRAS mutant patient-derived xenograft model that expresses HOXC10 was identified and established. Robust HOXC10 protein expression was confirmed by both immunoblot and immunohistochemistry (Figure 2-4C and D). Similar to the xenograft models, MEK and BET inhibitors also caused frank tumor regression in this PDX model (Figure 2-4E), underscoring the potential clinical utility of this combination.
Figure 2-4. HOXC10 overexpression marks a distinct set of NSCLCs and a PDX model is sensitive MEK/BET bromodomain inhibitors

(A) HOXC10 expression via the TCGA data set was plotted for normal lung tissue and KRAS-mutant lung adenocarcinoma tumors. Each dot represents a distinct sample within the dataset. Tumors with HOXC10 expression 2STDEV above normal tissue are highlighted in red and this class represents approximately half of all KRAS-mutant tumors.

(B) mRNA expression of HOXC10 of matched normal-tumor samples. Data derived from the provisional TCGA data set, expression normalized by HighSeq percentile (N=13).

(C) HOXC10 protein levels were assayed by immunoblot in three different PDX tumor samples. Alpha-tubulin was used as a loading control.

(D) Expression of HOXC10 by IHC in two of the PDX tumor models corresponds to the HOXC10 levels assayed by immunoblot.

(E) PDX #1 was implanted into a cohort of NSG mice. Tumors grew to approximately 150-200 mm$^3$ before enrollment in therapeutic study where they were treated daily with indicated treatments. A snapshot of the tumor response is presented from Day 22. The waterfall plot shows the log2 fold change in tumor volume from Day 22 to Day 0 on the left y-axis and the percent change in tumor volume on the right y-axis.

(F) Single sample GSEA was performed on KRAS-mutant tumors and divided into two categories, a group with high PRC2 activity (low PRC2 target expression) and a group with low PRC2 activity (high PRC2 target expression). HOXC10 expression was significantly higher in the group with low PRC2 activity (p<.0001, two-sided t-test with Welch’s correction).

(G) KRAS-mutant tumors were assayed for HOXC10 expression and divided into HOXC10 high and HOXC10 low groups. High expression was gated as two standard deviations above normal lung tissue expression, low expression was defined as two standard deviations below normal lung tissue expression. PRC2 target expression was assayed in these two groups by ssGSEA and PRC2 target expression was higher in the HOXC10-high group (p<.001, two-sided t-test with Welch’s correction).

(H) All KRAS-mutant/HOXC10-high lung adenocarcinoma tumors are represented in this pie chart (TCGA provisional, N=78). Within these samples, CNV loss of PRC2 obligate components SUZ12, EED, and EZH1/2 were assayed. Additional PRC2 defects were assessed including low expression of SUZ12 and EED (defined as two standard deviations below normal tissue) and low PRC2 activity as assayed by ssGSEA.
Figure 2-4 (Continued).
Relationship between HOXC10 and PRC2 defects in primary NSCLCs

While functional evidence indicated that PRC2 could regulate HOXC10 expression in vitro (Figure 2-3H), the relationship between HOXC10 and PRC2 activity in primary NSCLCs was further explored. Notably, HOXC10 expression levels inversely correlated with PRC2 activity in human tumors. Specifically, when KRAS-mutant tumors were stratified into those with de-repressed PRC2 targets (i.e. high expression, low PRC2 activity) versus those with repressed PRC2 targets (i.e. low expression, high PRC2 activity), HOXC10 is more highly expressed in “low PRC2 activity” tumors (Figure 2-4F). Conversely, when KRAS-mutant tumors were stratified by HOXC10 expression, PRC2 targets were significantly de-repressed in high HOXC10 tumors (Figure 2-4G).

These observations suggested that aberrant HOXC10 expression in NSCLC is largely regulated by defects in PRC2. Indeed, we found that approximately three-quarters of high-HOXC10 tumors exhibit measurable defects in PRC2 activity (Figure 2-4H). Thirty-five percent of these tumors harbored heterozygous mutations in at least one PRC2 component, thirteen percent exhibited low expression of PRC2 components, and an additional sixteen percent exhibited defects in PRC2 target gene repression due to a mechanism that remains to be determined (Figure 2-4H). However, HOX genes can also be deregulated in cancer via other mechanisms, for example through defects in trithorax group proteins and demethylases (reviewed in Mallo and Alonso, 2013). We anticipate that the remaining fraction of KRAS-mutant/high-HOXC10 tumors exhibit undetected defects in other epigenetic regulatory genes that remain to be discovered. Regardless, these observations suggest that HOXC10 is largely regulated by PRC2 defects in NSCLC, but that HOXC10 expression itself may be a superior predictive marker of sensitivity to MEK/BET bromodomain inhibitors.
**HOXC10 and MEK coordinately control the expression of pre-replication complex proteins**

Next, we investigated the mechanism by which HOXC10 suppression was regulating NSCLC death. Notably, while wildtype HOXC10 could inhibit cell death triggered by MEK/BET bromodomain inhibitors, a HOXC10 protein lacking the homeobox domain was unable to do so, underscoring the importance of HOXC10 regulated transcription in this response (Figure 2-5A, Figure A-4A). Therefore, we performed gene ontology (GO) enrichment analysis on the list of transcripts that were significantly downregulated by combined PD901 and JQ1 prior to cell death. Multiple gene sets related to DNA replication initiation, strand elongation, and replication checkpoints were the most significantly enriched (Figures 2-5B). Notably, these gene lists are not universally enriched in tumor cells treated with cytotoxic combinations (Malone et al., 2017). Genes associated with DNA replication initiation (the top scoring list), included many essential components of the pre-replication complex (pre-RC): CDC6 and MCM2-7.

To get a broad sense of the pathways that HOXC10 was regulating in this therapeutic context, we identified the genes that were both suppressed by MEK/BET bromodomain inhibitors and were also upregulated in HOXC10-expressing cells. We then ran Gene Ontology analysis on overlapping genes and found that the majority of top-scoring lists included genes crucial for pre-RC complex formation and cell cycle progression (Figure A-4B). Strikingly, the DNA replication initiation gene list was the most highly enriched and significant hit by this analysis (fold enrichment = 16.12, p-value = 1.40E-10, FDR = 7.77E-08; Figure 2-5C), raising the possibility that the suppression of these genes could be playing a causal role in cell death.

Pre-RC components bind DNA in the well-defined origin licensing process in the G1 phase of the cell cycle and mark all potential replication origins, a subset of which become activated in S phase (reviewed in Mechali, 2010). If insufficient numbers of origins are present, replication forks can stall, collapse, and trigger DNA damage (reviewed in Zeman and Cimprich, 2014). Indeed, we found that combined MEK and BET bromodomain inhibitors potently
suppressed the expression of multiple pre-RC genes that are essential for origin formation in eukaryotic cells (Figure 2-5D). Specifically, MEK and BET bromodomain inhibitors independently inhibited the expression of these genes to varying extents, but maximal inhibition was only observed in the presence of both agents (Figure 2-5E, Figure A-4C). The cooperative effects of MEK and BRD4 inhibitors on mRNA and protein expression were confirmed for the two most potently suppressed components, CDC6 and MCM5. (Figures 2-5E and -F). Together these results suggest that MEK and BET bromodomain proteins coordinately control the expression of pre-RC genes.

To confirm that HOXC10 suppression was functionally contributing to the decreased expression of pre-RC genes in tumor cells, ectopic HOXC10 was introduced into sensitive cell lines and transcriptional profiles were examined. In control LACZ expressing cells, combined MEK/BET bromodomain inhibitors potently suppressed the expression of all of these pre-RC components; however, ectopic HOXC10 expression substantially rescued the suppression of these genes (Figure 2-5G). These observations were confirmed in additional sensitive cell lines at the protein level (Figures 2-5H, Figure A-4D). It should be noted that HOXC10 expression did not completely prevent the suppression of these genes, but rather expression was restored to levels comparable to cells treated with MEK inhibitors alone. This observation suggests that HOXC10 is essential for coordinately regulating the expression of key replication proteins in these NSCLCs, together with the Ras-MEK-ERK pathway.

*MCM5* and *CDC6* genes are induced in the G1 phase of the cell cycle and mRNA levels peak at the G1/S boundary (Ohtani et al., 1998). To exclude the possibility that suppression was related to changes in cell cycle distribution, a cell cycle sorting experiment was performed. Importantly, *MCM5* and *CDC6* mRNA levels were suppressed in sensitive cells that were either in the G1 or the S phases of the cell cycle, in response to BRD4/MEK inhibitors (Figure 2-5I, Figure A-4E), suggesting that these changes were not a secondary consequence of effects on the cell cycle.
Figure 2-5. HOXC10 and MEK coordinately control the expression of pre-replication complex proteins

(A) Sensitive cell line NCI-H1573 was treated with indicated treatments for three days expressing four different constructs: Control, pHAGE-HOXC10-FLAG-Cterm, pHAGE-HOXC10-FLAG-Nterm, and pHAGE-HOXC10-del3(homeobox)-FLAG-Nterm. The deletion construct has no homeobox, which is responsible for HOXC10 transcriptional activity.

(B) GO analysis was performed on genes significantly downregulated (fold change < -2, p< .001) by combination treatment with GO biological process gene lists. The top five ranked gene groups are listed with corresponding fold enrichment.

(C) Genes downregulated by combination treatment and genes upregulated by HOXC10 expression were compared to create an overlap list of genes. GO analysis was then performed on this overlap gene list. Lists with 10-fold or higher enrichment were plotted by log10 of FDR versus log10 of p-value to find the most significantly enriched lists. DNA replication initiation scored the highest by this metric. Other high scoring lists were also important to DNA replication and DNA repair and are listed in Figure A-4B.

(D) The fold suppression of key pre-RC complex proteins upon combination treatment is listed in order of suppression. Data derived from microarray analysis of sensitive NCI-H1573 cell line following twenty-four hours of treatment with combined PD901 (500nM) and JQ1 (1000nM).

(E) CDC6 and MCM5 mRNA expression was analyzed by qPCR in response to indicated treatments. Expression was normalized to the housekeeping gene UBC and all fold changes presented are relative to DMSO. SD bars represent the standard deviation of three technical replicates.

(F) CDC6 and MCM5 protein expression in response to MEKi, BRD4i, and MEKi + BRD4i was assayed by immunoblot in sensitive cell line.

(G) NCI-H1573 cells expressing LACZ and HOXC10 were treated with DMSO or combined MEKi and BRD4i for twenty-four hours before RNA was harvested. RNA was subjected to microarray and each condition was analyzed for fold suppression in response to combination treatment. Suppression of pre-RC complex proteins including CDC6 and MCM5 is expressed in graph showcasing the rescue of suppression by HOXC10 overexpression.

(H) MCM5 and CDC6 protein expression was assayed in NCI-H23 cells expressing LACZ and HOXC10 in response to indicated treatments. Alpha tubulin was utilized as a control.

(I) NCI-H1573 cells were stained with Hoechst and then sorted by cell cycle phase. RNA was harvested from G1 and S phases for qPCR analysis of MCM5 and CDC6 expression across the cell cycle. Ct values were normalized to housekeeping gene UBC expression.

(J) CDC6 and MCM5 were transiently knocked down by siRNA SMARTpools and cells were then exposed to DMSO (black) or combined MEKi and BRD4i (red) for 72 hours. Two sensitive cells (left, NCI-H23 and NCI-H1573) and two resistant cells (right, NCI-H2030 and A549) are shown.
**Figure 2-5 (Continued).**

**B. GO biological process complete**

<table>
<thead>
<tr>
<th>Process</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA replication initiation (GO:0006270)</td>
<td>25</td>
</tr>
<tr>
<td>DNA strand elongation involved in DNA replication (GO:0006271)</td>
<td>22.58</td>
</tr>
<tr>
<td>DNA unwinding involved in DNA replication (GO:0006268)</td>
<td>21.71</td>
</tr>
<tr>
<td>DNA replication checkpoint (GO:0000076)</td>
<td>20.43</td>
</tr>
<tr>
<td>DNA strand elongation (GO:0022616)</td>
<td>18.82</td>
</tr>
</tbody>
</table>

**D. Gene Fold Suppression**

- CDC6: 7.08
- MCM5: 6.67
- MCM6: 6.37
- MCM7: 4.80
- MCM3: 4.18
- MCM4: 3.57
- MCM2: 3.51

**E. DNA replication initiation**

**F. MEKi, BRD4i, LACZ, HOXC10**

**G. Relative CDC6, MCM5 mRNA expression**

**H. LACZ, MEKi, BRD4i, MCM5, CDC6, tubulin**

**I. Relative CDC6, MCM5 mRNA expression**

**J. SENSITIVE vs. RESISTANT**

- siCNT, siCDC6, siMCM5

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**Figure 2-5 (Continued).**
The suppression of pre-RC components plays an active role in promoting cell death

Finally, to determine whether the suppression of pre-RC proteins was playing a causal role in cell death in response to these agents, either MCM5 or CDC6 were suppressed by siRNA sequences in both sensitive and resistant cell lines. In sensitive cell lines, further suppression of either CDC6 or MCM5 by RNAi potentiated the cytotoxic response of the combination (Figure 2-5J, left). By contrast, knockdown of MCM5 or CDC6 slightly suppressed the proliferation of resistant cells, but their suppression still did not induce cell death, even in the presence of MEKi/BRD4i (Figure 2-5J, right). Together these observations suggest that HOXC10-expressing cells are more sensitive to reduced levels of origin proteins, which are coordinately regulated by HOXC10 and the Ras/MEK pathway, and that the suppression of these pre-RC proteins plays an active role in triggering cell death.

BRD4/MEK inhibitors kill HOXC10 expressing NSCLC by inducing replication fork stalling and DNA damage

Suppression of pre-RC proteins has been shown to exert differential effects in different cells. In non-transformed cells, suppression of MCM5 and CDC6 levels cause a growth arrest and no cell death, due to a licensing checkpoint at the G1/S boundary which ensures that sufficient origins are licensed before proceeding to S phase (Blow and Gillespie, 2008). In this setting it is thought that death occurs because these cancer cells do not completely arrest in G1 and therefore proceed into S phase with too few origins of replication, resulting in replication fork collapse and ultimately DNA damage (Lau et al., 2009). Therefore, we examined the effects of MEK/BET bromodomain inhibitors on cell cycle dynamics in both sensitive and resistant cell lines. This combination induced a potent G1 cell cycle arrest in the resistant cell lines after twenty-four hours, consistent with suppression of proliferation and the absence of cell death observed in these cells (Figures 2-6A and 2-1D). By contrast, sensitive cells treated with the combined MEK and BRD4 inhibition did not completely arrest in G1 and a quarter
Figure 2-6. BRD4/MEK inhibitors kill HOXC10 expressing NSCLC by inducing replication fork collapse and DNA damage

(A) Resistant cells NCI-H2030 and A549 were treated with combined MEKi/BRD4i for twenty-four hours. Cells were then harvested, fixed, and stained with propidium iodide to observe cell cycle distribution via FACS. Representative cell cycle plots are shown and changes in the S/G2/M fractions are quantified.

(B) Sensitive cells NCI-H23 and NCI-H1573 were treated with combined MEKi/BRD4i for twenty-four hours. Cells were then harvested, fixed, and stained with propidium iodide to observe cell cycle distribution via FACS. Representative cell cycle plots are shown and changes in the S/G2/M fractions are quantified.

(C) DNA fiber analysis was performed on DMSO and combination treated NCI-H1573 cells. Cells were treated with drug for fourteen hours and then red fluorescently labeled EdU was spiked into media for incorporation into DNA. After two hours, the red labeled EdU was washed out and green-labeled EdU was added. The ratio of the length of green fibers vs. red fibers was quantified to determine the presence of stalled forks in each condition. The combination treatment has significantly smaller ratio indicating the presence of stalled replication forks in the population.

(D) Representative images of DNA fibers are shown from each condition.

(E) DNA damage was assayed over the course of a twenty-four hour combination treatment time course in sensitive cell line NCI-H23 compared to resistant cell lines NCI-H2030 and A549 by immunoblot of phospho-gamma H2AX. GAPDH is used as a loading control. These results are representative of at least two biological replicates per cell line.

(F) A time course experiment was performed on sensitive cell line NCI-H1573 and shows that CDC6 and MCM5 levels decrease ahead of the induction of DNA damage.
Figure 2-6 (Continued).
of the cells were still detected in S and G2/M phases of the cell cycle (Figure 2-6A). Taken with the kinetics of cell death shown in Figure 2-1D, this observation suggests that sensitive cells remain cycling as death is occurring.

Based on the paradigm described above, if these agents were triggering cell death by suppressing origins of replication, we would expect to see replication fork stalling in S phase and ultimately DNA damage, due to insufficient numbers of origins. We performed a DNA fiber analysis in which demonstrated that these agents induce profound replication fork stalling (Figure 2-6C and -D). By incubating cells with two different color-labeled EdU analogs, we could track the rate of DNA synthesis by measuring double-labeled fibers, specifically the length of the green part of the fiber (second incubation) divided by the length of the red part of the fiber (first incubation). By this metric, combination-treated cells exhibited a significantly decreased green to red ratio, indicating profound fork stalling due to treatment (Figure 2-6C). Representative fibers are shown in Figure 2-6D. While eukaryotic cells have a large reserve of replication origins to buffer against a reduction in their numbers, a catastrophic reduction of replication origins is sufficient to cause DNA damage in cells that continue to cycle through S phase despite this handicap (Ge et al., 2007; Ibarra et al., 2008; Kawabata et al., 2011). Accordingly, we found that MEK/BRD4 inhibitors potently induced DNA damage in sensitive cell lines but not in resistant cell lines (Figure 2-6E). Notably, the suppression of pre-RC proteins occurred within 8 hours and preceded phospho-gamma H2AX expression which was elevated at 12 hours and became maximal at 24 hours, further supporting a causal role for pre-RC protein suppression in cell death (Figure 2-6F).

**MEK and HOXC10 regulate pre-RC proteins by coordinately regulating E2F**

Finally, we sought to determine how HOXC10 was cooperating with the RAS-MEK-ERK pathway to regulate pre-RC gene expression. *MCM* genes and *CDC6* are well-established E2F
targets. As such, E2F levels dictate not only whether a cell can enter S phase, but are also required to produce the machinery necessary for replication (reviewed in Ren et al., 2001). Given the known role of many HOX genes in instructing proliferation versus arrest/differentiation during development, we investigated whether HOXC10 might coordinately control E2F levels, along with the Ras/ERK pathway in these cells since MEK inhibitors are known to suppress E2F genes in part by suppressing cyclin D1 levels (Lavoie et al., 1996; Winston et al, 1996; Resnitsky et al., 1995). As expected, MEKi suppressed E2F1 by more than 2 fold. We found that JQ1 alone also suppressed E2F1 expression by 1.5 fold and together both agents suppressed levels by 4.2 fold (Figure 2-7A). This pattern of cooperative suppression and restoration was also reflected in ssGSEA analysis of E2F target genes (Figure 2-7B), including MCM5 and CDC6. However, HOXC10 reconstitution restored more than half of the E2F1 expression in cells treated with MEKi/BRD4i, bringing E2F1 levels back to levels similar to those observed in response to MEKi alone (Figure 2-7C). Taken together, these data suggest that HOXC10 controls pre-RC gene expression in these NSCLCs by regulating E2F1 levels.

If HOXC10 was functioning by critically regulating the expression of E2F genes in these NSCLCs, then a reduction of E2F levels should prevent rescue by HOXC10. RNAi mediated suppression of the E2F1 gene alone was not sufficient to arrest these cells and had no effect on the potent cytotoxic effects of PD901/JQ1 (Figure 2-7D). As shown throughout, ectopic expression of HOXC10 substantially attenuated the cytotoxic effects of these agents; however, E2F1 suppression reversed these effects and restored sensitivity to PD901/JQ1 (Figure 2-7D). Together these studies demonstrate that BRD4 and MEK inhibitors function by coordinately suppressing the Ras pathway and HOXC10, which in turn suppress E2F genes and the replication machinery to levels which trigger replication defects, catastrophic DNA damage, and death.
Figure 2-7. MEK and HOXC10 regulate pre-RC proteins by coordinately regulating E2F
(A) Fold suppression of E2F1 RNA after twenty-four hour exposure to indicated treatments. 
(B) ssGSEA analysis of E2F1 target gene list after twenty-four hour exposure to indicated 
treatments. E2F targets are downregulated by MEKi alone and this downregulation is 
significantly enhanced when MEKi is combined with BRD4i (p < .05 two-sided t-test with 
Welch’s correction). Data for (A) and (B) is derived from microarray analysis of sensitive cell line 
NCI-H1573.
(C) Fold suppression of E2F1 in NCI-H1573 cell line stably expressing ectopic LACZ or 
HOXC10. Ectopic HOXC10 rescues suppression of E2F1 to levels that occur with MEKi alone in 
parental cell line (parental cell line data from Figure 2-7A).
(D) NCI-H1573 cell line was treated with indicated treatment for seventy-two hours. Data is 
represented as log2 fold change in cell number between Day 3 and Day 0. HOXC10 was stably 
expressed in NCI-H1573 and four conditions were analyzed: siCNT, siE2F1, siCNT + HOXC10, 
and siE2F1 + HOXC10.
DISCUSSION

Here we identify a promising therapeutic combination for KRAS-mutant NSCLCs, a tumor type for which there currently are no effective treatments. Specifically, we show that combined MEK and BET bromodomain inhibitors cause robust cell death in multiple human NSCLC cell lines, and trigger potent tumor regression in two human xenografts and one PDX model. The observation that these agents cause frank tumor regression rather than cytostasis, which is frequently described as a promising preclinical therapeutic response, is highly significant. In fact, responses here appear to be greater than those observed with a KRAS G12C inhibitor (Janes et al. 2018). While the development of this and other KRAS inhibitors certainly represent an invaluable advancement, the data presented here raise the possibility that, like MEK inhibitors, the effects of these agents could be further enhanced when combined with BET bromodomain inhibitors. BET bromodomain inhibitors are currently being evaluated in clinical trials and additional agents are being developed (reviewed in Stathis and Bertoni, 2018). Our data provide strong scientific rational for evaluating the combined effects of BET bromodomain inhibitors and MEK or other Ras pathway inhibitors in KRAS mutant NSCLC.

The clinical development of BET bromodomain inhibitors has been challenging because in most settings biomarkers that predict efficacy have not been identified. MYC expression is certainly one potentially important candidate, however early clinical findings suggest that MYC expression may not be predictive of efficacy. Moreover, BET bromodomain inhibitors do not always function by suppressing MYC (DeRaedt et al., 2014; Fong et al., 2015). In this study we identified a functional, predictive biomarker for this BET-bromodomain inhibitor-based combination in NSCLC. Specifically, we found that sensitive tumors uniquely and robustly express HOXC10.

HOX genes are master developmental regulators and are overexpressed in a variety of cancers. They have been proposed to contribute to tumor development by affecting diverse processes including proliferation, differentiation, survival, and metastasis (reviewed in Shah and
Sukumar, 2010). Because HOX genes control differentiation and cell fate during development, many HOX genes that are upregulated in specific cancers are normally expressed in that tissue during development (reviewed Abate-Shen, 2002). However, HOX genes that are not normally expressed in a specific tissue can also be “newly” expressed in cancers. Currently, there is no evidence that HOXC10 is expressed in human or mouse lung during development, although the possibility remains that it is expressed in developing human lung at a specific time point that has not yet been examined and/or in a specific cellular niche. Regardless, HOXC10 marks a new distinct subset of lung cancers that harbor a specific therapeutic vulnerability. The observation that HOXC10 suppression is essential for cell death suggests that it may also be useful as a therapeutic biomarker in post-treatment biopsies to assess target inhibition. In the future it will be important to understand whether or not HOXC10 marks lung cancers with a specific cell of origin and/or how HOXC10 may fully contribute to the development of NSCLC. It should be noted that HOXC10 expression is not restricted to KRAS mutant lung cancers (see Chapter 3) and therefore may confer additional vulnerabilities in other lung cancer subtypes.

We also show that HOXC10 is frequently, but not exclusively, deregulated in NSCLC as a consequence of defects in PRC2 genes. Interestingly, heterozygous loss of PRC2 components is common in NSCLC while homozygous loss is exceedingly rare. This observation is consistent with the growing appreciation that partial defects in PRC2 activity appear to play a role in the development of other tumor types (reviewed in Margueron and Reinberg, 2011). Regardless, these observations suggest that heterozygous alterations are selected for during NSCLC development, which may relate to the role of PRC2 in silencing the CDKN2A locus or other tumor suppressor genes (Collinson, et al. 2016).

Interestingly, EZH2 has also been shown to overexpressed in NSCLC (Zhang et al., 2016). Consistent with a dichotomous role for the PRC2 complex in NSCLC, both EZH2 overexpression and EED suppression have been shown to promote NSCLC development in mice. Interestingly, EED loss was shown to regulate EMT and promote the development of
invasive tumors in mice (Serresi et al., 2016). Fortunately, the data presented here suggest that while defects in the PRC2 complex may promote NSCLC progression, they also confer a therapeutic vulnerability.

While the complete function of HOXC10 in lung cancer is not known, this study suggests that it is critical for regulating the expression of E2F and pre-RC genes in these tumors. This observation makes teleological sense in that HOX genes are commonly involved in maintaining an undifferentiated and proliferative state during development. Therefore, in the context of NSCLC aberrant HOXC10 expression might override cues to stop proliferating. Regardless, here we show that combined MEK/BET bromodomain inhibitors cause a G1 arrest in NSCLCs that have an intact PRC2 complex and do not express HOXC10. By contrast those that harbor PRC2 defects and express HOXC10 are able to proceed through to S phase. However, because HOXC10 and pre-RC genes are substantially reduced by these agents, replication forks stall, ultimately causing DNA damage and cell death (see Figure 2-8 for model). These observations reveal a unique role for HOXC10, in conjunction with the Ras/MEK/ERK pathway, in regulating E2F and replication proteins in NSCLC. In addition, it suggests that these tumors have become dependent on HOXC10 for regulating these processes in certain settings, resulting in a unique therapeutic vulnerability to combined epigenetic and oncogenic inhibitors.
Figure 2-8. Model of therapeutic response to combined MEK and BRD4 inhibition
Schematic showing model in which both inhibitors cooperate to suppress Ras transcriptional output. Through suppression of HOXC10 and the Ras transcriptional output, both treatments cooperate to downregulate the E2F transcription factor which ultimately downregulates levels of key pre-replicative complex proteins. This coordinate downregulation of pre-RC genes leads to catastrophic DNA damage in treated cell lines due to lack of origin licensing. Ensuing DNA damage and replication stress are ultimately the cause of caspase-mediated cell death in KRAS-mutant/HOXC10 high lung adenocarcinoma tumors.
METHODS

Cell Lines and Reagents

Human lung cancer cell lines were purchased from the ATCC or obtained from Dr. William Hahn (Dana-Farber Cancer Research Center) in 2015. All cells were cultured in RPMI media supplemented with 10% FBS with the exception of NCI-H23, NCI-H1435, NCI-H1702, SKLU-1 which were all cultured in DMEM media supplemented with 10% FBS. Short interfering RNAs (siRNAs) ON-TARGET SMARTpools siRNA were purchased from Dharmacon to target KRAS, CDC6, MCM5, and E2F1, and transfected with lipofectamine RNAiMAX from Invitrogen. Lentiviral pLKO vectors were obtained from the RNAi consortium of the Broad Institute. shSUZ12 S1 (NM_015355.1-2076s1c1, target sequence 5'- GCTGACAATCAAATGAATCAT-3'), shSUZ12 S2 (NM_015355.1-668s1c1, target sequence 5’GCTTACGTTTACTGGTTTCTT-3’) and shSUZ12 S3 (NM_015355.1-501s1c1, target sequence 5’-CGGAATCTCATAGCACCAATA-3’) were used to target SUZ12. Antibodies were obtained from the following sources: Cell Signaling Technologies: vinculin (#4650), H3K27me (#9733), phoso-ERK (#9102), GAPDH (#2118); Sigma: α- tubulin (#T5168); AbCam: HOXC10 (#153904); Santa Cruz: SUZ12 (#46264), MCM5 (#165994), EMD Millipore: CDC6 (05-550), phospho-gamma-H2AX (#JBW301). Human HOXC10 was a pHAGE HA-FLAG-C-terminal tagged construct obtained from the Harvard PlasmID database. PD-0325901 was a gift from Kevin Shannon (University of California, San Francisco). JQ1 was synthesized by Chempartner in concert with Dr. Jun Qi (Dana Farber Cancer Institute)

Infections and Transfections

shRNA or cDNA constructs were prepared and virus was harvested as previously described (McLaughlin et al., 2013). Virus was incubated on target cells for 6 to 16 hours at a 1:2–1:10 dilution with 8-µg/mL polybrene. For LACZ and HOXC10-infected cells, infection was performed twice, first for 16 hours overnight, then for 6 hours the following day with full-strength virus, and cells were allowed to recover for 24 hours before selection. Infected cells were
selected in 0.5 to 2.0 µg/mL puromycin or 1 to 2 µg/mL blasticidin, depending on the construct and optimized for each cell line. Selections continued until uninfected control plates were killed, approximately 3-5 days depending on the cell line. For experiments with two constructs, infections were performed sequentially meaning first construct was infected and selected followed by second infection and selection. Constructs were in opposite selection markers so that all cells were selected by both puromycin and blasticidin. For transfections, cells were transfected for 16 hours with 10 µmol/L siRNA constructs using a 1:400 dilution of Lipofectamine RNAiMAX (Invitrogen, cat. # 13778-075) in antibiotic-free media.

**Cellular proliferation assays**

To perform cellular proliferation assays, 75,000-100,000 cells per well were seeded onto 6-well plates in appropriate media. Approximately 24 hours later, day 0 counts were taken and for inhibitor experiments, drug treatments were started on day 0. Media was not replaced over the course of 72 hours and day 3 counts were completed in triplicate on day 3 to determine the change in cell number compared to day 0. Proliferation experiments that included siRNA knockdown were performed on cells approximately 24 hours after initial transfection.

**Incucyte Cell Proliferation and Death Assay**

Cells were infected with IncuCyte Nuclight red (mKate2) reagent (#4625) and selected in puromycin to create stable cell lines. Nuclight versions of each cell line from Figure 1-2C were plated at 3000-4000 cells/well in a 96 well plate. Approximately 24 hours later, the media was removed and medium containing 1:1000 green caspase3/7 apoptosis assay reagent (#4440) and appropriate drug concentration was added. The 96 well plates were then placed in the Incucyte instrument and images were taken every two hours over the course of five days. The Incucyte software was then trained to count the cells based on the number of red-expressing nuclei in the field of view. Four images were taken per well and averaged as technical replicates. Cell death is assayed by the presence of green signal (caspase reagent) overlapping with red signal (nuclei). Cell number is assayed as the number of red nuclei minus the number
of dead overlapping nuclei. Cell number and caspase counts are calculated for every Incucyte reading and normalized to Day 0 to create the graphs seen in Figures 2-1C and -D.

**Analysis of Public Data**

Public expression, mutational, and copy number data was downloaded from the TCGA-LUAD project. Expression of HOXC10 in normal tumors (samples containing -11 in sample name) was assayed for median and standard deviation. Tumor samples were then stratified according to HOXC10 expression levels relative to the normal tissue. Overexpression was called as two standard deviations over the median of normal tissue. Matched KRAS-mutant tumor-normal samples were identified for the analysis in Figure 2-4B. Expression data of high-HOXC10 and low-HOXC10 tumors was also obtained from the TCGA and single sample gene set enrichment analysis (ssGSEA) was performed on the data to determine the relative level of PRC2 target expression in each group of tumors. Similarly, the same ssGSEA analysis was performed on all normal and tumor samples. Samples were then stratified according to high and low PRC2 target expression (two standard deviations over normal tissue) and then corresponding HOXC10 expression was assayed. These analyses are presented in Figure 2-4.

Public expression, mutational, and copy number data was also downloaded from the Broad Institute Cancer Cell Line Encyclopedia via the UCSC cancer genome browser. Mutational and copy number data was assayed on the cBioPortal visualizer (http://www.cbioportal.org/). Sensitive and resistant cell lines were isolated and expression data was compared using the BRBAArrayTools Version 4.5.1 plug-in in Microsoft Excel 2016. A two-class comparison analysis was performed at a corrected p-value of .001 to obtain the differentially expressed genes shown in Figure 3.

**Expression analysis via qPCR**

RNA was extracted from cells after indicated treatments using Trizol (Invitrogen) and was extracted by phenol-chloroform extraction. Primers for MCM5 were (5′–3′):

TCACCAAGCAGAAAATACCC (forward) and GTCCATGAGTCCAGTGAG (reverse). Primers
for CDC6 were (5′–3′): GACCTCAAGAAGGAACTG (forward) and
ATACCTCTTCTGACAAATCTC (reverse). Primers for HOXC10 were (5′–3′):
CTATCCGCTCTACCTCTCGC (forward) and ACATGCAGAGACATTCTCC (reverse). All
samples were normalized to human GAPDH or UBC. Primers for GAPDH were (5′–3′)
GCCGGCTCCGTTATGG (forward) and AACCTGTTTCATCATCAGT (reverse). Primers
for UBC were (5′–3′) TGCTTGCATTCTCGATG (forward) and
ATTTGGTCGCGGGTTCTTG (reverse).

Cell-cycle-specific analysis of gene expression

Cells were seeded onto 15-cm plates overnight. The next morning, plates were treated
with either DMSO or combined treatment. Following 24 hours of treatment, cells were
trypsinized and resuspended at a concentration of <1x10^6 cells/mL in complete media
containing 10ug/mL of Hoechst stain (BDBiosciences #33342). Cells were incubated for 1 hour
at 37 degrees and then pelleted and re-suspended in 1X PBS prior to sorting. Each treatment
group was analyzed on a BD Aria Sorter. Cell populations were fractionated into G1, S, and
G2/M populations based on Hoechst staining. At least 100,000 cells were collected per cell
cycle fraction. RNA was extracted from resulting fractions using an RNeasy kit from Qiagen.
qPCR analysis was performed on isolated RNA according to protocol defined above. Cell cycle
fractions were confirmed by qPCR analysis of Cyclin D, E, and B (see Figure A-4E).

Flow Cytometry Analysis of Cell Cycle Stages

Cells were plated to 60-70% confluence on 10-cm plates. The next day, media was changed
with appropriate concentration of drug and cells were incubated for 16 or 24 hours. After drug
treatment, cells were trypsinized, resuspended, and washed in 1X PBS. Cells were then
suspended in propidium iodide staining media, incubated for thirty minutes at room temperature,
then analyzed on a DPX11 analyzer. FlowJo software was used for cell cycle analysis and
figure creation.
**DNA Fiber Analysis**

Cells were plated at 50% confluence onto 15-cm plates. Media was changed the next day and new media with appropriate drug concentrations was added. After fourteen hours, red-labeled EdU was added to the media. Following a two-hour incubation with the red-labeled EdU, media was washed out, cells were washed, and new media containing drugs and green-labeled EdU was added. Cells were then incubated for two more hours. In total, cells were exposed to drug for 18 hours. In this cell line, NCI-H1573 death does not begin until 24 hours so the processes described from this experiment take place prior to cell death.

**Human Xenograft Assays**

Animal procedures were approved by the Center for Animal and Comparative Medicine in Harvard Medical School in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. Xenograft experiments were performed in Nu/Nu mice purchased from Charles River laboratories. Briefly, $2.5 \times 10^6$ cells in 50% matrigel/media were injected into the left and right flank of each mouse. Thus, each mouse contributed two tumors to each arm of the study such that there were five mice and ten tumors per treatment arm. Treatment commenced at day 0 when tumors reached an average size of 300-500 mm$^3$, approximately 3-4 weeks after initial injection.

**Drug treatments, dosing schedule, and tumor volume measurements**

For all mouse experiments including two human xenografts and one patient-derived xenograft, PD-0325901 was administered at 1.5 mg/kg daily by oral gavage (vehicle: 0.5% (w/v) methylcellulose solution with 0.2% (v/v) polysorbate 80 (Tween 80)). JQ1 was administered at 45 mg/kg intraperitoneally daily in a 10% (2-hydroxypropyl)-β-cyclodextrin solution (C0926, Sigma). Compounds given in combination were administered sequentially. Mice were started on treatment when tumor size reached 200-300 mm$^3$. Tumor size was measured every 2-3 days by calipers. Tumor volume was calculated using the standard formula $L \times W^2 \times 0.52$. 
**Patient-derived xenograft**

In collaboration with the Belfer Institute and the BWH Experimental Therapeutics Core, we obtained three PDX tumor samples from patients with KRAS-mutant lung adenocarcinoma. These tumors were crushed and prepared in 1% SDS boiling lysis buffer. HOXC10 protein levels were assayed by western blot and levels were compared to high and low expressing HOXC10 human lung cancer cell lines. A HOXC10-high PDX was selected for further study. This PDX tumor was expanded in NOD-SCID mice and these expanded tumors were then harvested and re-implanted into experimental mice. Ten tumors were enrolled per treatment arm when volume reached approximately 150-200 mm$^3$. Mice were sacrificed when tumor volumes reached $>2000$mm$^3$. All dosing was done as described above. The Experimental Therapeutics Core conducted all animal care and tumor measurements.

**Statistics**

For quantitative measurements, graphs represent mean ± SD. Where indicated, data are presented as fold change or log$_2$ fold change over initial measurements. Changes in tumor volume are presented in a waterfall plot with each bar representing the change in tumor volume of an individual animal in the study. ANOVA followed by Bonferroni’s multiple comparison tests, 2-tailed unpaired $t$ tests, and Mann-Whitney $U$ tests was used to compare data sets where indicated, and $P$ values are shown. A $P$ value less than or equal to 0.05 was considered significant. Data were graphed and analyzed using GraphPad Prism v.6.
Chapter 3: Characteristics of KRAS-mutant tumors with PRC2 defects and/or high HOXC10 expression
PREFACE

This chapter contains unpublished data that is meant to provide context for the work presented in Chapter 2 and serve as a launching point for future investigations into KRAS-mutant lung tumorigenesis.

INTRODUCTION

KRAS-mutant tumors represent the largest category of lung adenocarcinomas and a large amount of heterogeneity has been observed among patients with these malignancies. This heterogeneity is clinically manifested by: 1) variable aggressiveness/metastatic potential and 2) differential sensitivity to standard-of-care treatments and new targeted therapies, making predicting response and selecting optimal treatment courses challenging. In order to alleviate this problem, investigators have attempted to differentially categorize KRAS-mutant tumors with the hope of identifying biomarkers that may ultimately predict therapeutic responses. Certainly, part of this heterogeneity may be due to specific KRAS-mutant alleles (reviewed in Chapter 1), but co-existing mutations also help explain these differences (Haigis, 2017; Skoulidis et al., 2015).

A number of KRAS-mutant subtypes are already characterized by co-occurring tumor suppressor mutations including KRAS-TP53 (KP) mutant, KRAS-STK11 (KL) mutant, and KRAS-CDKN2A (KC) mutant tumors (Skoulidis et al., 2015). Each of these subtypes exhibits unique features and therapeutic sensitivities.

The most well studied KRAS-mutant subtype exhibits co-occurring loss-of-function mutations in TP53. One-third of KRAS-mutant tumors fall into this category. The tumorigenic properties of mouse KP tumors have been well categorized through the widespread study of a genetically engineered mouse model (GEMM) of KRAS-mutant lung adenocarcinoma (the KrasLSL-G12D/+ p53floxflox model). This mouse is a conditionally driven model of KRAS-mutant lung tumorigenesis that is triggered by Cre expression. Survival of KP mice is reduced approximately
twofold compared to their KRAS-mutant-only counterparts. The decreased survival of KP mice is due to a greater growth rate of tumors rather than an increased number of tumors or metastatic disease (DuPage et al., 2009). Consistently, studies have also demonstrated that TP53 is able to limit malignant transformation of KRAS-mutant tumorigenesis, largely via its ability to suppress MAPK signal amplification (Cicchini, et al. 2017; Feldser et al., 2010). These data demonstrate that TP53 loss leads to enhanced growth and proliferation of tumor cells, but does not increase tumor cell aggressiveness or metastatic ability. This was further supported by a study that found TP53 loss alone was insufficient to drive dissemination of tumor cells in an in vivo metastatic model of lung cancer (Caswell et al., 2014). Consistently, patients with KP tumors have a significantly longer RFS compared to patients with KL or KC tumors, which have higher metastatic potential. In humans, KP tumors are also enriched for high inflammation, active immunoediting, and high underlying mutation rates, suggesting that these tumors could be the most sensitive to immunotherapy agents among the KRAS-mutant subtypes (Skoulidis et al., 2015).

**KRAS-STK11** mutant tumors (KL tumors) are becoming increasingly appreciated as a distinct therapeutic subtype, especially after TCGA characterized a distinct expression subtype, termed Proximal Proliferative, which is enriched for mutation of KRAS and inactivation of STK11 (TCGA, 2014). This Proximal Proliferative expression subtype is one of three new expression subtypes categorized by TCGA via unsupervised hierarchical clustering of expression datasets. Mutated in approximately one-quarter of all KRAS-mutant tumors, STK11 codes for the protein LKB1, a negative regulator of AMPK. KL tumors are also classified as “immune inert” which may be a result of the lower rate of somatic mutations in these tumors as compared to KP tumors, suggesting that KL tumors may be less sensitive to immune checkpoint-blocking therapies (Skoulidis et al., 2015). Furthermore, LKB1 loss in the KRAS-mutant context has been shown to result in decreased sensitivity to BRD4 inhibitor monotherapy and increased sensitivity to FAK...
and MEK inhibitor monotherapy (Shimamura et al., 2013; Gilbert-Ross et al., 2017; Mahoney, et al. 2009).

**KRAS-CDKN2A/B** defective tumors (KC tumors) represent approximately 10% of KRAS mutant tumors (TCGA, 2014). Complete loss of CDKN2A/B accelerates murine KRAS-mutant lung adenocarcinoma, leading to a loss of differentiation, increased metastatic disease, and decreased overall survival (Schuster et al., 2014). Loss of differentiation is also reflected in the KC subtype as these tumors are enriched for invasive mucinous carcinoma, a histological subtype not found in other genetic settings (Skoulidis et al., 2015). As loss of stemness or induction of differentiation has been shown to affect therapeutic sensitivities in leukemia, this observation could be significant in stratifying the patient population for treatment (reviewed in de Thé, 2017).

Beyond co-occurring tumor suppressor mutations, co-occurring expression changes have also been studied in conjunction with KRAS mutation. The best example of this class is KRAS mutant tumors with concomitant NKX2-1 downregulation. In fact, within the KC subtype defined by Skoulidis and colleagues, low NKX2-1 expression was common. In a study of Kras<sup>LSL-G12D/+ p53<sup>flox/flox</sup></sup> mice, metastatic tumors invariably exhibited downregulation of this gene when compared to primary tumors. Further experiments confirmed its role in controlling differentiation and limiting metastatic potential in part by repressing Hgma2 expression (Winslow et al., 2011). This example showcases how deregulation of a homeobox gene is able to modify lung tumorigenesis.

Despite the advent of tumor sequencing and large publically available datasets, additional subtypes of KRAS-mutant lung adenocarcinoma have yet to be characterized in a clinical setting. This could be related to a general reliance on mutational status rather than expression or copy number variation to categorize tumors. My work in Chapter 2 reveals a unique subset of KRAS-mutant lung adenocarcinomas that are sensitive to dual MEK and BRD4 inhibition. This subset is characterized by a defective PRC2-HOXC10 gene axis that I defined in
KRAS-mutant human lung adenocarcinoma cell lines. Interestingly, when I explore clinical data available from the TCGA, this subset is not entirely overlapping. In other words, while the majority of tumors with high HOXC10 expression are regulated by PRC2 defects, a subset of HOXC10-high tumors exist without PRC2 heterozygous loss and vice versa. This is unsurprising because HOXC10 is regulated by multiple epigenetic mechanisms including the opposing function of Trithorax proteins. Therefore, in order to better understand the relationship between HOXC10 and PRC2 defects in the context of KRAS-mutant lung tumorigenesis, I examined these two KRAS-mutant categories, those with PRC2 defects and those with high HOXC10 expression, as individual entities.

In this data chapter, I will share preliminary studies related to the therapeutic response to combined MEK and BRD4 inhibition in a related aggressive mouse model of KRAS-mutant lung adenocarcinoma. Importantly, this preliminary work sets the stage for exploring PRC2 loss and metastasis in vivo. I will also discuss observations related to PRC2 loss in vitro and in silico, as a means of generating hypotheses related to the complex’s role in tumor aggressiveness, Ras pathway regulation, and stemness. Moreover, I will discuss HOXC10’s potential role in KRAS-mutant NSCLC based on additional preliminary studies, and dataset analyses, examining its relationship to DNA replication and stemness. I will also present data from an in silico analysis that may provide a basis for identifying direct HOXC10 targets. Throughout the chapter, I will comment on relevant clinical data in KRAS-mutant tumors with high HOXC10 expression and/or PRC2 heterozygous loss as a means of considering their relevance as a clinical biomarker.

RESULTS

**Dual MEK and BRD4 inhibition triggers dramatic tumor regression in an aggressive KRAS-mutant NSCLC mouse model**

To further demonstrate that combined MEKi and BRD4i treatment is able to trigger in vivo tumor regression, we tested this combination in a mouse allograft of KRAS-mutant lung
adenocarcinoma. This model utilized a KRAS-mutant lung cancer cell line derived from a fast-growing primary tumor of the Kras\textsuperscript{LSL-G12D/+} p53\textsuperscript{flox/flox} GEMM (from Winslow et al., 2011). We transduced these cells with a luciferase construct and injected them into the mouse tail vein so that their in vivo lung growth could be visualized by BioLuminescent Imaging (BLI). These cells established tumors in the mouse lung and grew rapidly over the course of 2-3 weeks. As outlined in Figure 3-1A, tail vein injection of these luciferase-modified cells was followed by 2-3 weeks of BLI monitoring every three days. Daily treatment with vehicle, MEKi, BRD4i, or combination treatment was initiated when the average tumor radiance reached a threshold level of radiance units ($10^6$ units). As we’ve previously observed in other mouse models, single MEK inhibitor treatment is sufficient to slow tumor growth, while only combined treatment was able to shrink tumors (Figure 3-1C,D). Results of the BLI surrogate metric were confirmed by measuring tumor burden via H&E staining endpoint tumors at day 10 (Figure 3-1E). Representative BLI and H&E slides are shown for each treatment group (Figure 3-1D,E). This mouse allograft model is powerful because 1) the tumor cells grow and establish very rapidly due to their aggressive nature, 2) tumor growth can be monitored in the same mouse over time via the surrogate BLI metric, 3) unlike other xenografts, these tumors grow within the lung tissue niche, and 4) the cells can be modified easily to assay a variety of experimental questions.

To investigate the mechanism of action in this mouse model, we performed analyses similar to those conducted in human tumor cells. Specifically, we began by analyzing downregulated transcripts following twenty-four hours of treatment. We identified both similarities and differences when we compared the responses of mouse and human NSCLC to combined MEK and BRD4 inhibitors. Similar to human cells, we found that these agents, suppress many of the same signatures, including KRAS transcriptional outputs, PRC2 targets, and E2F1/RB signatures (Figure 3-2A). We also found that pre-RC complex components are highly downregulated and that this downregulation depended on the cooperation between both arms of the combination treatment (Figure 3-2B).
Figure 3-1. Dual MEK and BRD4 inhibition triggers dramatic tumor regression in an aggressive KRAS-mutant NSCLC mouse model
(A) Schematic showing timeline of experiment which consists of injection of mouse lung cancer cell line followed by BLI monitoring of tumor growth until tumors are established in the lungs. Once established, mice are enrolled into treatment arms and scanned every three days. Endpoint for experiment was ten days as vehicle mice become very sick at this point.
(B) Waterfall plot shows change in average tumor radiance between day 10 and day 0 for each treatment arm. Each bar represents one mouse.
(C) Quantification of H&E staining of all mouse tumors after day 10 sacrifice. Tumor burden is quantified as tumor area/total area of lung.
(D) Representative BLI scans of one mouse from each treatment group showing the change in average radiance between Day 0 and Day 10.
(E) Representative H&E slides from each treatment group showing tumor burden within lung after ten days of treatment.
Figure 3-1 (Continued).
Figure 3-2. Transcriptional changes in 482T1 mouse lung cancer cells
(A) 482T1 mouse lung cancer cell lines were exposed to combination treatment for twenty-four hours. RNA was harvested and subjected to microarray analysis. GSEA analysis was then performed on expression profiles comparing combination treatment to DMSO treatment. Top GSEA signatures downregulated by combination treatment are displayed in chart.
(B) Pre-replicative complex genes were along the most highly downregulated genes following combination treatment. This downregulation was the result of cooperative suppression by both treatment arms. The fold suppression of DMSO versus combination treatment is shown in chart.

Unlike the human setting, HOXC10 was not expressed in this mouse allograft model. There are many possibilities that may explain the lack of HOXC10 expression here such as: 1) this one allograft model is more similar to the 50% of human tumors that lack HOXC10, 2) aberrant HOXC10 expression may be related to the cell of origin, which may be different in this model or altogether different in mice, or 3) another homeobox or developmental gene could be more relevant to this system. Nevertheless, we were surprised to see that the combination treatment was still efficacious in this low-HOXC10 context. There are many possibilities to explain the observed sensitivity to combined MEK/BRD4 inhibitors including 1) an alternative homeobox or developmental protein may be suppressed by this combination, 2) another protein that links BRD4/MEK inhibitors to the Pre-RC machinery may be suppressed, 3) the deeper suppression of the Ras pathway may be sufficient to mediate a therapeutic response in this model, and 4) relatedly, the therapeutic response might be even more enhanced in tumors in which HOXC10 or another homeobox gene is expressed. Examination of these possibilities is
ongoing. It should also be noted that I performed these studies before knowing that copy number alterations in PRC2 were contributing to the sensitivity of these agents in human cells and we did not find any obvious evidence of PRC2 defects in these cells though we were unable to perform copy number analysis to confirm.

**Knockdown of PRC2 component SUZ12 increases tumor metastasis and decreases survival**

After we discovered that decreased PRC2 activity correlated with response to combined MEK and BRD4 inhibition, we were next interested in investigating the consequences of PRC2 suppression in KRAS tumorigenesis. To address this question, we took the luciferase-expressing KRAS-mutant primary tumor cell line and knocked down SUZ12 expression with shRNA sequences. We then assessed tumor/metastasis development in the two cohorts of mice, those injected with cells expressing non-targeting or SUZ12-targeting constructs. We performed BLI monitoring every three days to measure tumor progression and found that it took the same amount of time for the lung tumors to establish (as assayed by first detected BLI signal in chest) between the two cohorts (data not shown).

However, it quickly became clear that the shSUZ12 cohort fared much worse than their shSCR counterparts as the shSUZ12 group exhibited significantly decreased overall survival (Figure 3-3A). Strikingly, the shSUZ12 cohort not only had tumors establish in their lungs, but also throughout their abdomen, suggesting an increased metastatic ability of the cells. In fact, sixty percent of the shSUZ12 cohort exhibited abdominal metastases at four weeks whereas the vast majority of mice from this shSCR cohort and the previous experiment did not (Figure 3-3B). The shSUZ12 cohort had metastases that exhibited more signal (and thus, higher tumor burden) than the established lung tumor. In fact, the signal from the shSUZ12 lung tumors was often blown out by the metastatic tumors’ signal (Figure 3-3C).
Figure 3-3. Knockdown of PRC2 component SUZ12 increases tumor metastasis and decreases survival

(A) Survival curve comparing two cohorts of mice (N=10, each). (B) Percent of mice with detectable metastases (defined as radiance signal above $10^4$ units outside of chest area). Sixty percent of shSUZ12 cohort developed metastases compared to sixteen percent of mice without SUZ12 knockdown (shSCR cohort and cohort from Figure 3-1). (C) Representative images of metastatic tumor burden in mice from the shSUZ12 cohort. In most cases, the radiance from the metastatic tumors was so bright that the primary tumor was undetectable at relevant exposures. (D) Western blot analysis of the 482T1 cell line used in the mouse allograft. Following knockdown of SUZ12 or EED, an increase in EMT markers was observed.

Together, this data suggests that decreased PRC2 activity in a KRAS-mutant setting enhances the metastatic ability of the tumor. This hypothesis was supported by increased expression of key markers of the epithelial to mesenchymal transition (EMT) following knockdown of SUZ12 or EED (Figure 3-3D).
**PRC2 suppression triggers induction of EMT markers, upregulation of proliferation signatures, and re-deployment of early development programs**

To determine whether PRC2 might also mediate EMT in human lung adenocarcinoma, we knocked down SUZ12 and EED in a human lung cancer cell line with normal PRC2 activity (i.e. no mutation or heterozygous loss of PRC2 components). Again, we observed increased expression of key EMT markers (Figure 3-4A). Strikingly, the morphology of the cells also changed with cells becoming more spindly and mesenchymal in appearance (Figure 3-4B).

![Western blot analysis of A549 cell line showing the induction of EMT markers following knockdown of SUZ12 and EED.](image1)

![Representative images of cells following SUZ12 knockdown show cells adopting a more mesenchymal cell shape.](image2)

**Figure 3-4. PRC2 suppression triggers induction of EMT markers**
(A) Western blot analysis of A549 cell line showing the induction of EMT markers following knockdown of SUZ12 and EED.
(B) Representative images of cells following SUZ12 knockdown show cells adopting a more mesenchymal cell shape.

To further investigate how PRC2 loss affects the overall cellular environment we assessed transcriptional changes in an isogenic KRAS-mutant cell line following stable knockdown of SUZ12. By performing GSEA analysis we found that knockdown of SUZ12 induced expression signatures that correspond with proliferative states including those generated by RB knockdown and E2F1 or E2F3 overexpression (Figure 3-5A). Other signatures of note include the highest scoring signatures consistent with activated Wnt signaling...
(LEF1_UP), raising the possibility that SUZ12 may play a role in suppressing stemness and Wnt signaling in KRAS-mutant lung tumors (Figure 3-5A). Consistently, when we perform GO analysis on the individual genes upregulated by more than two-fold following SUZ12 knockdown, we find that the majority of GO-hits correspond to lists important for early development and morphogenesis (Figure 3-5B). Together, these data suggest that PRC2 complex could play a role in suppressing proliferation and stemness/developmental signatures in KRAS-mutant lung cancer.

We next examined the publically available expression profiles of KRAS-mutant human tumors with and without PRC2 heterozygous loss to determine if any of these themes hold true in actual patient tumors. First, we confirmed that PRC2 HET tumors showed de-repression of PRC2 targets as compared to their PRC2 WT counterparts (Figure 3-5C). Upon further GSEA analysis in this setting, we found that tumors with PRC2 heterozygous loss were enriched for signatures corresponding to RB knockdown and E2F1/E2F3 overexpression (Figure 3-5C). We also analyzed signatures that correspond to early lung progenitor states and AT1/AT2 cell type markers. We find that early lung progenitor signatures are upregulated in tumors with PRC2 heterozygous loss and that AT1/AT2 differentiation signatures are suppressed in this same setting (Figure 3-5D). These signatures were first developed by single cell RNA-seq of mouse lung progenitor cells, specifically those at very early stages of development (early lung progenitor, E13.5 days) and those that have begun to differentiate into specialized AT1/AT2 cell types (AT1/AT2 markers, E18.5 days) (Treutlein et al., 2014). These data raise the possibility that PRC2 heterozygous loss may enhance the stemness of KRAS-mutant tumors by re-activating early progenitor pathways and suppressing differentiation markers found in specialized subtypes. More work is needed to determine the specific lung differentiation markers that may be relevant to the tumorigenic development of these PRC2-heterozygous tumors (Figure 3-5D).
Figure 3-5. PRC2 suppression upregulation of proliferation signatures and re-deployment of early development programs

(A) RNA was harvested from NCI-H2030 cells stably expressing shSCR or shSUZ12. Microarray analysis was performed on RNA and expression was analyzed by GSEA. Relevant genesets enriched in the shSUZ12 are listed in the table.

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<tr>
<td>RB_P107_DN,V1_UP</td>
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<td>0.035</td>
<td>0.183</td>
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</table>

(B) GO analysis with biological processes lists was performed on all genes significantly upregulated (fold change > 2) following SUZ12 suppression. Out of the top forty enriched GO biological processes lists, the majority were associated with early development, morphogenesis, and cell migration.

(C) GSEA was performed on publically available expression data comparing KRAS-mutant tumors with PRC2 heterozygous loss to KRAS-mutant tumors with WT PRC2. Genesets enriched in the PRC2 heterozygous tumors are listed in table.

<table>
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<th>NAME</th>
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(D) A gene list compiled from early lung progenitor cells was found to be significantly upregulated in PRC2 heterozygous tumors.

(E) Two gene lists compiled from more differentiated lung cells (bipotential progenitors and AT1 cells) were significantly suppressed in PRC2 heterozygous tumors.
Figure 3-6. HOXC10 expression leads to upregulation of EMT markers
(A) Ectopic HOXC10 expression in A549 cell line leads to strong upregulation of EMT markers
(B) Ectopic HOXC10 expression in this same cell line increases migration of the cells in a scratch wound assay.

Potential function of HOXC10 in KRAS-mutant lung adenocarcinoma

HOXC10 expression is an important biomarker that can be used to predict response to our epigenetic-based combination treatment. We also found that HOXC10 is largely, but not exclusively, upregulated by PRC2 defects, which we show can drive EMT phenotypes. Therefore, we investigated whether HOXC10 expression might also promote EMT in human lung adenocarcinoma. To address this question, we overexpressed HOXC10 in a cell line lacking HOXC10 expression. We found that, like with PRC2 knockdown, HOXC10 overexpression led to the upregulation of EMT markers (Figure 3-6A). Furthermore, HOXC10 overexpression increased in vitro migration compared to control LACZ-expressing cells (Figure 3-6B). These signaling and phenotypic results are consistent with the possibility that HOXC10 expression may mediate a more aggressive phenotype, however more work must be done to confirm these results, perhaps in an in vivo model of lung tumorigenesis.
To determine which signaling pathways might be influenced by HOXC10 expression, I performed GSEA analysis on cells stably expressing a control LACZ construct versus cells stably expressing HOXC10. HOXC10 overexpression in this context leads to increased enrichment of E2F targets and markers of cell cycle progression including the GO list representing all key players of DNA replication initiation (Figure 3-7A,B). To complement these observations, we also compared HOXC10-high and HOXC10-low KRAS-mutant tumors from the TCGA and found that HOXC10-high tumors were enriched for signatures important for cell cycle progression, defective polycomb complex regulation, and stemness (Figure 3-7C). Together, this data suggests that HOXC10’s baseline transcriptional activity involves upregulating signatures important for DNA replication and repair, an unsurprising observation considering its role in mediating these processes in the therapeutic response to dual MEK and BRD4 inhibition (Chapter 2). Many of the same themes found in the PRC2 heterozygous tumors hold true in this HOXC10-high setting, adding further functional connection between the two subtypes.

**Strategies to identify potential HOXC10 transcriptional targets**

HOXC10 is a master transcription factor, but identification of specific targets has proven elusive due to the redundancy among paralogous HOX genes and the high proportion of HOX binding sites present throughout the genome. Further, HOX genes share very similar binding affinities and binding protein partners, making untangling their specific targets even more challenging (reviewed in Svingen and Tonissen, 2006). Utilizing a predictive binding tool that analyzes large ChIP-Seq datasets, we identified both the human and mouse predicted HOXC10 binding sites (Figure 3-8A,-B). Both predicted sites were comparable in sequence. Interestingly, the predicted HOXC10 binding site was slightly different than the consensus HOX binding site sequence, (C/G)TAATTG, suggesting distinct gene regulation by HOXC10 compared to other HOX genes. We then assayed the presence of this predicted binding site.
throughout the genome to identify putative HOXC10 targets utilizing a database called FIMO, Finding Individual Motif Occurrences, from the MEME suite. The top fifty hits include HOXA5, one of the HOX genes normally expressed in human lung development (Figure 3-8C). None of the predicted pre-RC complex components or E2F1 was significantly enriched for a HOXC10 binding site in their promoter, suggesting that the regulation of these genes by HOXC10 may be indirect. However, this analysis only examined regions 1-1.2 kb away from the transcriptional start site so if HOXC10 binds at distant enhancers, the relationship would be lost in this assay.

Since HOX genes are often expressed in concert with other HOX family members and studies suggest cross-regulation of HOX genes within HOX clusters, we also analyzed the presence of other predicted HOX binding sites in the promoters of the pre-RC and E2F proteins. We found that CDC6, MCM3, and MCM7 were recurrently enriched for HOX binding sites upstream of their transcriptional start sites (Figure 3-8D). This suggests that the HOX code may

![Figure 3-7. HOXC10 expression leads to upregulation of proliferative genesets](image)

(A) HOXC10-overexpressing (HOXC10-OE) cells were compared to cells expressing LACZ. Genesets enriched in HOXC10-OE cells are shown in the table.

(B) Representative GSEA blot shows that E2F1 target genes are significantly expressed in HOXC10-OE cells as compared to LACZ-expressing cells.

(C) Publically available expression data from TCGA was analyzed and two groups of KRAS-mutant tumors were formed. High-HOXC10 tumors (N= 65) and low HOXC10 tumors (N=32). The two groups were compared by GSEA and gene sets significantly upregulated in high HOXC10 tumors are listed in table.
converge to regulate these genes during development, presenting an opportunity for their aberrant upregulation during tumorigenesis. While we did not observe aberrantly high expression of any of these HOX genes in the sensitive KRAS-mutant cell lines, global HOX downregulation was induced by BETi (Figure A-5). Further, high expression of other HOX genes does trend with high HOXC10 expression, suggesting similar modes of expression regulation (data not shown). We hypothesize that HOXC10 may trigger a signaling cascade of HOX genes

### Figure 3-8. Strategies to identify potential HOXC10 transcriptional targets

(A) Predicted mouse HOXC10 binding site
(B) Predicted human HOXC10 binding site
(C) List of top fifty genes with predicted HOXC10 binding site 1-1.2 kb away from the transcriptional start site.
(D) List of pre-RC genes with HOX binding sites 1-1.2 kb away from their transcriptional start sites.

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that coordinately regulates these DNA pre-replicative (pre-RC) complex components. More work is needed to refine the relationship between HOXC10, HOX genes, and the pre-RC complex.

**HOXC10 as a clinical biomarker**

Very recently, high HOXC10 expression was found to be associated with decreased overall and progression free survival in a small study of lung adenocarcinoma patients (Tang et al., 2017). In an analysis of a larger data set from the TCGA dataset, a similar trend was observed when comparing patients with HIGH versus LOW HOXC10 but was not significant (data not shown). More detailed analysis of data corresponding to different tumor subtypes is needed to further understand the relationship of HOXC10 to clinical outcomes. Regardless, our functional studies have suggested that HOXC10 may promote EMT and invasion, which is thought to underlie metastasis and tumor aggressiveness. In addition, our work has shown that HOXC10 is a predictive biomarker for response to combined MEK and BRD4 inhibitors so we began to characterize its expression via immunohistochemistry (IHC) in patient tumors.

IHC staining of patient tumor slides showcased diverse patterns of HOXC10 expression (Figure 3-9). Of the sixty-one KRAS-mutant tumor samples analyzed, 67% exhibited some positive HOXC10 staining. Of this group, 39% exhibit strong nuclear HOXC10 staining, comparable to the proportion of tumors we find with high mRNA expression in the TCGA dataset (Figure 3-9A). This strong staining aligns with the clinical standards that a pathologist would deem positive. Weak nuclear staining was observable across an additional thirty percent of samples (Figure 3-9B). A negative HOXC10 tumor sample is shown on the rightmost side of Figure 3-9B for comparison purposes. Overall, this preliminary IHC analysis of a KRAS-mutant panel suggests that HOXC10 expression could be a promising clinical biomarker.
Figure 3-9. HOXC10 IHC analysis of patient tumors
(A) Series of three different patient tumors showing diverse patterns of strong HOXC10 expression
(B) Two tumors show weak HOXC10 expression (left) and one tumor showing negative HOXC10 expression (rightmost).
(C) Summary of HOXC10 expression in 61 KRAS-mutant patient tumors.
(D) Contingency tables comparing STK11 mutation to HOXC10 expression, strong (top) and weak (bottom). In both analyses, HOXC10 expression is significantly enriched in the STK11-mutant setting.
Figure 3-9 (Continued).
We used data from this small cohort to determine if any clinical characteristics correlate with positive HOXC10 expression. We found no associations with differentiation status (poor, moderate), pattern (solid, acinar, papillary, lepidic), or amount of tumor infiltrating lymphocytes (data not shown). Each tumor was classified as KRAS-mutant only, KRAS-TP53 mutant, or KRAS-STK11 mutant. Strikingly, HOXC10 expression significantly co-occurs with STK11 (LKB1) mutation (Figure 3-9D). This result was unexpected as we did not see any such correlation in our sensitive versus resistant cell lines or in analysis of the high HOXC10 tumors in the TCGA. Moreover, LKB1 mutation has been shown to confer resistance to BRD4i monotherapy while HOXC10 leads instead to sensitivity to combined MEK and BRD4 dual therapy (Shimamura et al., 2013). Further exploration of this relationship to determine its functional and clinical relevance is warranted.

Lastly, we examined HOXC10 expression levels across all lung adenocarcinoma subtypes to see whether combining BET inhibitors with kinase inhibitors may be useful in KRAS-WT settings. Interestingly, tumors with high HOXC10 expression (expression > 2STDEV above normal tissue) are found across all lung adenocarcinoma subtypes with particularly high enrichment for these tumors in ROS1 and EGFR-mutant tumors, though the small pool of data available for ROS1-mutant tumors limits the analysis (Figure 3-10). Further study of combined MEK and BET inhibitors is warranted in NF1-mutant tumors as thirty-eight percent of this Ras-driven subtype exhibits high HOXC10 expression; especially since the only NF1-mutant cells analyzed in Chapter 2 had low HOXC10 expression. This data provides the basis for exploring additional epigenetics-based therapies in lung adenocarcinoma as described in the discussion of Chapter 4.
Figure 3-10. HOXC10 expression in other lung adenocarcinoma subtypes
HOXC10 expression was assayed in publically available data from the TCGA. High HOXC10 was called as two standard deviations over the normal tissue mean. Each subtype is listed above, N= number of samples in subtype.

DISCUSSION and FUTURE DIRECTIONS

Further examination of mouse allograft model

The in vivo data presented in this chapter utilized a unique allograft system that allows the investigator to examine lung tumor dynamics in its appropriate anatomical niche with a non-invasive bioluminescent monitoring system. We saw that knockdown of SUZ12 in this setting leads to a more aggressive progression of lung tumorigenesis. While PRC2 knockdown may correspond to more invasive disease, we find this feature also confers sensitivity to our combination treatment in human tumors. To test whether this observation is consistent in this mouse setting, we would next like to perform a treatment intervention study comparing the response to combined MEK and BET inhibition in shSCR and shSUZ12 mouse allograft models. We expect that the shSUZ12 cohort will be more sensitive to the combination treatment in vivo.
and that this will manifest as improved tumor shrinkage and/or increased treatment durability. We could also use this model and other *in vivo* models to further study HOXC10 in lung tumorigenesis.

**Further examination HOXC10 in lung tumorigenesis**

The work that we presented so far for defining HOXC10’s role in lung tumorigenesis is very preliminary. More study is needed both *in vitro* and *in vivo* to tease apart the relationship between HOXC10 and oncogenesis. First, HOXC10 overexpression and knockdown should be further studied in migration, soft agar, and invasion assays to see how the homeobox gene modifies the behavior of KRAS-mutant cancer cells. These studies should be undertaken in human lung cancer cell lines, but also in a transformed lung epithelial cell line (Lundberg et al., 2002). This lung epithelial cell line can be transformed with a mutant KRAS construct and further modified with a HOXC10 overexpression construct. This will allow us to examine the contributions of each of these alterations in a clean background.

More importantly, we are interested in developing a mouse model of lung tumorigenesis with HOXC10 expression. One simple experiment is to perform a mouse allograft or human subcutaneous xenograft experiment with cells expressing HOXC10 at high levels versus those without HOXC10 (and also the converse experiment with HOXC10 knockdown). Features such as primary tumor growth and metastatic capabilities should be measured. Another possibility is to develop a genetic model utilizing CRISPR technologies. Perhaps the best way to do this would be to utilize a CRISPR system developed in the Jacks lab that somatically activates oncogenic KRAS combined with CRISPR/Cas9 mediated genomic editing (Sánchez-Rivera et al., 2014, Platt et al., 2014). With this method, we would use the Kras^{LSL-G12D/+} p53^{floxed/floxed} model and design a CRISPR homologous recombination construct with Cre expression such that delivery of this construct to the lung intratracheally would lead to mutant KRAS expression, loss of *TP53*, and overexpression of HOXC10. We could then monitor tumor dynamics in tumors with
and without HOXC10 expression. These studies would lead to more insight into how HOXC10 modifies tumor progression and sensitivity to our combination treatment.

**Direct HOXC10 transcriptional targets in KRAS-mutant setting**

The data presented above to identify direct transcriptional targets of HOXC10 is a pilot study based only on ChIP-Seq reports in normal, noncancerous cells. While informative, more work is needed to define HOXC10 targets. There are a number of ways to address this question.

First, we are currently performing ChIP-seq experiments pulling down endogenous HOXC10 in KRAS-mutant lung adenocarcinoma to show where HOXC10 binds in the genome. Unfortunately, it is unclear whether currently available HOXC10 antibodies are amenable to pulling down endogenous HOXC10. Thus, this ChIP-seq experiment is also being performed in parallel in an exogenous setting comparing FLAG-tagged WT HOXC10 to FLAG-tagged HOXC10 lacking the homeobox. Other options include ChIP-PCR at relevant loci including CDC6 and the MCM proteins. However, since *in silico* data does not predict HOXC10 binding directly near these promoters, it is unclear where we should focus for PCR amplification. All of these experiments should be performed alongside other HOX genes as a control such as HOXA5 (which has a different predicted binding motif) to ensure that results are specific to HOXC10 activity.

We are also interested in further defining the relationship between HOXC10, E2F, and pre-RC component genes. Accordingly, we can perform E2F reporter assays to show that HOXC10 directly causes the induction of this pathway.

**HOXC10 as a biomarker**

The IHC analysis in this chapter suggests that the HOXC10 antibody can serve as a strong and specific biomarker in the clinic. More work is needed to refine this staining process
and to determine criteria for deeming a sample “positive”. Such criteria can include percentage of positive tumor cells on the slide, strength of nuclear stain over cytoplasmic staining, and specific tumor staining over normal tissue. One challenge with this work is the diversity of expression patterns that we obtained in our initial panel of human tumors. For example, there were tumors that had strong nuclear expression, those with weak nuclear expression, and others with strong nuclear expression in only focal parts of the tumor. It is unclear in which situation whether a pathologist should call such staining positive. We plan to work closely with our collaborators in the Santagata lab to understand how we can best categorize positive and negative HOXC10 signals.

It would also be informative to assess matched tumors before, on, and off treatment to see if HOXC10 expression is re-deployed in resistant tumor outgrowths. This type of experiment would require additional mouse experimentation and longer treatments to harvest tumors on and during treatment as well as resistant tumor outgrowths. Such work would further explore HOXC10’s role in the therapeutic response.

Lastly, I found a very strong co-occurrence of LKB1 mutation in samples with high HOXC10 levels. The co-occurrence of LKB1 mutations and HOXC10 expression was not seen in the sensitive versus resistant cell lines. Further exploration of this relationship via mutational analysis of the TCGA data set will be conducted as this could be helpful knowledge for selecting patients.

**METHODS**

*Cell Lines and Reagents*

The 482T1 mouse lung cancer cell line was a gift from Monte Winslow (Stanford University) and was isolated from (Winslow et al., 2011). A549s were purchased from ATCC. The authors performed no further authentication of the cell lines. Cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10%).
Antibodies were obtained from the following sources: Cell Signaling Technologies: vinculin (#4650), Slug (#9585), Snail (#3879); BD Transduction Laboratories: N-cadherin (#610920); Sigma: α- tubulin (#T5168); AbCam: HOXC10 (#153904); Dako: vimentin (#M7020); Santa Cruz: SUZ12 (#46264). Non-targeting short interfering RNAs (siRNAs) ON-TARGET SMARTpools siRNA were purchased from Dharmacon to target human HOXC10 (L-017601-00-0005). A non-targeting control pool was also purchased from Dharmacon (D-001810-10). siRNAs were transfected with lipofectamine RNAiMAX from Invitrogen. Lentiviral pLKO vectors were obtained from the RNAi consortium of the Broad Institute. shSUZ12 (NM_015355.1-2076s1c1, target sequence 5′- GCTGACAATCAAATGAATCAT-3′) was used to target SUZ12. Human HOXC10 was a pHAGE HA-FLAG-C-terminal tagged construct obtained from the Harvard PlasmID database. PD-0325901 was a gift from Kevin Shannon (University of California, San Francisco). JQ1 was synthesized by Chempartner in concert with Dr. Jun Qi (Dana Farber Cancer Institute). The MSCV-luc-IRES-GFP construct used in mouse allograft studies was a gift from Tyler Jacks (Massachusetts Institute of Technology).

Mouse Allograft Models

482T1 cells derived from a primary KRAS-TP53 mutant mouse tumor were infected with the MSCV-luc-IRES-GFP for six hours. Cells were FACS-sorted to obtain a GFP+ population. These GFP-luciferase+ cells were then injected by tail vein into 129S1/SvImJ mice (10K cells/mouse). Following injection, mice were monitored every three days by Bioluminescent imaging on an IVIS Lumina III. For the experiments in Figure 3-3, the GFP+ cells were infected with shSCR and shSUZ12 constructs. Following puromycin selection (1:1000 of 2mg/ml stock) and confirmation of SUZ12 knockdown, cells were injected as described above.

Tumor Volume Measurements

Tumor volume was measured indirectly via bioluminescent imaging using the IVIS Lumina III. To measure size via tumor radiance, mice were injected with luciferin and imaged approximately seven minutes following injection. Mice were enrolled in the study into random
treatment arms when radiance readings in the chest region of interest (ROI) passed a threshold of $1 \times 10^7$ radiance units (p/s). After enrollment, mice were imaged every three days until endpoint.

**Drug Treatments and Dosing Schedule**

Animal procedures were approved by the Center for Animal and Comparative Medicine in Harvard Medical School in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. 129S1/SvImJ mice were purchased from Jackson Laboratory. PD-0325901 was administered at 1.5 mg/kg daily by oral gavage (vehicle: 0.5% (w/v) methylcellulose solution with 0.2% (v/v) polysorbate 80 (Tween 80)). JQ1 was administered at 45 mg/kg intraperitoneally daily in a 10% (2-hydroxypropyl)-β-cyclodextrin solution (C0926, Sigma). Compounds given in combination were administered sequentially. For survival curve in Figure 3-3A, mice were sacrificed when signs of distress including ruffled fur, hunched back, and labored breathing were observed.

**Migration Assay**

For migration assays, A549 cells were grown to 95-100% confluence and treated with mitomycin C to inhibit cell division. Wounds were generated with a pipette tip and images of wound healing (migration) were taken at indicated time intervals.

**Microarray**

RNA was isolated using Trizol extraction from cells stably expressing either LACZ or HOXC10 and shSCR or shSUZ12. RNA was purified by an RNeasy kit (Qiagen) according to manufacturers instructions with an in-solution DNAse digestion step. RNA was hybridized to the Affymetrix Human 2.0 STS array chip by the Molecular Biology Core Facilities at Dana-Farber Cancer Institute. To determine genes and gene sets differentially expressed between groups, a class comparison analysis was performed using the Broad Institutes Gene Set Enrichment Analysis tool.
Cell proliferation studies

Approximately 100,000 cells per well were seeded into 6 well plates. For siRNA experiments, cells were seeded 12-26 hours after transfection. Twenty-four hours after plating, day 0 counts were taken using a hemocytometer. For drug treatment experiments, inhibitors were added at this time. Final cell counts were taken 72 hours after day 0 counts, again by hemocytometer.

Acknowledgments

We would like to thank Roderick T. Bronson for histologic confirmation of all lung adenocarcinomas in the mouse allograft model.

Research Contributions

Stephanie Louise Guerra- Planned and performed experiments and data analysis
Shannon Coy- Performed and analyzed IHC staining of KRAS-mutant patient tumors
Emily Gaudiano- Performed EMT signaling and migration in vitro experiments during rotation project
Kian Kock- Performed all in silico HOXC10 and HOX binding analyses
Thomas De Raedt- Trained S. Guerra to perform data analysis
Sandro Santagata- Supervised the IHC analysis of KRAS-mutant patient tumors.
Karen Cichowski- Supervised and helped develop the project
Chapter 4: Conclusions and Future Directions
Targeting Cancer at the Intersection of Epigenetics and Signaling
Stephanie L. Guerra\textsuperscript{1,2} and Karen Cichowski\textsuperscript{1,2,3}

\textsuperscript{1}Genetics Division, Department of Medicine, Brigham and Women’s Hospital, \textsuperscript{2}Harvard Medical School, \textsuperscript{3}Ludwig Center for Dana-Farber/Harvard Cancer Center

\textbf{Author Attributions}
Stephanie L. Guerra- Researched and co-wrote the article.

Karen Cichowski- Supervised, researched and co-wrote the article.

The text and figures from the section “Targeting cancer at the intersection of signaling and epigenetics” as presented here serves as the basis for a publication in Annual Reviews of Cancer in April 2018.
SUMMARY

The goal of my thesis work has been to identify an efficacious treatment combination for patients with KRAS-mutant lung adenocarcinoma. This patient population represents an unmet clinical need as there are no approved targeted treatments or effective chemotherapies available and advanced KRAS-mutant disease is largely lethal. In my thesis I not only identified combined MEKi and BETi as a novel targeted treatment regimen, but also discovered a clinically relevant biomarker and characterized a new subset of KRAS-mutant lung adenocarcinoma. Importantly, this combination treatment can be readily translated to patients.

Treatment of KRAS-mutant lung cancer is challenging because KRAS itself is difficult to target directly and efforts to inhibit its downstream effector pathways is limited by feedback activation of the RAS-MEK-ERK signaling cascade and parallel pathways. In Chapters 2 and 3, I demonstrate that combined MEK and BET inhibition is efficacious in four different preclinical models of KRAS-mutant disease. Demonstrable regression of established tumors was observed across all models including a patient-derived xenograft. Potent tumor regression in preclinical models of KRAS-mutant cancer is rare, making our results exciting for future clinical development.

In our efforts to identify the therapeutic mechanism of action, we performed a global transcriptional analysis and demonstrated that BET inhibitor alone was sufficient to downregulate a subset of downstream Ras transcriptional targets. Combined MEK and BET inhibition more deeply suppressed this pathway output, an observation that is consistent with our work in NF1-mutant nervous system tumors (De Raedt et al., 2014). However, we also find that deep suppression of the Ras transcriptional output is not sufficient to induce a complete cytotoxic response, indicating that BET inhibitors add an additional layer of functional activity in addition to its effects on Ras (Figure 2-3B).

Only half of KRAS-mutant cells tested were sensitive to this unique therapeutic combination. In Chapter 2, we used this disparity in sensitivities to further characterize the
therapeutic mechanism of action. First, we find that sensitive cells are enriched for defects in PRC2 activity largely via heterozygous loss of one or more obligate PRC2 components and that BETi is able to reverse the effects of these PRC2 defects. By comparing the two groups, those that die in response to treatment and those that arrest in response to treatment, we also identified HOXC10 as a uniquely enriched expressed gene in the sensitive group of cells. We believe this unbiased comparison strategy will prove useful to identifying mechanisms of action in other therapeutic contexts. I also found that HOXC10, a canonical PRC2 target, is overexpressed in almost half of all KRAS-mutant lung adenocarcinomas but has yet to be characterized in this context making this discovery exciting and potentially impactful. In both Chapters 2 and 3, we find that HOXC10 works to transcriptionally regulate the expression of pre-replicative (pre-RC) complex components, crucial proteins responsible for initiating DNA replication origins prior to S-phase. The conclusion of Chapter 2 demonstrates that HOXC10 exhibits these effects alone and also in concert with the RAS-MEK-ERK pathway, likely through regulation of E2F1.

Importantly, we believe that HOXC10 can be used as a clinically relevant biomarker. First, HOXC10 expression was clearly ON or OFF in the sensitive versus resistant groups of KRAS-mutant lung adenocarcinoma cell lines. Second, we show that an antibody against HOXC10 can be used in immunohistochemical staining of patient tumor samples. The possible clinical utility of these observations was discussed in Chapter 3. Together these findings add additional support that combined MEK and BET inhibition could be readily translated to a clinical environment.

In Chapter 3, I explore the relationship between PRC2 defects and high HOXC10 expression in KRAS-mutant lung adenocarcinoma. The loss of PRC2 activity in this setting can lead to increased metastatic activity in vivo and an increase in markers responsible for the epithelial to mesenchymal (EMT) transition. PRC2 defects are difficult to characterize as many factors may lead to low PRC2 activity including low expression of components, copy number
loss, or mutation. Thus, throughout the paper we have utilized a signature of PRC2 targets to help define defective PRC2 activity. While this thesis and other studies suggest that PRC2 defects promote tumor progression, our findings also suggest that these defects confer a therapeutic vulnerability.

Almost half of all KRAS-mutant tumors harbor high HOXC10 expression. My work shows that HOXC10 mRNA levels from large-scale analyses largely correlate with observed protein expression, an important observation that lends more clinical utility to HOXC10 as a putative biomarker. Further, previous studies have shown that high HOXC10 activity correlates with poor survival outcomes or increased clinical aggressiveness, and small correlative studies in lung cancer also suggest this association (Tang et al., 2017). Other features of high-HOXC10 tumors correspond with our findings in PRC2 heterozygous tumors, namely high expression of hallmark E2F targets, genesets related to high MYC expression, and stemness signatures (Chapter 3).

In conclusion, this thesis (1) provides a strong base of evidence for pursuing combination MEK and BET inhibition in KRAS-mutant lung cancer patients, (2) suggests exploration of this treatment combination in other Ras-driven settings, and (3) characterizes two KRAS-mutant lung cancer subsets (low PRC2 and/or high HOXC10). It is my hope that this work will encourage further investigation of these features in lung tumorigenesis and will spark future studies that target cancer at the intersection of signaling and epigenetics.

**FUTURE DIRECTIONS**

**Clinical Outlook for combined MEKi and BRD4 in NSCLC**

My work in Chapter 2 characterizes combined MEKi and BETi in KRAS-mutant lung adenocarcinoma patients and we believe that the combination should be readily translated to patients. Utility of BET inhibitor monotherapy is not practical in solid tumor settings as many standard of care regimens rely on a base of chemotherapy drugs or kinase inhibition. Ethically it makes the most sense to introduce BET inhibitors as therapeutic add-ons in advanced solid
tumors. In fact, most of the Phase Ib/II clinical trials of BET inhibitors (BETi) in solid tumors are studies that test this drug in combination with hormonal therapies such as fulvestrant and enzalutamide in advanced breast and prostate cancer (NCT02964507, NCT03150056). We expect this trend in study design to continue for other cancers.

At least fifteen BET inhibitors are under active clinical study and are currently being tested for safety and tolerability across advanced malignancies including multiple myeloma, lymphoma, castration-resistant prostate cancer (CRPC), and KRAS-mutant non-small cell lung cancer (NSCLC) (Table 1-2). OTX-015/MK-8628, developed by Oncoethix and purchased by Merck, is furthest in clinical development and has reported results for two safety trials in hematological malignancies and advanced solid tumors (NCT02259114, NCT01713582). In preclinical and clinical studies, MK-8628 works by inhibiting the NFKB/TLR/JAK/STAT pathways as well as MYC- and E2F1-regulated genes (Boi et al., 2016). GSK525762 is another BET inhibitor that is in early clinical testing across many tumor types. One safety study in 70 cancer patients showed good tolerability to daily dose of the drug and partial response or stable disease in 6/10 patients with NUT midline carcinoma (NMC, NCT01587703). Clinical efficacy data on solid tumors besides NMC are extremely limited. Overall, preliminary clinical response to BET inhibitor monotherapy in non-NMC solid tumors has been underwhelming with cytostatic outcomes, extremely low response rates, and frequent tumor relapse, suggesting that intrinsic and acquired resistance to BET inhibitors is common (Massard et al., 2016). Efforts to increase the clinical impact of BET inhibitors by combining them with other targeted treatments have been rampant, which makes this dissertation highly relevant to the current clinical environment.

A major clinical concern for BET inhibitors is adverse events partly because BET proteins BRD2 and BRD4 have been shown to be essential in mice (Houselstein et al., 2002; Gyuris et al., 2009). While side effects such as thrombocytopenia, anemia, neutropenia, and GI toxicities have been reported during early clinical trials, they are often reversible with treatment interruption (Berthon et al., 2016; Amorim et al., 2016; Stathis et al., 2016). The initial concern
for toxicity issues was largely sparked by testing of the BET inhibitor, BAY 1238097, whose clinical study was halted due to severe adverse events at sub-therapeutic doses (Postel-Vinay et al., 2016). But overall, other late-stage BET inhibitors such as MK-8628 and GSK525762 have been well tolerated in preliminary studies and show a favorable safety profile in early phase clinical trials in solid tumors (Massard et al., 2016).

The quickest route for future clinical testing of MEKi and BRD4i in patients with KRAS-mutant lung cancer may be to initiate trials with the BET inhibitor, GSK-525762, since GlaxoSmithKline produces one of the few FDA-approved MEK inhibitors, trametinib. GSK-525762 is furthest in GlaxoSmithKline’s clinical BETi pipeline and is already being tested in combination with hormone therapies in advanced prostate and breast cancers (NCT02964507, NCT03150056). In 2014, Merck acquired OncoEthix to obtain control of their BET inhibitor, MK-8628 (formerly OTX015). The company also has MEK inhibitors in their clinical pipeline so assuming approval of both these MEK and BET inhibitors, this company is another option for future clinical development. A more distant clinical collaborator could be Genentech as they have both BET inhibitors and MEK inhibitors (cobimetinib) in preclinical and clinical development, respectively (Lee et al., 2016; Lieu et al., 2017).

Another opportunity for the treatment of KRAS-mutant lung cancer is to combine BETi with the newly developed inhibitor of G12C-mutant KRAS. This inhibitor induces modest tumor regression as a monotherapy in preclinical KRAS-mutant xenograft and patient derived xenograft models (Janes et al., 2018). Since it targets the RAS-MEK-ERK pathway at the Ras signaling node rather than at downstream MEK, it makes sense that this drug can elicit tumor regression alone unlike MEK inhibitor monotherapy. However, the tumor regression in xenografts that we see with combined MEK and BET inhibition is more potent than demonstrated with this KRAS inhibitor monotherapy, suggesting that the clinical impact can be increased with an additional layer of this epigenetic treatment. Further, resistance to targeted
kinase inhibitors is inevitable so perhaps BETi can increase the treatment’s durability. This is an exciting prospect that warrants further study.

An additional option is to combine our dual treatment with an additional layer of immunotherapy, generating a triple combination strategy. Clinical trials testing the utility of nivolumab (PD-1 inhibitor) monotherapy versus chemotherapy are underway in advanced NSCLC and thus far, demonstrate improved overall survival (Vokes et al., 2018). Unfortunately, there is no clear predictor of response to immune checkpoint inhibitors in NSCLC making selecting patients for this therapy difficult. Clinical studies combining MEKi and immune checkpoint inhibitors have not yet been initiated yet for KRAS-mutant NSCLC, but seem likely based on the fact that similar studies have been completed successfully in melanoma patients (Ribas et al., 2015; Hwu, et al. 2016; NCT02027961, NCT02130466). Our work here suggests that more potent responses could come from the triple combination treatment and we believe combined inhibition of MEK, BET, and PD-1 could be well tolerated and effective in patients. In fact, unpublished work from our lab shows that this triple combination is well tolerated and effective in NF1-mutant tumors (data not shown). As immunotherapies continue to provide benefit to patients with NSCLC, it seems likely that future clinical trials will also rely on a base of checkpoint inhibitors. Overall, the prospect for combined inhibition of the RAS-MEK-ERK pathway and BET activity is promising and may also extend to additional tumor settings.

**Efficacy of MEKi and BRD4i in other tumor settings**

BET inhibitors are currently being tested for safety in early phase clinical trials and while the safety profile in these early trials is promising, preliminary efficacy results show partial responses in few patients with solid tumors besides NUT midline carcinoma. This is perhaps unsurprising, as preclinical studies with BETi in lung, breast, and prostate cancers have invariably demonstrated cytostatic responses (Shimamura et al., 2013; Shu et al., 2016; Asangani et al., 2014). Preclinical studies currently focus on combining BET inhibitors with
kinase inhibitors, with the expectation that BETi will enhance initial therapeutic response and improve treatment durability. In Chapter 2, I showed that combined MEK and BETi were efficacious in triggering cell death and tumor shrinkage in KRAS-mutant lung adenocarcinoma with HOXC10 overexpression. Because of the broad Ras-activation found across lung adenocarcinoma subtypes and the high level of HOXC10 expression exhibited among these subtypes (see Figure 3-10), it is possible that BRD4 inhibitors may be efficacious in other lung adenocarcinoma settings such as EGFR-mutant tumors.

Patients with EGFR-mutant lung cancer have benefited from the development of EGFR-targeting small molecules. These drugs increase overall survival and later generations of these drugs are active against a common mechanism of resistance, namely secondary mutations in EGFR (i.e. T790M mutations). EGFR inhibitors are now in their third generation, but concerns still surround the development of resistance via re-activation of the RAS-MEK-ERK pathway outside of EGFR mutation. In fact, a recent preclinical study demonstrated that the durability of EGFR inhibitors is improved when used in combination with the FDA-approved MEK inhibitor trametinib (Tricker et al., 2015). This combination works by more deeply and durably suppressing RAS-MEK-ERK signaling output. My work in Chapter 2 shows that BRD4i alone is able to suppress Ras transcriptional targets and that combined MEK and BRD4i leads to even more potent suppression. Since BRD4 works globally and can prevent resistance through multiple signaling nodes, future work could focus on combining EGFR and BET inhibition to see if it is comparable to dual EGFR and MEK inhibition. Further, a triple combination treatment strategy with EGFRi, MEKi, and BETi could prove efficacious and tolerable, as more studies characterizing the use of three targeted treatments are emerging (Singleton, et al. 2017; Corcoran et al., 2018). Assessing whether HOXC10 is a predictive biomarker in this setting will also be important.

Another cancer where BETi-based treatment combinations have proven efficacious is in BRAF-mutant melanoma. Melanoma is a deadly skin cancer with poor overall survival rates,
especially for metastatic disease. Patients with tumors harboring BRAF mutations respond well to BRAFV600E-specific inhibitors (response rates are greater than 50%) and there are three FDA-approved inhibitors currently used in the clinic. However, survival of patients is limited as resistance to BRAF inhibitor monotherapy invariably develops via the re-activation of the RAS-MEK-ERK signaling cascade or parallel pathways. Phase III clinical trials testing combined BRAF and MEK inhibition versus BRAF inhibition alone showed significantly improved survival (Robert et al., 2014; Johnson et al., 2014). Unfortunately, progression free survival is still limited by resistance and preclinical studies find this resistance largely attributable to re-activation of the RAS-MEK-ERK pathway yet again (Lu et al., 2017). Our lab determined that triple therapy (MEK, BRAF, and BET inhibition) could improve treatment durability and response in several preclinical BRAF-mutant in vivo models and our collaborators found that this response depended largely on MYC suppression (Singleton et al., 2017). Within melanoma, other common subtypes include NRAS- and NF1-mutant tumors. Again, considering the impact of BET inhibitors on oncogenic signaling output, future work can and should focus on layering BET inhibitors on top of MEK inhibitor standard-of-care in these settings. It will also be important to assess whether clinical efficacy in other melanoma subtypes also depend on MYC suppression. Preliminary results in the lab demonstrate that this combination treatment in NRAS- and NF1-mutant tumors is effective and mechanistic studies are underway (data not shown).

In BRAF-mutant colon cancer, single agent BRAF inhibitors have demonstrated a surprising lack of efficacy in patients with a response rate of only 5% to BRAF inhibitor vemurafenib, as compared to typical melanoma primary responses of >50% (Hyman et al., 2015; Kopetz et al. 2015). Intrinsic resistance to this treatment has been partially attributed to feedback activation of EGFR and MEK (Corcoran et al., 2012). In fact, response rates jump to 10% and 21% when combined BRAF and MEK inhibitors or triple combination treatment of BRAF, EGFR, and MEK inhibitors are used, respectively (Corcoran, 2015; Corcoran et al., 2018). Another factor that may underlie the observed lack of clinical efficacy is that Ras effector
inhibitors do not suppress Wnt pathway activity, a major driver of colorectal cancer via the loss of APC (reviewed in Polakis et al., 1999). As epigenetic therapies, including BETi, have been demonstrated to shift differentiation states and regulate cellular identity, it is possible that BETi could inhibit Wnt signaling in this environment (Shu et al. 2016; Saenz et al., 2017). Our lab’s preliminary in vitro works shows that combined BETi and BRAFi triggers increased cell death compared to BRAFi monotherapy in BRAF-mutant colorectal cancer (data not shown). Thus, testing combined BRAF and BET inhibitors in BRAF-mutant colorectal cancer is an exciting therapeutic area and mechanistic studies are ongoing.

My work suggests that BET inhibitors have the potential to improve the impact of a wide variety of standard-of-care treatments. We expect that the use of BET inhibitors in these diverse settings will rely on different mechanisms of action and biomarkers, but that each therapeutic exploitation will contribute more information about the tumorigenic process. As a global probe of signaling and cellular identity, BET inhibitors hold promise for changing the research and clinical landscape. Moreover, examining how BETi works beyond its traditional role in MYC suppression is important.

*BET inhibitors and MYC suppression*

BETi use in solid tumor settings was sparked by the finding that inhibition of BRD4 leads to the downregulation of MYC and its downstream targets in hematological settings (Delmore et al., 2011; Puissant et al., 2013). While many studies have attributed BET efficacy to this indirect suppression of the ‘undruggable’ MYC transcription factor, rigor should be applied throughout research studies before such a claim is made in other tumors. Two of the pioneering studies showing BETi activity in multiple myeloma and MYCN-amplified neuroblastoma showed clear downregulation of c-MYC and MYCN in multiple therapeutic contexts and that this downregulation was primarily responsible for the therapeutic effects of JQ1 (Delmore et al., 2011; Puissant et al., 2013). These findings have sparked other studies using BETi that have
not been as rigorous in their characterization of therapeutic activity, a problematic trend which could lead to decreased knowledge of BETi functional output across cancer contexts.

In fact, there are many pre-clinical studies that show that BETi can function in MYC-independent ways. These studies either demonstrate that BETi does not downregulate MYC or that MYC downregulation is not sufficient or necessary to induce its therapeutic effects. For example, in my work with BETi, I find that MYC RNA and protein levels are not downregulated by BETi (Figure A-1A). Similar lack of MYC suppression has been observed in NF1-mutant nervous system tumors and leukemia (De Raedt, et al., 2014; Fong et al., 2015). Further, high MYC protein levels in cell lines do not predict response to BETi in prostate cancer or triple negative breast cancer (Asangani et al., 2014; Shu et al., 2016). And despite the fact that BETi attenuates MYC protein levels in a recent prostate cancer study, subsequent knockdown of MYC did not phenocopy BETi’s effects on cellular proliferation nor did overexpression rescue them. Thus, the study authors concluded that MYC was not the primary target of BETi’s anti-tumor effects (Asangani et al., 2014). A similar conclusion was made in SPOP-mutant prostate cancer where investigators found that BETi downregulated MYC in both sensitive and resistant lines, suggesting that MYC does not play a role in the therapeutic response (Fong et al., 2017). Even clinically, one preliminary trial found no association between MYC expression and sensitivity to BETi as c-MYC mRNA was not downregulated in any patients, including responders (Berthon et al., 2016; NCT01713582)

Together, these examples indicate that it is not sufficient to solely demonstrate MYC suppression by BETi to claim a MYC-dependent mechanism of action. Instead, rigorous study should demonstrate the key role MYC plays in BET inhibitor response. Some key experiments include knockdown of MYC in an attempt to phenocopy the drug’s effect as well as ectopic expression of MYC to reverse it. In melanoma, researchers first found that MYC expression is commonly reactivated in diverse models of BRAFi resistance including ERK reactivation, PI3K pathway activation, and NOTCH1 pathway activation. They then show that BETi is able to
cooperate with BRAFi to prevent resistance and improve durability. This activity is dependent on BETi's effects on MYC, as demonstrated by ectopic MYC expression, which reverses the effects of BETi. They also demonstrated that knockdown of MYC phenocopied the effects of JQ1 in \textit{in vitro} and \textit{in vivo} models. Beyond their characterization of BETi's effects on MYC, they also delved deeper to determine that the MYC axis triggers metabolic dependencies within these resistance cell lines, suggesting further therapeutic opportunities (Singleton et al., 2017).

Overall, their work shows that MYC can be an essential part of the response to BETi but also that further examination of this dependency can propose novel therapeutic study. Investigators should consider these lessons when designing their own experiments with BETi combinations. Overall, these studies demonstrate that BET inhibitors and other epigenetic-based therapies uniquely adapt to the transcriptional environment of the target tumor, acting in a tailored way from one setting to another.

\textit{Targeting cancer at the intersection of signaling and epigenetics}

\textit{Introduction}

Precision medicine and the subsequent targeted therapy revolution have increased survival outcomes for a wide variety of tumors. However, targeted treatments against oncogenic kinases are often limited by resistance mechanisms featuring re-activation of the signaling cascade via mutations within the same or parallel pathways. Work from this thesis and other studies demonstrate that combining epigenetic therapy with standard of care kinase inhibitor treatments leads to (1) enhanced tumor regression via cooperative suppression of oncogenic signaling output and (2) more durable responses through prevention of signaling-based resistance mechanisms. Inhibitors of BET and EZH2 provide clear examples of both paradigms of cooperation and directly suggest this therapeutic strategy for further clinical study.
**BETi + combination treatments**

BET bromodomain proteins are epigenetic readers that bind acetylated histones and recruit key transcription factors to active promoters and enhancers (Jang et al., 2005; Hargreaves et al., 2009; Yang et al., 2008). BET inhibitors largely work by displacing BET bromodomain protein, BRD4, from these active promoters and enhancers, preventing its ability to activate gene expression at these sites. This effect is specific to the genes that are highly expressed within the cell (and thus have high enhancer activity) and these genes often correspond to signaling pathways that the tumor is relying on for its growth. As stated previously, in the setting of KRAS-mutant lung cancer, BET inhibition largely downregulates downstream Ras transcriptional output, demonstrating how epigenetic inhibition can affect the signaling environment. Other studies also show that BETi can cooperate with other targeted inhibitors to downregulate oncogenic signaling. In fact, BRD4 cooperates with the androgen receptor (AR) transcription factor to mediate AR targets, suggesting that AR and BET inhibitors would lead to concomitant suppression of these targets in prostate cancer (Asangani et al., 2014). In another study, AR overexpression is shown to induce genome-wide chromatin relaxation such that combining BETi with AR-targeting agents induces a double-whammy of AR target gene suppression, with BETi inducing chromatin compaction at AR target loci and ARi blocking AR transcriptional activity (Urbanucci et al., 2017). These studies have prompted at least two different active Phase Ib/II combination therapy clinical trials in advanced prostate cancer (NCT03150056, NCT02607228).

Other reports have characterized BET inhibitors in downregulating heterogeneous resistance to kinase-targeting agents. For example, lapatinib resistance occurs primarily through kinome reprogramming when multiple receptor tyrosine kinases (RTKs) are upregulated in response to treatment. Finding one drug to consistently target all possible resistance mechanisms is difficult with standard targeting agents, but BET inhibition works by broadly suppressing these lapatinib-induced kinases (Stuhlmiller, et al., 2015). Similarly, PI3K inhibition
is not sufficient to induce tumor regression in a model of metastatic breast cancer but conversely leads to re-activation of PI3K signaling and compensatory upregulation of RTK expression. BETi blocks these resistance responses by shifting the transcriptional environment, leading to a more durable therapeutic response (Stratikopoulos, et al., 2015).

In *BRAF* mutant melanoma, resistance mechanisms ultimately converge on *MYC* activation (Singleton et al., 2017). Because of BETi’s classic role as a suppressor of MYC, the study layers BETi on top of the standard of care (BRAFi and MEKi) to create a triple combination treatment that prevents resistance-mediated MYC activation. BET inhibitors have also been used to prevent resistance in hematological malignancies. In T cell acute lymphoblastic leukemia (T-ALL), a recent study modeled clinical resistance to γ-secretase inhibitors, often used to prevent NOTCH1 activation in this setting (Knoechel et al., 2014). Investigators found that knockdown of BRD4 is essential for persister viability and that BETi could reverse the resistance phenotype by combining γ-secretase inhibitors and BETi to induce apoptosis.

**EZH2i + combination treatments**

EZH2 inhibitors are active in clinical development in both hematological and solid tumor settings (Table 4-1). One particular inhibitor from Epizyme, EPZ-6438, is under study in over ten active clinical trials being tested in lymphomas, ovarian cancers, and other advanced solid tumors. EZH2 is the enzymatically active subunit of the PRC2 complex, a histone methyltransferase that triggers chromatin compaction and transcriptional repression. The epigenetic writer was first appreciated in oncogenesis when its overexpression was correlated with poor survival outcomes in prostate cancer (Varambally et al., 2002). EZH2 inhibitors work by inducing loss of global H3K27me3 repressive mark and concomitant upregulation of gene expression.
Like drugs targeting BET, EZH2 inhibitors can also reverse signaling-based resistance mechanisms. A recent study in CML patients demonstrated that residual disease relies on EZH2 for resistance to tyrosine kinase inhibitors (TKIs), finding that EZH2 inhibitors sensitize these persistent cells to TKI treatment. Importantly, EZH2i or TKI alone induces upregulation of EZH2-repressed targets, but only combination treatment durably potentiates them, triggering the loss of TKI-persistent stem cells in an in vivo CML model (Scott et al., 2016). Similarly, EZH2i reverses TKI resistance in renal cell carcinoma by regulating TKI-induced kinome reprogramming, decreasing global RTK phosphorylation, and increasing the expression of key tumor suppressors DAB2IP and PTPN3 (Adelaiye-Ogala et al., 2017). Combination EZH2i and TKI treatment in this setting leads to decreased metastases and tumor volume in renal cell models. Importantly, EZH2i has an advantage over other kinase-targeting agents due its ability to globally combat the resistance response, making it a useful tool for overcoming heterogeneous modes of resistance.

EZH2i can also reverse resistance to hormonal pathway inhibitors. In tamoxifen-resistant breast cancer, EZH2 normally mediates silencing of estrogen co-factor GREB1, an action that converts the tamoxifen drug into an ER agonist. By inhibiting EZH2, this negative epigenetic consequence is blocked and tumor progression is slowed (Wu, et al., 2017). In prostate cancer, EZH2 acts as a co-activator for the androgen receptor (AR) transcription factor, a study that
directly suggests dual ARi and EZH2i treatment for the treatment of castration-resistant prostate cancer and has sparked early phase clinical trials (Xu et al., 2012). Thus, these two epigenetics-based therapies (BETi and EZH2i) consistently add therapeutic flexibility to standard of care treatments.

Cooperative suppression of resistance mechanisms triggered by epigenetics-based therapy

The efficacy of epigenetic monotherapy can be limited by intrinsic resistance mechanisms due to oncogenic signaling activity. Adding an additional layer of kinase inhibitors in these contexts lead to more impactful therapeutic responses in preclinical models. For example, preclinical studies in ovarian cancer find that BETi monotherapy response is limited by subsequent activation of RTK signaling, suggesting usage of kinase inhibitors MEKi, PI3Ki, FGFRi, or ERKi in combination with BET inhibitors (Kurimchak et al., 2016). Termed ‘kinome reprogramming’, this study again demonstrates the interplay between signaling and epigenetic environments. In acute myeloid leukemia (AML), resistance to BET inhibitors occurs via increased Wnt/β-catenin signaling because increased Wnt activity re-triggers the expression of BRD4 target genes in resistant clones, negating BETi efficacy. By layering Wnt inhibitor on top of BETi, resistance is prevented and combined inhibition significantly improves survival in preclinical mouse models (Fong et al., 2015).

Intrinsic resistance to BET inhibitors in triple negative breast cancer depends on posttranslational features of BRD4, such as its phosphorylation and its interaction with MED1. This observation suggests that combining BET inhibitors with modulators of BRD4 phosphorylation including CK2 inhibitors and PP2A (serine phosphatase targeting BRD4) activators to reverse treatment-induced phosphorylation will restore sensitivity to BETi (Shu et al., 2016). Interestingly, intrinsic resistance to BET inhibitors is also prominent in SPOP-mutated prostate cancer. Mutations in SPOP, an E3 ubiquitin ligase adapter protein that normally triggers degradation of BET proteins, lead to accumulation of BET proteins and prevent PI3K
pathway suppression. Researchers find that this SPOP-mutant resistance can be overcome by combining BETi with AKT inhibitors in prostate cancer (Zhang et al., 2017). Overall, BETi monotherapy is limited by resistance mechanisms that can be reversed by adding an additional layer of oncogenic kinase suppression, suggesting another way that oncogenic pathways and epigenetic regulation can be co-targeted for therapeutic efficacy.

**Caution with epigenetic-based combination treatments**

When designing epigenetic-based therapies, it is particularly important to select relevant patient populations as many epigenetic regulators can act as oncogenes or tumor suppressors, depending on the setting. For example, EZH2 inhibitors have been designed to target tumor environments that depend on mutations or amplifications of EZH2 function. However, EZH2 gain-of-function is not the only tumorigenic role of the PRC2 complex as copy number loss of SUZ12 and EED (PRC2 components) has also been tied to increased tumor aggressiveness in glioblastoma, NF1-mutant nervous system tumors, and T-ALL (DeRaedt, et al. 2014; Ntziachristos et al., 2012). Similarly, low EZH2 correlates with poor prognosis in acute myeloid leukemia (AML) and induces resistance to TKIs and other cytotoxic drugs. Thus, EZH2 inhibition may have the unintended effect of inducing resistance rather than reversing it in primary AML (Gollner, et al., 2017). A similar trend has been observed in breast cancer where EZH2 inhibition has differential effects depending on the tumor’s co-occurring mutations. EZH2 inhibition sensitizes cells to topoisomerase inhibitors in the setting of EGFR or SMARCA4 mutations but conversely promotes resistance in tumors that lack these mutations. Together, these observations suggest caution and finesse when selecting patients for EZH2i clinical trial participation and these lessons should be extended to other epigenetic-based combination therapies.
Conclusion

More studies are needed to characterize epigenetic-based combination treatments because of their 1) demonstrated efficacy across a wide range of tumor types, 2) ability to downregulate heterogeneous modes of resistance and 3) limited practicality as monotherapies in solid tumor settings.

Targeting HOX genes

As presented in Chapter 3, HOX genes, including HOX genes expressed during lung development (HOXA5, HOXB5, and HOXC5), are globally downregulated by BET inhibitors in this KRAS-mutant setting (Figure A-5). Moving forward it will be interesting to assess whether global HOX downregulation, especially downregulation of these re-deployed developmental HOX genes is therapeutically relevant.

Future work will focus on whether BETi’s ability to downregulate HOXC10 and other HOX genes is a result of direct regulation at the HOX locus. Direct ChIP-PCR of BRD4 is this therapeutic setting will tell us more about the regulation of H3K27 marks at this locus. We expect BRD4’s regulation of HOX genes is direct, implying that BET inhibitors may be able to alter HOX gene expression in other settings and could have therapeutic implications in HOX-driven settings such as prostate cancer and AML.

As outlined in Chapter 1, efforts to target HOX genes are underway in AML and prostate cancer studies since both of these cancers are characterized by aberrant HOXA9 or HOXB13 activity, respectively. Future studies should focus on testing BET inhibitors in these cancers to determine if they can therapeutically downregulate their respective oncogenic homeobox activities.

In AML, efforts to target HOX indirectly with MLL-based inhibition such as menin and DOT1L inhibitors are under active study. Future work could assess whether these inhibitors can downregulate HOXC10 in KRAS-mutant lung cancer. While it is unlikely that menin and DOT1L
inhibitors would be able to synergize with MEK inhibitors to induce tumor regression, usage of these inhibitors can help to further tease out the role of HOXC10 in a KRAS-mutant background.

**Cell cycle dynamics and therapy**

In this thesis, I described how the therapeutic efficacy of combined MEK and BET inhibition converge on the regulation of DNA replication initiation. Specifically, we find that HOXC10-expressing cancer cells are sensitive to global downregulation of pre-replicative complex (pre-RC) components including CDC6 and the MCM helicase proteins. CDC6 is an origin-licensing factor that is responsible for marking putative origins of replication and recruiting the MCM helicase complex to these origin sites during early G1. As outlined in Chapter 2, this process of origin licensing is necessary to ensure proper and efficient DNA replication once throughout the cell cycle. Previous studies have indicated that strong knockdown of CDC6 leads to G1-S cell cycle arrest in normal cells, but that this same perturbation in cancer cells leads to a G1-S block and the induction of cell death during mitosis (Lau et al., 2009). Cell death occurs in cancer cells because these cells have a higher threshold for cell cycle checkpoints, likely due to acquired defects during early tumorigenesis. Thus, cancer cells with too few origins of replication are not uniformly arrested at the G1/S cell cycle block, but rather progress into S phase where they incompletely replicate DNA and acquire stalled forks. Cell death is then induced due to the acquisition of catastrophic DNA damage. Cancer cells seem uniquely poised to die when faced with treatments that affect their DNA replication capabilities.

In Chapter 2, we demonstrated that the sensitive cell lines exhibit many of these same features in response to the combination treatment. Sensitive cells show downregulation of pre-RC proteins within twelve hours of treatment, subsequent DNA damage as assayed by phospho-gamma-H2AX, and limited cell cycle arrest after treatment exposure. Conversely, the resistant cells essentially act as normal cells in this scenario: they exhibit no DNA damage and are severely arrested following just sixteen hours of treatment.
These observations imply that the design of combination treatments should take into account how the cell cycle is affected by one or more of the inhibitors. Our work shows that severe cell cycle arrest by one of the treatment arms can limit drug-induced cell death if the mechanism for this death relies on S-phase entry. Thus, perhaps it is best to find therapeutic combinations that do not have such strong arrest of the cell cycle if the ultimate goal is DNA-damage induced cell death. This thesis helps us think about how combination treatment must cooperate on multiple levels to maximize therapeutic responses.

More work is needed understand how HOXC10 is hijacked to regulated replication. It is unclear exactly why the sensitive and resistant cells exhibit such different responses to the combination treatment. Our work in Chapter 2 suggests that the expression of HOXC10 in sensitive cells induces or marks a highly proliferative state that is not easily arrested. This hypothesis is supported by work in Chapter 3 that indicates that high-HOXC10 cells are enriched for E2F1 and E2F3 target signatures as activity of these E2F transcription factors are required for cellular proliferation (Humbert et al., 2000). We examined checkpoint signaling between the two populations of cells and have seen no differences in CHK1 or RB phosphorylation that can explain the disparity in cell cycle arrest (data not shown). Importantly, our studies may be limited by our examination of an asynchronous population of cells since many of these phosphorylation events occur at different stages of the cell cycle. Teasing apart the therapeutic relevance cell cycle checkpoint signaling has been challenging due to the limitations of asynchrony, but the work has significant implications for our lab as we consider how to design rational combination therapies.
**Bench to Bedside Translation and Preclinical Tumor Regression**

There are many promising preclinical studies that have generated clinical trials in KRAS-mutant lung cancer including treatments that combine MEK inhibitors with chemotherapy or with inhibitors of PI3K, BCL-XL, or CDK4/6 (Table 1-1). Unfortunately, as we have seen with preliminary patient studies, the promise of preclinical studies is not always recapitulated in the clinic. One reason for failed bench to bedside translation is that we have yet to determine a threshold for preclinical response that accurately dictates clinical outcomes. Additionally, preclinical studies often claim efficacy for treatments that trigger barely, if any, tumor regression or cell death. These therapies also often lack rigor in heterogeneous settings, failing to characterize additional biomarkers beyond KRAS mutation. This is problematic as recent studies have demonstrated that secondary mutations can modify therapeutic sensitivities (Chen et al., 2013; Skoulidis et al., 2015). Finally, a practical but highly relevant reason for lack of translation is that toxicity issues often are not comparable between mice and humans. More efforts should be made to monitor toxicity (pharmacokinetics) and target inhibition (pharmacodynamics) in both settings.

Overall, cell death and tumor regression in preclinical models should be prioritized over treatments that solely induce cell cycle arrest and tumor stasis. Indeed, in our study of cancer treatments, we select for treatments that induce tumor regression in established tumors (300-500 mm$^3$) and have identified multiple promising combinations that fit this standard (De Raedt et al., 2011; De Raedt et al., 2014; Malone et al., 2017).

Recent bench to bedside examples in KRAS-mutant lung adenocarcinoma illustrate these points, specifically in MEK combined with PI3K inhibitors or chemotherapy. In preclinical testing of MEKi plus PI3Ki, tumor regression was observed in a KRAS-mutant GEMM of lung adenocarcinoma (Engelman et al., 2008). In the preclinical study, tumors were established and observed by MRI prior to starting treatment. Investigators found that tumors regressed over the course of two weeks of treatment. However, tumors also regressed to a similar degree in the
MEKi inhibitor alone arm, making the added benefit of PI3K inhibitors unclear in this setting. Clinical trials for combined MEK and PI3K treatment are ongoing in advanced tumors but have been underwhelming in KRAS-mutant lung cancer due to toxicity concerns and questionable in vivo target inhibition at tolerable doses (Bedard et al., 2012; Infante et al., 2012; Khan et al., 2012). While we cannot conclusively say that the lack of true efficacy in pre-clinical models predicted these disappointing patient studies, more work is needed to determine how and why pre-clinical combinations do not translate well to patients. Indeed one follow-up report indicates that lack of Bcl-xL inhibition is one reason for treatment failure, suggesting that cell death induction should be a prerequisite for therapeutic translation in KRAS-mutant disease (Hata, et al. 2014). This report further suggests that future investigations should account for heterogeneity in KRAS-mutant tumors to maximize clinical success. In our studies, we tested combined MEK and BET inhibitors in multiple KRAS-mutant settings and were able to identify HOXC10 as a biomarker that predicts response. We are hopeful that this biomarker will help launch this treatment to clinical success.

Along these lines, another emerging trend in preclinical studies is to test and examine effects of treatments in heterogeneous murine co-clinical trials. This strategy allows the study of secondary mutations within KRAS-mutant tumors and possible patient stratification upon clinical examination. Investigators performed such a trial for combined MEKi and chemotherapy in multiple models of KRAS-mutant disease including those with secondary mutations in TP53 and LKB1. This study found that tumors with mutation in LKB1 had a priori resistance to the combination treatment, suggesting that this factor is important during the analysis of the concurrent human clinical trial results (Chen et al., 2012). In fact, while Phase II trial results were promising with combined MEKi and chemotherapy, larger Phase III trial did not show improved overall survival as compared to chemotherapy alone (Janne et al., 2013). The concurrent mouse study presents a possible explanation, demonstrating how this approach can provide beneficial information to clinical trial investigators before trials begin.
Other preclinical studies in KRAS-mutant lung adenocarcinoma have yet to be tested in patients but present varied responses in mouse models. Combined MEK and BCL-XL inhibition has been tested in a KRAS-mutant lung cancer GEMM model where each single treatment caused 30-40% tumor regression, but combined treatment caused 70% tumor regression (Corcoran, et al. 2013). This study poses the question of how much cooperativity we should expect for a combination treatment to warrant further study. Clinical trials are underway for this combined treatment so we will soon be able to understand how these factors translate to the clinic (NCT02079740). Another treatment regimen is combined MEK and CDK4/6 inhibition. The efficacy of this treatment combination was first suggested when a synthetic lethal interaction between KRAS and CDK4 was identified in a NSCLC mouse tumor model (Puyol et al., 2010). Accordingly, co-administration of drugs targeting MEK and CDK4/6 has been tested in KRAS-mutant lung cancer models (Tao et al., 2016). Response to this dual treatment in vivo has shown only modest slowing of tumor growth. While radiotherapy plus MEK and CDK4/6 inhibition leads to increased tumor stasis in vivo, tumor regression is not robust. A clinical trial combining trametinib and palbociclib has been initiated for KRAS-mutant lung cancer but based on the result of this recent study, strong clinical efficacy is unlikely.

While the design of combination targeted treatments is an emerging preclinical practice, the summary presented above suggests that meta-analyses and comparisons of preclinical responses should be undertaken to ensure the most efficacious therapies make it to patients quickly. Moreover, we have outlined a list of guidelines that should be followed when feasible to ensure that preclinical testing in mouse models is as effective as possible (Table 4-2). In my target ID study, I followed many of these guidelines though more work is necessary to more closely track toxicity concerns and develop dosing strategies before successful translation to the clinic.
All together, the findings presented in this dissertation, as well as the topic areas of this discussion, aim to provide promising pre-clinical data for a combination epigenetics-based treatment and expand our understanding of the therapeutic targeting of KRAS-mutant lung cancer. We hope that this work will spark additional preclinical studies of treatments that effectively and durably target both the signaling and epigenetic environments across solid tumor settings.

Table 4-2. Guidelines for Successful Preclinical Studies

<table>
<thead>
<tr>
<th>Guidelines for Successful Preclinical Studies</th>
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<tr>
<td><strong>Rationale:</strong></td>
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<tr>
<td><strong>Heterogeneity:</strong></td>
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<tr>
<td><strong>Cytotoxicity:</strong></td>
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<tr>
<td><strong>In vivo validation:</strong></td>
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<td><strong>Intervention studies only:</strong></td>
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<td><strong>Dosing:</strong></td>
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<td><strong>Toxicity:</strong></td>
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<td><strong>Biomarker:</strong></td>
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Appendix A: Supplementary Figures for Chapter 2 and 3
Figure A-1. MYC regulation by combination treatment and mouse toxicity study

(A) MYC protein levels are not substantially regulated by BRD4i in two sensitive cell lines. In fact, MEKi appears to downregulate MYC more so than BRD4i, an observation that holds true in qPCR analysis of NCI-H1573.

(B) Healthy NSG mice were treated with full doses of combination MEKi and BRD4i for fourteen days and their weight was monitored daily. All mice exhibited either stable weight or weight gain during treatment, indicating that toxicity is likely not a concern in this setting. More toxicity studies must be done to confirm.
Figure A-2. Analysis of key tumor suppressor/oncogene alterations in KRAS-mutant panel

(A) We analyzed the presence of mutations, amplifications, or CNV in key tumor suppressors and oncogenes that have been shown to modify response to either MEKi or BRD4i monotherapy. No significant enrichment of any of these modifications is observed.
Figure A-3. Supplemental panels to Figure 2-3

(A) Ras transcriptional output is suppressed in inherently resistant cell line NCI-H2030.
(B) HOXC10 mRNA is suppressed by BRD4i and combination treatment in two sensitive cell lines.
(C) BRD4i suppresses the expression of PRC2 targets
(D) ImmunobLOTS showing corresponding HOXC10 and pERK expression for Figure 2-3G
(E) ImmunobLOTS showing expression for Figure 2-3J
Figure A-4. Supplementary panels to Figure 2-5
(A) Immunoblot showing expression for Figure 2-5A
(B) List of gene sets enriched at fold enrichment >10 for GO analysis performed in Figure 2-5C
(C) Fold change for each treatment arm for each pre-RC complex component listed in Figure 2-5D
(D) Another immunoblot from NCI-H1573 cells showing rescue of MCM5 protein following HOXC10 overexpression
(E) Cyclin expression levels in G1 and S fractions collected for Figure 2-5I, confirming proper segregation of cell cycle stages
(F) Immunoblots showing knockdown of CDC6 or MCM5 for experiments in Figure 2-5J
A. | No construct | HOXC10-Cterm | HOXC10-Nterm | HOXC10-del3 |
---|---|---|---|---|
HA-tag | ![Image] | ![Image] | ![Image] |
tubulin | ![Image] | ![Image] | ![Image] |

B. | Gene | Fold Change vs. DMSO | raw \(p\) value | FDR |
---|---|---|---|---|
MEKi | MEKi BRD4i COMBO | 3.53 1.19 7.08 | \| | |
BRD4i | 2.41 1.96 6.67 | \| | |
COMBO | 2.70 1.33 6.37 | \| | |
CDC6 | 2.91 1.40 4.80 | \| | |
MCM5 | 2.64 1.25 4.18 | \| | |
MCM3 | 2.28 1.13 3.57 | \| | |
MCM4 | 2.15 1.52 3.51 | \| | |
MCM2 | | | | |

C. | Gene | Fold Change vs. DMSO | raw \(p\) value | FDR |
---|---|---|---|---|
CDC6 | MEKi | BRD4i | COMBO | 3.53 1.19 7.08 | \| | |
MCM5 | siCNT | siCDC6 | siMCM5 | 2.41 1.96 6.67 | \| | |
MCM6 | siCNT | siCDC6 | siMCM5 | 2.70 1.33 6.37 | \| | |
MCM7 | siCNT | siCDC6 | siMCM5 | 2.91 1.40 4.80 | \| | |
MCM3 | siCNT | siCDC6 | siMCM5 | 2.64 1.25 4.18 | \| | |
MCM4 | siCNT | siCDC6 | siMCM5 | 2.28 1.13 3.57 | \| | |
MCM2 | siCNT | siCDC6 | siMCM5 | 2.15 1.52 3.51 | \| | |

D. | Gene | mRNA expression |
---|---|---|
LACZ | DMSO COMBO 24HR | + + + | + + + |
HOXC10 | DMSO COMBO 24HR | + + + | + + + |

E. | Gene | mRNA expression |
---|---|---|
MCM5 | DMSO COMBO 24HR | + + + | + + + |
GAPDH | DMSO COMBO 24HR | + + + | + + + |

Figure A-4 (continued).
Figure A-5. BETi induces downregulation of other HOX genes in KRAS-mutant NSCLC
(A) HOX qPCR of NCI-H1573 cell line shows that HOX genes are globally suppressed by combination treatment, largely mediated by the BRD4 arm. Each row of Heatmap is normalized to itself.
(B) Individual qPCR was also performed on HOXA5, HOXB5, and HOXC5 (HOX genes expressed during lung development) to show that BETi also mediates downregulation of these genes.