BMI1 is a context-dependent tumor suppressor that is a barrier to dedifferentiation in non-small cell lung adenocarcinoma

by

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ABSTRACT

Predictive value is expected when preclinical models of disease are used for research. However, not all models appropriately mimic the disease progression or the treatment paradigm in the clinic. This thesis addresses an epigenetic regulator, *Bmi1*, which acts in stem cells to maintain their proliferative and self-renewal capacity primarily through silencing of the *Ink4a/Arf* locus. *Bmi1* has been proposed as a good therapeutic candidate in cancer because of its presumed role in maintaining tumor propagating cells (TPCs). This conclusion is based on the observed tumor suppressive effects of *Bmi1* deletion in *in vitro* cell culture models, *in vivo* transplant models, and autochthonous models in which *Bmi1* was absent throughout development. However, to date, no one has assessed the consequences of deleting *Bmi1* in existing autochthonous tumors, to mimic patient treatment in the clinic. To accomplish this, we have generated a mouse model that allows induction of autochthonous lung adenocarcinoma, driven by oncogenic *Kras* and *Tp53* loss (KP LUAD), and subsequent deletion of *Bmi1* specifically within the tumor cells once more than half the tumors progress to grade 3 or higher. We confirmed that this model yielded *Bmi1* loss that was tumor-specific and almost complete. We then aged tumor bearing mice for up to seven weeks post *Bmi1* deletion to determine the impact on LUAD. Unexpectedly, *Bmi1* deletion did not yield significant tumor suppression. Instead, gene expression analyses of *Bmi1* deficient tumor cells revealed upregulation of a gastric gene expression program that is a known marker of lung tumor progression towards a more aggressive state in the KP LUAD model. Additionally, single cell sequencing showed that Bmi1 deficient tumors contained a higher frequency of cells that expressed previously described markers of TPCs and metastasis. We also extended these findings to colorectal cancer where we show that deletion of *Bmi1* is not tumor suppressive in either *in vitro* organoids or orthotopic transplants. Given these findings, we conclude that deletion, or inhibition, of BMI1 in existing tumors will be ineffective for cancer treatment in the contexts examined, and potentially deleterious because it can enable acquisition of alternate differentiation states that promote tumor progression.

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CHAPTER 1: INTRODUCTION

Bmi1, a part of polycomb repressive complex 1 (PRC1), is a known epigenetic regulator of self-renewal and proper differentiation of stem cells. There has been extensive interest in *Bmi1* as a therapeutic candidate given its presumed role in self-renewal of tumor propagating cells, and the tumor suppressive effects observed upon *Bmi1* deletion in cancer cell lines and mouse models of cancer. However, remarkably, the consequences of deleting *Bmi1* in existing tumors has never been tested. In this chapter, I will briefly introduce tumor propagating cells and their roles in cancer progression, and then describe the broad mechanisms by which the polycomb complexes regulate gene expression and influence differentiation. I will then focus on *Bmi1*, describing its known functions in stem cell biology and differentiation, and its role in cancer. Finally, I will describe lung cancer development and progression, and genetically engineered mouse models that enable its study, and are the major context in which I have investigated *Bmi1*'s role.

Cancer tumor propagating cells (TPCs) and their regulators

Cancer is a huge medical unmet need. In the United States alone, cancer will claim an estimated 600,000 lives in 2019 (Siegel et al., 2019). This estimated death toll varies largely by cancer tissue of origin. However, the trend of worsening 5-year survival rate as cancer stage at diagnosis increases is very consistent for all cancer types. Cancers that have spread to distant sites have worse prognosis than locally restricted tumors or regionally disseminated cancer. This is largely because most local tumors and lymph node metastases can be surgically resected, but treatment of distant metastases relies upon non-surgical cancer therapies. These non-surgical therapies are usually less effective than surgical resection because cancer cells

often develop resistance, leading to relapse. Consequently, cancer cells that are able to spread to distant secondary organ sites, and cells that are chemoresistant and cause relapse, are the main culprits of cancer-related deaths.

It has been postulated since the 90s that not all cancer cells are equal, and that a hierarchy exists amongst cancer cells that is reminiscent of the stem cell hierarchy (Bonnet & Dick, 1997). According to this model, only a subpopulation of cancer cells have the potential to divide indefinitely and to give rise to all cell types within the tumor. These cells have been dubbed cancer stem cells or tumor propagating cells (TPCs). TPCs are functionally defined by their ability to produce tumors upon serial transplantations, similar to reconstitution assays for stem cells. As an extension of this functional definition, TPCs are also thought to be responsible for forming metastases at distant sites, and contributing to tumor relapse post-therapy (reviewed in Desai et al., 2019). The first identification of TPC in human cancer was in the context of acute myeloid leukemia (Bonnet & Dick, 1997; Lapidot et al., 1994). These cells were rare in the population, but could be enriched by specific cell surface markers, and were able to generate leukemia when transplanted into immunocompromised mice, (Bonnet & Dick, 1997; Lapidot et al., 1994). Since then, TPCs have been identified in various human solid cancers including breast cancer (Al-hajj et al., 2003), brain cancer (Singh et al., 2003), pancreatic cancer (C. Li et al., 2007), colon cancer (O'Brien et al., 2007), and ovarian cancer (S. Zhang et al., 2008). Since TPCs are responsible for cancer relapse and metastases, which account for most cancer-related deaths, there has been a big push in the field to understand TPC biology and hopefully exploit TPC vulnerabilities for cancer therapy.

One approach to understanding TPC biology has been to investigate the role of genes and pathways important for maintaining stemness potential in normal stem cells. This has been fueled by the observation that poorly differentiated aggressive human tumors tend to gain gene expression signatures reminiscent of embryonic stem cells, including core pluripotency genes such as *Nanog*, *Oct4*, *Sox2*, and *c-Myc* (Ben-Porath et al., 2008). Moreover, signaling pathways that have been well-established to be important in normal stem cells, such as *Hedgehog*, *Notch*, and *Wnt*, are all exploited in various TPCs (reviewed in Desai, Yan, & Gerson, 2019). Numerous studies have also established the role of epigenetic regulators in maintaining the self-renewal potential and undifferentiated state of stem cells (reviewed in Avgustinova & Benitah, 2016). These epigenetic regulators are of key interest in TPC biology. One such epigenetic regulator is BMI1, which is a component of polycomb repressive complex 1 (PRC1). In advanced cancer, PRC1-regulated genes with documented importance in stem cell biology are preferentially repressed by hypermethylation, suggesting that they have acquired a more stem-like state (Ben-Porath et al., 2008; Widschwendter et al., 2007). This has fueled interest in the field of cancer biology to understand the role of PRC1 and Bmi1 in TPCs. This first requires a thorough understanding of role of PRC1 and Bmi1 in normal cellular context, which is detailed in the sections below.

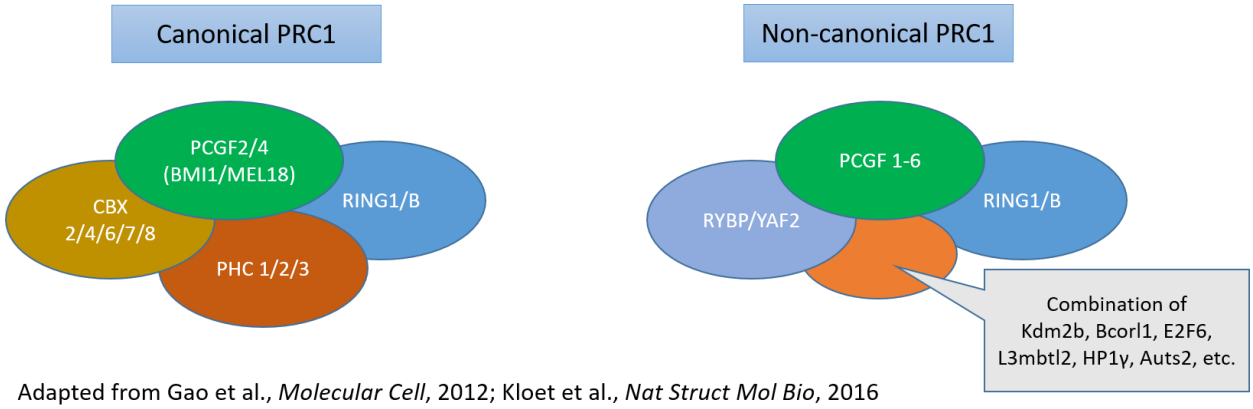
Polycomb Repressive Complex 1

Even though all the cells in an organism have the same genetic material, they are able to control the genetic expression patterns to create diversity in cell types. These patterns include the genes that are turned on, and equally importantly, the genes that are turned off. The

initiation and maintenance of these genetic expression patterns are controlled by both transcription factors that dictate lineage specificity and epigenetic modulators that control chromatin structure and histone modifications. One group of such epigenetic modulators are the polycomb proteins, which were first identified as regulators of expression of *Hox* gene expression in *Drosophila melanogaster* via a screen for body segmentation patterning during development (Lewis, 1978). Subsequent studies established the existence of two different polycomb complexes with different core functions. Polycomb repressive complex 1 (PRC1) has the enzymatic function of placing a single ubiquitin molecule on histone H2A at K119 amino acid (Cao, Tsukada, & Zhang, 2005; de Napoles et al., 2004; Ve, 2005; H. Wang et al., 2004). Additionally, PRC1 has a non-enzymatic role in compacting polynucleosomes (Francis, Kingston, & Woodcock, 2004). Polycomb Repressive Complex 2 (PRC2), in contrast, acts to methylate histone 3 at K27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002). In this section, I will primarily focus on PRC1 with additional discussion on PRC2 whenever pertinent to the function of PRC1.

The catalytic core of PRC1 is comprised of an E3 ligase, RING1A or RING1B, along with one of the PCGF(1-6) subunit (Buchwald et al., 2006; Gao et al., 2012; Kloet et al., 2016; Li et al., 2006). This catalytic core binds to many combinations of auxiliary proteins to give rise to variations in PRC1 complex structure and biological function. One diversification of function comes from competition between CBX proteins and RYBP or YAF2 in binding to the C terminal domain of RING 1A/B (Wang et al., 2010). Based on the participating binding partner, PRC1 is broadly classified into canonical or non-canonical PRC1 complexes (Figure 1.1) (Gao et al., 2012; Kloet et al., 2016). The canonical PRC1 contains CBX proteins along with polyhomeotic-like proteins

(PHC 1/2/3) and either PCGF2 (MEL18) or PCGF4 (BMI1). The non-canonical PRC1 complex contains RYBP or YAF2 together with any of the six known PCGF proteins, including BMI1. The catalytic activity of PRC1 is specific to the non-canonical PRC1 complex containing RYPB or YAF2, suggesting a catalysis-independent role for canonical PRC1 (Blackledge et al., 2014; Tavares et al., 2012). Importantly, despite the variation within PRC1, its function mostly revolves around repressing gene expression. It is still a field of intense research to understand how these complexes target chromatin sites and how they exert their gene silencing effect. Since BMI1 is part of both canonical and non-canonical PRC1, much of the mechanistic and functional analyses of PRC1 is relevant to BMI1. Below I will discuss our understanding of PRC1, which is directly applicable to our understanding of BMI1.



Adapted from Gao et al., *Molecular Cell*, 2012; Kloet et al., *Nat Struct Mol Bio*, 2016

Figure 1.1. Schematic illustrating components of the two variants of polycomb repressive complex 1 (PRC1): canonical PRC1 with CBX subunit, and non-canonical PRC1 with RYBP or YAP2 subunit. Canonical PRC1 contains either PCGF2 (MEL18) or PCGF4 (BMI1), and one of the PHC subunits. Non-canonical PRC1 contains any one of the 6 PCGF subunits. Different variants of non-canonical PRC1 is dictated by additional binding partners shown in the grey box.

How PRC1 is targeted to chromatin sites

The first understanding of how PRC1 is targeted to genes came from the observation that PRC1 and PRC2 are often colocalized to polycomb domains containing H2AK119ub1 and H3K27me3

marks (reviewed in Blackledge et al., 2015). Subsequent studies in *D. melanogaster* showed that PRC1 can be recruited to the H3K27me3 mark, which is laid down by PRC2, and this recruitment is mediated by the chromobox containing subunits (CBX) in PRC1 (Figure 1.2) (Min et al., 2003; L. Wang et al., 2004). Based on our current knowledge, this PRC1 recruitment mechanism can only be applicable to canonical PRC1 complex, as CBX proteins are limited to this variant of PRC1 (Gao et al., 2012; Kloet et al., 2016). Notably, loss of PRC2 doesn't abolish PRC1 occupancy in mouse ES cells, and the levels of H2AK119Ub are unchanged (Tavares et al., 2012), supporting the existence of a different recruitment mechanism for PRC1, presumably non-canonical PRC1. Moreover, while these observations explained how PRC1 is targeted to genes, it did not shed any light on how PRC2 is recruited in the first place.

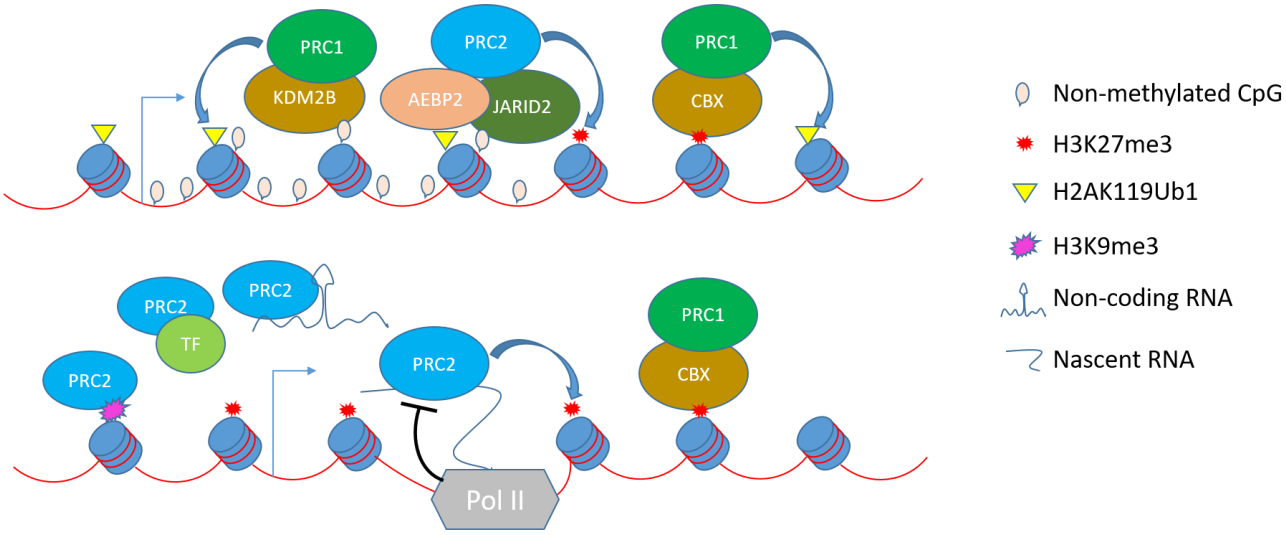
In *D. melanogaster*, early evidence demonstrated that polycomb complexes are targeted to the genome by polycomb repressive elements (PREs) (reviewed in Steffen & Ringrose, 2014). However, PRE equivalents do not appear to exist in vertebrates, leading to an ongoing search for the mechanism(s) of PRC recruitment (reviewed in Blackledge et al., 2015). For a few examples, transcription factors have been shown to recruit polycomb complexes to specific loci in the genome (Figure 1.2) (Arnold et al., 2013; Dietrich et al., 2012; Gao et al., 2012; M. Yu et al., 2012). However, it is unclear whether this is a general targeting mechanism for polycomb complexes. There is also evidence that non-coding RNAs can target PRC2, but whether this mechanism is example-specific or general to this complex is still highly debated (Figure 1.2) (reviewed in Brockdorff, 2013).

In absence of locus-specific targeting mechanisms for polycomb complexes, there has been a lot of research in examining the generic targeting mechanisms for recruitment. Early insight

into a generic mechanism came from the observation that polycomb complexes occupied sites are enriched for CpG islands devoid of activating motifs (Figure 1.2) (Ku et al., 2008; Mikkelsen et al., 2007). This correlative evidence was supported by sufficiency experiment *in vivo* in which an artificial CpG island was able to recruit polycomb complex (Mendenhall et al., 2010). Recent evidence that KDM2B, a component of PRC1, is able to bind non-methylated CpG dinucleotides through its zinc finger CXXC DNA binding domain provides a potential mechanism for recruitment of PRC1 to non-methylated CpG islands (Farcas et al., 2012; He et al., 2013; Wu, Johansen, & Helin, 2013). Notably, KDM2B is part of a variant of non-canonical PRC1 containing PCGF1 (Gao et al., 2012; Kloet et al., 2016), thus providing the first mechanism for recruitment of non-canonical PRC1 to the genome. Similarly, JARID2, a known component of PRC2, has been shown to bind to GC-rich DNA, providing an analogous mechanism for PRC2 targeting and the subsequent recruitment of canonical PRC1 (Figure 1.2; G. Li et al., 2010).

Another mechanism of polycomb targeting has been proposed to be promiscuous binding of PRC2 to RNA (Figure 1.2; Davidovich et al., 2015; Davidovich et al., 2013). This model suggests that PRC2 randomly samples the genome and binds to nascent RNA to increase its local concentration at sites of transcription (Kaneko et al., 2013). PRC2 is then either excluded by the productive transcription machinery and histone marks at transcriptionally active sites, or retained at transcriptionally silent regions with short abortive RNAs (Kanhere et al., 2010; reviewed in Klose et al., 2013). This is consistent with evidence that cessation of transcription precedes PRC2 recruitment and deposition of H3K27me3 marks (Hosogane et al., 2013; Riising et al., 2014). An alternate possibility is that polycomb complexes could be recruited to transcriptionally silent loci via interaction with other silencing marks, as suggested by loss of

PRC2 at target sites upon loss of G9A, a histone methyltransferase for H3K9 (Mozzetta et al., 2014). The PRC2-catalyzed H3K27me3 marks could then recruit PRC1 for the establishment of polycomb domains at these transcriptionally silent loci, which would inhibit further stochastic activation of transcription and enable a locking in of the silenced state.



Adapted from Blackledge et al., *Nature Reviews Molecular Cell Biology*, 2015

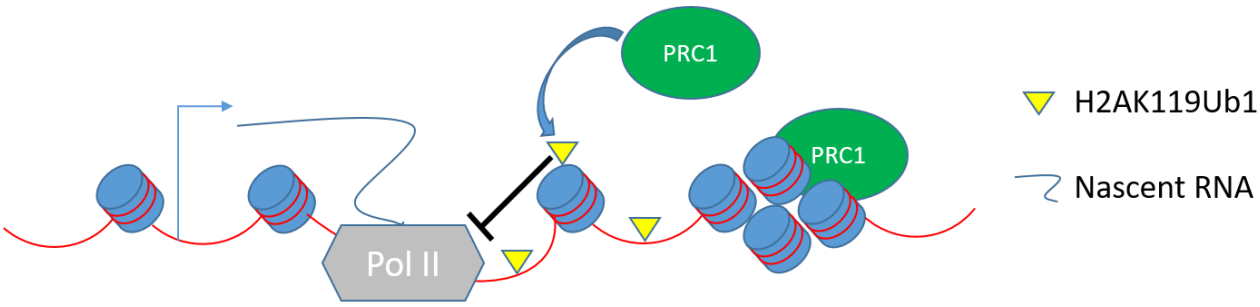
Figure 1.2. Schematic illustrating different modes of recruitment of PRC1 and PRC2 to the chromatin. The top schematic illustrates recruitment of non-canonical PRC1 to non-methylated CpG islands via interaction facilitated by the KDM2B subunit, and subsequent monoubitinylation of H2AK119. PRC2 is recruited either by non-methylated CpG islands via JARID2 or by H2AK119Ub1 via AEBP2/JARID2, leading to subsequent methylation of H3K27. This H3K27me3 mark can further recruit canonical PRC1 via its CBX subunit. The bottom schematic illustrates other modes of PRC2 recruitment, including: other repressive marks on histones, transcription factors, non-coding RNA, or short abortive nascent RNA while being inhibited by productive transcription. After recruitment, PRC2 places the H3K27me3 mark, which subsequently recruits canonical PRC1 to the chromatin.

How PRC1 exerts its gene silencing function

Since PRC1's enzymatic activity to ubiquitinate H2AK119 was first discovered, there has been interest in understanding how this histone mark is coupled with repressive mechanisms. There are some hints that H2AK119ub1 restrains poised polymerase at the promoter (Figure 1.3)

(Stock et al., 2007), however, no direct mechanisms have been elucidated to date (reviewed in Simon & Kingston, 2013). Evidence for the ubiquitin mark having a repressive role comes from studies where enzymatic activities of RING proteins were specifically ablated. In the context of mouse ES cells, mutation of RING protein led to deregulation of many of the PRC1 target genes (Endoh et al., 2012). However, classic PRC1 targets such as *Hox* genes were only partially deregulated suggesting that other mechanisms of repression are in play.

Evidence for another repression mechanism came from the finding that PRC1 is able to compact nucleosomal arrays (Figure 1.3) even in the absence of histone tails, suggesting this is independent of PRC1's enzymatic function (Francis et al., 2004). This compaction is able to resist chromatin remodeling by other chromatin remodelers such as SWI/SNF (King, Francis, & Kingston, 2002). Importantly, subsequent studies showed that this chromatin compaction is able to repress genes such as *Hox* genes *in vivo*, and it remains intact in the context of catalytically inactive RING1B (Eskeland et al., 2010). Instead, chromatin compaction has been attributed to the PHC subunit of PRC1, which can catalyze auto polymerization via its SAM domain (Isono et al., 2013). Accordingly, loss of PHC leads to loss of PRC1 selectively at sites marked by H3K27me3. This leads to a model in which CBX protein mediated recruitment to H3K27me3 and SAM mediated polymerization act together to maintain polycomb domains at silenced genes.



Adapted from Blackledge et al., *Nature Reviews Molecular Cell Biology*, 2015

Figure 1.3. Schematic representing modes of gene repression by PRC1. The monoubiquitination mark placed by PRC1 on H2AK119 can restrain poised polymerases, while PRC1 mediated compaction can block access to the transcriptional machinery.

Our current understanding of polycomb biology can be summarized in the following model. PRC2 can be targeted to specific loci by transcription factors, non-coding RNA, or accumulation of PRC2 at transcriptionally silent loci by random genome sampling. Retention of PRC2 at these loci then results in deposition of methylation marks at H3K27. PRC2 can recognize the H3K27me3 through its EED subunit, which also acts as an allosteric activator of the enzymatic activity of PRC2 (Margueron et al., 2009). This has been proposed to enable spreading of the H3K27me3 mark along the genome and also as a mechanism for maintaining these marks during replication (Hansen et al., 2008). The H3K27me3 marks are also recognized by CBX proteins in canonical PRC1. This leads to recruitment of canonical PRC1, which can then polymerize using its PHC subunit to compact the chromatin and establish the polycomb repressive domain. As an alternate mechanism, the non-canonical PRC1 are recruited to non-methylated CpG islands via its KDM2B subunit. Upon recruitment to chromatin, this complex deposits ubiquitin marks on H2AK119 to establish the repressive domain. Recently a new feedback loop has been elucidated where PRC1 mediated monoubiquititylation of H2AK119 is

able to recruit PRC2 and subsequently deposit H3K27me3 mark (Blackledge et al., 2014; Cooper et al., 2014). This could be driven by a variant of PRC2 containing AEBP2 and JARID2, which has been shown to bind to the H2AK119ub mark *in vitro* (Kalb et al., 2014). Thus, *de novo* recruitment of non-canonical PRC1 could lead to recruitment of PRC2 and subsequent recruitment of canonical PRC1. It is still unclear whether this mechanism is in play *in vivo*.

PRC1's role in self-renewal and differentiation

To understand the functional relevance of the repressive function of PRC1, the major components of PRC1, RING1A and RING1B, were deleted *in vivo* and assessed for phenotype. Both homozygous and heterozygous deletions of RING1A led to anterior transformation and defect in axial skeletal patterning, however, the mice were still viable (del Mar Lorente et al., 2000). This suggests that RING1A is important for late stages of murine development. In contrast, deletion of RING1B/RNF2 resulted in a more severe developmental phenotype that is characterized by gastrulation arrest (Voncken et al., 2003). Notably, this is similar to the phenotype seen in PRC2 deficiency that results from the deletion of EED, EZH2, or SUZ12 (Faust et al., 1998; Faust et al., 1995; O'Carroll et al., 2001; Pasini et al., 2004). In contrast to RING1A, no phenotype is observed upon heterozygous deletion of RING1B. These results tell us that the targets of RING1A and RING1B complexes are not entirely overlapping.

Regardless of phenotype of deletion occurring in the early or late stages of development, RING1A and RING1B are both important to ES cell potency. RING1B deletion in ES cells leads to deregulation of repressed genes that are part of differentiation pathways and organismal development (van der Stoop et al., 2008). These genes exhibit promoters that are bivalent and

also CpG-rich. However, binding studies for RING1B in ES cells revealed that only a small subset of the genes bound by RING1B are derepressed. Moreover, these ES cells maintain the expression of pluripotency regulators such as OCT4 and NANOG. This suggests that additional mechanisms are used to repress most genes that are bound by RING1B. The additional mechanism could very well be other variants of PRC1, because complete loss of PRC1 by deleting both RING1A and RING1B leads to loss of self-renewal of ES cells and eventual differentiation (Endoh et al., 2008). This tells us that even though RING1A KO mice have defects in later stages of development, RING1A still contributes to gene regulation in ES cells.

In addition to the maintenance of self-renewal in ES cells, PRC1 is also important for proper differentiation. Upon differentiation of ES cells, chromatin states and chromatin bound proteins are stabilized leading to more heterochromatin formation and gene silencing (Mattout & Meshorer, 2010; Meshorer et al., 2006). Simultaneously, we also observe upregulation of members of canonical PRC1 such as CBX2, CBX4, CBX8, PHC2, and BMI1 (Kloet et al., 2016; Morey et al., 2012). Since canonical PRC1 lack enzymatic activity and repress genes by mediating chromatin compaction, their upregulation as ES cell programs are downregulated makes sense. Direct evidence that PRC1 subunits MEL18, RYBP and PCGF6 dictate mesodermal lineage specification further confirms the role of PRC1 in proper differentiation (Morey et al., 2015; Ujhelly et al., 2015; Zdzieblo et al., 2014).

Much of the descriptive role of PRC1 in stem cells and differentiation come from studies conducted in ES cells and in utero. Outside of this context, there are very few studies looking at the role of PRC1 in adult stem cells, and most of these delete components of PRC1 prenatally and then assess the effect on adult stem cells in postnatal animals (reviewed in Avgustinova &

Benitah, 2016). It is important to ask whether postnatal tissue lineage restricted stem cells still requires PRC1 for their self-renewal and multipotency maintenance. New insights were revealed when RING1A and RING1B were deleted simultaneously in adult intestinal stem cells (Chiacchiera, Rossi, Jammula, Chiacchiera, et al., 2016). Upon deletion of PRC1, intestinal stem cells were unable to maintain their self-renewal and tissue homeostasis. Mechanistically, deletion of PRC1 led to derepression of many non-intestine lineage-specific transcription factors that negatively affect WNT/b-CATENIN pathway activity, which is essential for intestinal stem cells maintenance (Chiacchiera et al., 2016). This provided new insights into the ability of PRC1 to repress non-lineage genes and provide lineage commitment. Similar insight into PRC1 being important to maintain lineage identity and repress non-lineage genes have been recapitulated in experiments deleting other subunits of PRC1, such as CBX4 (Mardaryev et al., 2016). Notably, most of the remaining studies on PRC1 function in adult stem cells come from deleting Bmi1 in various contexts.

BMI1

Bmi1 (*B-cell-specific Moloney murine leukemia virus integration site 1*) was originally discovered as an oncogene that cooperated with *Myc* in driving B cell and T cell lymphomagenesis (Ygal Haupt et al., 1993; van Lohuizen, Verbeek, et al., 1991). Based on sequence similarity, it was quickly realized that *Bmi1* was a member of the *posterior sex combs (psc)* gene family identified in *Drosophila melanogaster* (Brunk et al., 1991; van Lohuizen et al., 1991). *Psc* is a PRC1 component that is required to appropriately regulate *Hox* genes expression and segmentation patterning in *D. Melanogaster* (Paro, 1990; Struhl & Akam, 1985; Wedeen et al., 1986). This led

to analyses of the role of BMI1 in vertebrate development through the generation of germline *Bmi1* knockout (KO) mice (N. M. van der Lugt et al., 1994). These *Bmi1* KO mice were viable but displayed three major phenotypes: a profound hematopoiesis defect, cerebellar neurological abnormalities, and posterior transformation of the axial skeleton. Mice with overexpression of *Bmi1* were subsequently generated, and these animals demonstrated the converse axial skeletal phenotype; anterior transformation (Alkema et al., 1995). These axial skeleton phenotypes are accounted for by deregulation of *Hox* genes along the strict boundaries required for proper morphogenesis, and BMI1 was shown to be involved in repression of subset of the *Hox* genes (Alkema et al., 1995; N. M. T. van der Lugt et al.,1996).

Bmi1 in stem cells

The focus of *Bmi1* research shifted towards the phenotype in hematopoiesis and neurogenesis in germline KO mice when it was shown that *Bmi1* was highly expressed in the most primitive bone marrow cells and the expression was minimal in mature blood cells (J Lessard et al.,1998). This led to the hypothesis that *Bmi1* is important for maintenance of the hematopoietic and neuronal stem cell compartments in both prenatal and postnatal animals. As a first step in this direction, it was shown that *Bmi1* deficient mouse embryonic fibroblasts (MEFs) and lymphoid cells had increased levels of both *p16* (*Ink4a*) and *p19* (*Arf*) expression (Jacobs et al., 1999). *Ink4a* and *Arf* are two major tumor suppressor genes, expressed from the same locus (*Cdkn2a*), which regulate cellular senescence and apoptosis (Figure 1.4; Ivanchuk et al., 2001; Sharpless & DePinho, 1999; Sharpless & Chin, 2003). INK4A is an inhibitor of cell cycle. It inhibits the binding of CYCLIND to CDK 4/6, which results in mono-phosphorylation of the retinoblastoma protein,

pRB. Mono-phosphorylated pRB can bind to E2F and represses E2F mediated transcription. This leads to G1 phase arrest and senescence. ARF, on the other hand, inhibits MDM2, which would have ubiquitinated TP53 and led to its degradation. Inhibition of MDM2 stabilizes the tumor suppressor TP53, which can induce cell cycle arrest, senescence, or apoptosis (Ivanchuk et al., 2001; Sharpless & DePinho, 1999). Importantly, loss of the *Ink4a/Arf* locus *in vivo* was able to largely rescue the *Bmi1* KO hematopoietic and neurological phenotypes, again confirming that BMI1 plays a critical role in repressing *Ink4a/Arf*, and controlling cell cycle, senescence, and cell survival (Jacobs, Kieboom, et al., 1999).

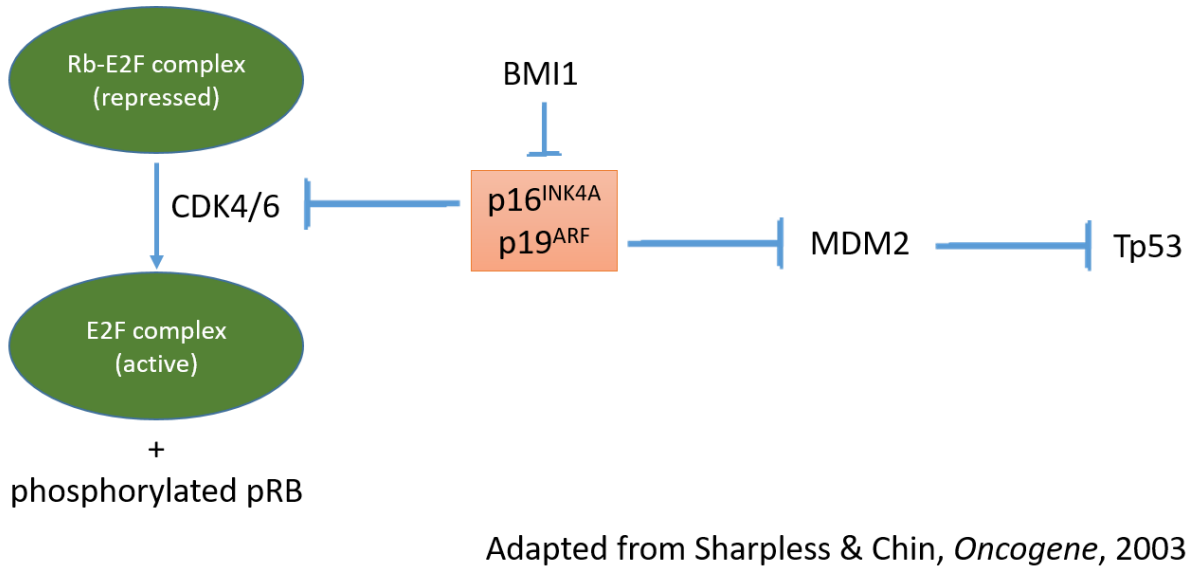


Figure 1.4. Schematic illustrating the BMI1 and INK4A/ARF axis. BMI1 represses two potent tumor suppressors INK4A and ARF that are expressed from the same locus. INK4A is an inhibitor of CDK4/6, which is required for phosphorylation of pRB and subsequent activation of E2F complex. ARF is an inhibitor of MDM2, which ubiquitinates TP53 for degradation.

Following the stem cell hypothesis, further research was conducted in the hematopoietic and neuronal lineages, because this is where the defects were observed in *Bmi1* KO animals. *Bmi1* KO animals were found to have normal number of hematopoietic stem cells (HSCs) in the fetal

liver but reduced HSC numbers in postnatal animals (Park et al., 2003). This suggests that the hematopoietic phenotype is not because of problem of lineage specification early in development, but rather after HSCs have been established in the fetal liver. Transplant experiments demonstrated that both fetal liver HSCs and bone marrow HSCs from *Bmi1* KO mice were only transiently able to contribute to hematopoiesis, indicating a defect in self-renewal. Indeed, *Bmi1* KO HSCs are impaired in self-renewal and proliferation and have increased levels of *Ink4a/Arf* expression (Julie Lessard & Sauvageau, 2003; Park et al., 2003).

The first description of *Bmi1* KO mice and its hematopoietic phenotype had noted that *Bmi1* KO mice had decreased number of hematopoietic cells in bone marrow, and the vacant space was filled by adipocytes. Thus, it was possible that the hematopoietic phenotype could be cell autonomous or microenvironment dependent (N. M. van der Lugt et al., 1994). Subsequent studies showed that both are true. Specifically, loss of *Ink4a/Arf* rescued the HSC self-renewal phenotype in *Bmi1* KO mice, but the bone marrow microenvironment remained impaired and it resulted in sustained depletion of HSCs in postnatal animals (Oguro et al., 2006). Accordingly, the bone marrow of *Bmi1* KO recipients could not support transplanted *Bmi1* WT HSCs (Oguro et al., 2006). Conversely, overexpression of *Bmi1* in mice caused HSCs to have enhanced self-renewal ability, reinforcing the role of *Bmi1* in maintaining self-renewal capability in stem cells (Iwama et al., 2004; A. Rizo et al., 2008).

Similar to the role of *Bmi1* in HSCs, *Bmi1* is also important in neural stem cells. *Bmi1* has been shown to be important for self-renewal capability but not the survival or differentiation of neural stem cells (Molofsky et al., 2003). The reduced self-renewal capability upon *Bmi1* loss is also largely accounted for by increased expression of *Ink4a/Arf*, as loss of *Ink4a* partially

rescues the phenotype (Bruggeman et al., 2005; Molofsky et al., 2005). This phenotype is restricted to neural stem cells, as neuronal progenitor cells do not exhibit a phenotype upon *Bmi1* loss (Molofsky et al., 2003). The role of *Bmi1* is also conserved in epithelial tissues. *Bmi1* KO mice are able to undergo normal lung development, but putative bronchioalveolar stem cells (BASCs) have impaired self-renewal capacity and proliferation *in vitro*, and post injury *in vivo* (Dovey et al., 2008). This phenotype is also partially rescued by loss of *Arf* (Dovey et al., 2008). Similarly, *Bmi1* KO mice have a mammary epithelium growth defect that is rescued upon loss of *Ink4a/Arf* (Pietersen et al., 2008). Other examples of *Bmi1* KO phenotype in stem cells include a self-renewal defect in satellite cells, which are the resident stem cells of skeletal muscle (Robson et al., 2011). Upon *Bmi1* loss, the stem cell pool decreases and progenitor cells pool increases. The remaining stem cells also enter a pre-senescence state and are unable to proliferate even when stimulated by high serum *in vitro*. This is reminiscent of the phenotype seen in stem cells of geriatric mice, which is caused by de-repression of *Ink4a* (Sousa-Victor et al., 2014).

Even though *Bmi1* is important for self-renewal of stem cells in multiple tissue types, its expression is not restricted to just the stem cells. In mammary glands, *Bmi1* is expressed in all cells, with the expression highest in luminal cells (Pietersen et al., 2008). Similarly, *Bmi1* is expressed in both basal and non-proliferative suprabasal cells in the epidermis (K. Lee et al., 2008). In the intestine, *Bmi1*'s expression is present in both the stem cells at the bottom of the crypt and most of the transient amplifying cells (Itzkovitz et al., 2011). The first hint consistent with this expanded expression was the finding that *Bmi1* is important for subsequent fate specification. In HSCs and multipotent progenitors (MPPs), genes that regulate B cell lineage

development, such as *Ebf1* and *Pax5*, are repressed by bivalent domains. Loss of *Bmi1* results in activation of these genes and aberrant lymphoid specification, resulting in increased B cell differentiation (Oguro et al., 2010). Since *Bmi1* is important for maintenance of bivalent domains controlling lineage specification, it is plausible that *Bmi1* needs to stay expressed not only in stem cells, but also in multipotent or bipotent progenitors. Similar phenotypes are also seen in the intestinal tissue where loss of *Bmi1* perturbs the normal balance of absorptive and secretory lineages (Lopez-Arribillaga et al., 2015; Maynard & Lees, unpublished observation).

The role of *Bmi1* in repressing *Ebf1* and *Pax5* also establishes a role for *Bmi1* beyond repression of the *Ink4a/Arf* locus. It was noted early on that even though deletion of *Ink4a/Arf* rescued some of the phenotypes of germline *Bmi1* KO mice, the double KO mice were still smaller in size than WT mice (Jacobs *et al*., 1999) and the early lethality phenotype of *Bmi1* loss remains (Molofsky *et al*., 2005). Careful examination of the neurological and hematopoietic phenotype showed that loss of *Ink4a/Arf* yielded only partially rescue, even though the self-renewal phenotype of stem cells were almost fully rescued *in vitro* (Molofsky *et al*., 2003; Oguro et al., 2006). This is at least partially explained by the role of *Bmi1* in fate specification: specifically, transplant of *Bmi1* and *Ink4a/Arf* double KO bone marrow cells into WT recipient mice showed that these cells still retained the *Bmi1* KO differentiation defect of early lymphoid specification and increased differentiation towards B cell lineage (Oguro et al., 2010). These observations establish that *Bmi1* has additional targets beyond *Ink4a/Arf* that are also important for maintenance of self-renewal capability in stem cells.

Bmi1 in cancer

Since *Bmi1*'s discovery through its ability to cooperate with *Myc* in inducing lymphomagenesis, its role has been studied extensively in the context of cancer. *Bmi1*'s oncogenic potential was first identified in mice with forced expression of *Bmi1* in lymphoid compartment, which resulted in mostly T-cell lymphoma (Y Haupt *et al*., 1993). Combining *Bmi1* and *Myc* transgenes resulted in pre-B and B cell lymphoma (Y Haupt *et al*., 1993). Mutational analysis of *Bmi1* showed that the N terminal RING domain and central portion of BMI1 protein is essential for the lymphomagenesis phenotype, while the C terminal region is dispensable (Alkema *et al*., 1997). This result is clearer under the light shed by structural studies on BMI1-RING1B complex, where BMI1 and RING1B interact using the RING domain, and the central part of BMI1 loops around RING1B to hug it (Buchwald et al., 2006; Li et al., 2006). In contrast, the C terminal region of BMI1 acts as a negative regulatory domain, and thus its deletion causes BMI1 to have increased oncogenic function (Yadav *et al*., 2010). The function of *Bmi1* as an oncogene and a cooperating partner in *Myc*-driven lymphomagenesis comes primarily from its ability to repress the *Ink4a/Arf* locus and block *Myc*-induced apoptosis (Jacobs, Scheijen, *et al*., 1999a). In the context of MEFs, expression of *Myc* also results in upregulation of *Arf* and leads to apoptosis, which is inhibited by *Bmi1* (Jacobs, Scheijen, *et al*., 1999a). This first description of oncogenic role of *Bmi1* by repressing *Ink4a/Arf* locus shaped the next decade of research on *Bmi1*'s function in cancer.

Identification of *Bmi1* as an oncogene that represses two very important tumor suppressor genes, *Ink4a* and *Arf*, led to the hypothesis that cancer cells could employ upregulation or

amplification of *Bmi1* as a mechanism to repress *Ink4a/Arf*. This led to the hunt for expression patterns and amplification of *BMI1* in human cancers. Since the field was focused on *Bmi1*'s role in lymphomagenesis until that point, it made sense for researchers to look at human hematological malignancies. Early research showed that *BMI1* locus amplification is present, but not common, in human mantle cell lymphoma and non-Hodgkin lymphoma (Beà et al., 2001). However, some hematopoietic malignancies still upregulate BMI1 expression regardless of amplification status. Contrary to results in mice, BMI1 and INK4A/ARF expression levels did not show a simple inverse relationship in human cancer (Beà et al., 2001). Despite this finding, the expression levels of BMI1 correlated with an increase in grade of the malignancy, at least in the case of B-cell non-Hodgkin lymphomas (van Kemenade et al., 2001). The expression of BMI1 also correlated with KI67 expression, which is a marker of proliferating cells. This was even more impressive given that normal dividing B-cells do not express BMI1 (van Kemenade et al., 2001).

In addition to lymphomas, increased levels of BMI1 in human tumor samples compared to normal tissue of same origin has been shown in multiple solid tumors including medulloblastomas (Leung *et al*., 2004), gastrointestinal tumors, pituitary and parathyroid adenomas (Sánchez-Beato *et al*., 2006), chronic myeloid leukemia (CML) (Mohty *et al*., 2007), neuroblastoma (Nowak et al., 2006), and many other tumor types (reviewed in Sauvageau & Sauvageau, 2010). Consistent with previously described literature, BMI1 was also found to be higher in advanced cancer, as in the case of CML, where advanced-phase patients had higher expression of BMI1 than chronic-phase patients (Mohty et al., 2007). As expected from increased expression in advanced cancer, *BMI1* mRNA level was also found to be a marker of

poor prognosis in various tumors types such as CML (Mohty et al., 2007), bladder cancer (Qin et al., 2009), pediatric brain cancer (Farivar et al., 2013), esophageal squamous cell carcinoma (Hwang et al., 2014), and many others.

It was inferred that since BMI1 expression was higher in cancer compared to normal tissues, and this increase correlated with increased grade, *BMI1* must be contributing to cancer progression. However, it is also possible that *BMI1* is a tumor suppressor that cancer cells upregulate with increased oncogenic insult to try and mitigate the damage. This alternative hypothesis is similar to the observation in murine model of non-small cell lung cancer (NSCLC) that tumor suppressive *Ink4a/Arf* expression is upregulated during the transition from adenoma to adenocarcinoma (Feldser et al., 2010). The role of *BMI1/Bmi1* has been addressed using human and murine cancer cell lines, and murine models. Early studies showed that *Bmi1* is important for proliferation and survival of leukemia cells in murine models (Julie Lessard & Sauvageau, 2003). This result has been reiterated in numerous other cancer cell lines including glioma (Godlewski et al., 2008), gastric carcinoma (Li et al., 2010), multiple myeloma (Jagani et al., 2010), cervical cancer (Chen et al., 2011), breast cancer (Xu et al., 2011), Ewing sarcoma (Hsu & Lawlor, 2011) and many more. Genetically engineered mouse models (GEMMs) also support the notion that *Bmi1* is oncogenic. In a hepatocellular carcinoma model, forced expression of *Bmi1* in purified hepatic stem/progenitor cells was sufficient to initiate tumorigenesis when these cells were transplanted in mice (Chiba et al., 2007). In a melanoma model, *Bmi1* had no effect on the primary tumor but it enhanced dissemination of tumor cells and metastatic growth (Ferretti et al., 2016). This is consistent with the observation in human cancers that *Bmi1*'s expression increases with tumor progression. Conversely, germline deletion

of *Bmi1* impaired the dissemination of tumor cells in the melanoma model (Ferretti et al., 2016). Importantly, these tumor suppressive effects of *Bmi1* deletion in germline or tissue specifically during development has been observed in other GEMMs of cancer as well. In murine pancreatic cancer models, *Bmi1* is required for initiation of neoplasia (Bednar et al., 2015). In the context of *Apc* loss driven murine intestinal cancer models, *Bmi1* KO animals had lower tumor numbers and burden compared to WT animals. This phenotype was dependent upon *Bmi1*'s function to repress *Arf*, as combining *Arf* loss in the intestinal cancer model partially rescued the *Bmi1* KO phenotype (Maynard et al., 2013).

Given *Bmi*1's positive role in maintaining self-renewal potential in stem cells and *Bmi1*'s positive role in cancer, both via *Ink4a/Arf* locus repression, it was not long before researchers started to focus on *Bmi1*'s role in TPCs. Given that the first description of TPCs was in leukemia (Bonnet & Dick, 1997), the first studies were conducted in leukemia tumor propagating cells (L-TPCs). *Bmi1* KO in L-TPCs arrested proliferation and yielded signs of apoptosis, and the L-TPCs were unable to maintain their undifferentiated state or transplant potential (Julie Lessard & Sauvageau, 2003). As with the *Bmi1* deficient HSCs, the L-TPCs also showed upregulation of *Ink4a/Arf* (Aleksandra Rizo et al., 2009). Examination of the role of *Bmi1* during reprogramming of normal cells to L-TPCs also showed its importance at early differentiation stages (Yuan et al., 2011). *Bmi1* KO granulocyte/macrophage progenitors (GMPs) could be transformed by introduction of oncogenes but fewer L-TPCs were retained and these showed increased differentiation. Moreover, when transplanted into recipient mice, these cells could not establish leukemia. Interestingly, this phenotype was only partially rescued upon *Ink4a/Arf* loss, and it revealed derepression of lineage inappropriate transcription factors including *Tbx15*

(Yuan et al., 2011). Moreover, reintroduction of *Bmi1* could not rescue the phenotype, indicating that the effect of *Bmi1* loss is permanent, most likely pushing the cells to a more differentiated state from which L-TPCs cannot arise (Yuan et al., 2011).

Bmi1's role in TPCs have also been established in solid cancers. One such example is human colorectal cancer. The standard assay of TPC frequency is limiting dilution *in vivo*, where cells are transplanted at various doses into mice and the frequency of tumor development is measured. Using this assay, knockdown of *BMI1* was shown to decrease TPC frequency in both cancer cell lines and primary tumor samples from patients (Kreso et al., 2014). Similarly, in a murine model of head and neck squamous cell carcinoma (HNSCC), *Bmi1* positive tumor cells were shown to have higher enrichment for TPCs compared to *Bmi1* negative tumor cells in a limited dilution assay (D. Chen et al., 2017). These *Bmi1* positive tumor cells were also enriched in the tumor upon treatment of cisplatin, suggesting that they are more chemoresistant, which is one of the hallmarks of TPCs.

Even though most of the literature is consistent with the role of *Bmi1* in tumor promotion through its ability to repress *Ink4a/Arf* locus, there is evidence for alternate mechanisms. In a murine model of pancreatic cancer, loss of *Bmi1* is tumor suppressive. However, this phenotype is independent of *Ink4a/Arf* and the data point to reactive oxygen species dysregulation instead (Bednar et al., 2015). Similarly, *Bmi1* deficient immortalized GMPs have low replating efficiency in colony forming assays that is independent of *Ink4a/Arf* status (Yuan et al., 2011). This phenotype is phenocopied by overexpression of *Tbx15*, a lineage inappropriate transcription factor that is upregulated upon loss of *Bmi1* (Yuan et al., 2011). This suggests that *Bmi1*'s role of

repressing lineage specifying transcription factors is also important for TPC potential, similar to repression of *Ebf1* and *Pax5* being important for HSC function (Oguro et al., 2010).

Lung adenocarcinoma (LUAD)

LUAD in patients

According to the Cancer Facts & Figures published in 2019, lung and bronchus cancer is the second leading cause of estimated new cancer cases, and the first leading cause of estimated cancer deaths, in the United States. Even though lung and bronchus cancer accounts for only 12.9% of total newly diagnosed cancer cases, it accounts for 23.5% of total estimated cancer deaths, making it a huge unmet medical need (Siegel et al., 2019). According to the National Cancer Institute's Surveillance, Epidemiology, and End Results Program (SEER), the five-year survival rate for lung and bronchus cancer is 19.4%. This survival rate is very dependent on the stage at which the disease was diagnosed; if at the localized tumor stage, the 5-year survival rate is 57.4%, but diagnosis at the distant metastasis stage, which is true for more than half of lung cancer patients, decreases the 5-year survival rate to a meager 5.2% (SEER, 2019).

Even though the national statistics lump lung and bronchus cancer into one category, lung cancer is a collection of various different malignancies (Chen et al., 2014; Reck et al., 2013). Based on histological differences, lung cancer can be broadly classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Even within NSCLC, there are various subtypes, with adenocarcinoma and squamous cell carcinoma predominating, and a small fraction of NSCLC being categorized as large-cell carcinoma or other miscellaneous categories (Howlader et al., 2019). Sequencing analyses of large numbers of adenocarcinomas and

squamous cell carcinomas showed that the recurring mutations, amplifications, and deletions differ greatly between the two subtypes, reinforcing the histological classifications as different malignancies (Campbell et al., 2016).

Lung adenocarcinoma (LUAD) arises mostly in distal lung and is associated with the marker NKX2.1/TTF1. It is characterized by glandular histology and/or the presence of mucin (Davidson et al., 2013). IASLC/ATS/ERS classification of adenocarcinoma, based on invasiveness, breaks down the adenocarcinoma subtype into adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIC), and invasive adenocarcinoma, with the first two having effectively 100% 5-year survival rate post resection. Invasive adenocarcinoma is further subdivided based on histological patterns, such as lepidic, acinar, papillary, micropapillary, and solid (Travis et al., 2011). This is important because certain histological features, such as micropapillary, are associated with poor prognosis (Travis, 2011).

In recent years, a lot of effort has been placed on understanding the molecular drivers of LUAD and in using the information about genetic alterations to inform therapeutic approaches. Comprehensive profiling from The Cancer Genome Atlas (CGA) Research Network has provided extensive insights in that regard (Collisson et al., 2014). *TP53* has been identified as the most mutated gene (46%), closely followed by *KRAS* (33%). Interestingly, *EGFR* mutations in 14% of patients were mutually exclusive to *KRAS* mutations, suggesting that there is a strong selective pressure to activate the EGFR-RAS pathway in LUAD. Identification of *EGFR* mutations led to the use of *EGFR* inhibitors in lung cancer. Other targeted therapies approved for LUAD target alterations in BRAF and ALK. The CGA study also show that LUAD has a relatively high mutational burden of 8.87 mutations per megabase of DNA. Consequently, LUAD is rife with

neoepitopes, some of which are recurrent among patients (Campbell et al., 2016). Consequently, there is great interest in the use of immune checkpoint inhibitors in LUAD, and to date four anti-PD1/PD-L1 antibodies have received FDA approval for treatment of LUAD (CRI, 2019).

Genetically engineered mouse models (GEMMs) of LUAD

With the detection of vast array of genetic alterations in human LUAD comes the challenge of identifying driver mutations in cancer versus passenger mutations that have little functional consequences. This is particularly challenging for patients with smoking history who have an especially high mutational burden. Researchers have relied on preclinical models to validate putative driver mutations and also assess the therapeutic efficacy of targeting these alterations. GEMMs have been widely used as relevant preclinical model of cancer. GEMMs allow for generation of autochthonous tumors that arise and progress in their own native microenvironment, thus, recapitulating a more complete picture of tumor biology. Conditional alleles and site-specific recombinase systems such as Cre-loxP system and Flp-frt system made possible GEMMs in which candidate driver genes are expressed at the endogenous level in any cancer type (reviewed in Branda & Dymecki, 2004). These systems allow for whole body or tissue specific deletion or activation of any gene of interest, by either flanking exons with recognition sites for the recombinase, or inserting flanked STOP cassettes that can be removed by the recombinase, respectively. Newer generation versions of these technologies include the ability to temporally control the deletion or activation of any gene by allowing a small molecule to control recombinase activity. The most notable example is the CreERT2 system, where the

Cre recombinase nuclear import and activity is controlled by 4-hydroxy-tamoxifen (reviewed in Branda & Dymecki, 2004). Coupling different recombinases and inducible systems can allow for temporal and spatial control over genetic alterations. For instance, tumor initiation can be triggered by one recombinase system, while deletion of another gene of interest in established tumors can subsequently be triggered by a second inducible recombinase system.

In the field of LUAD, multiple GEMMs have been established that use various recurring mutations in patient samples as the driver mutations. One of the most commonly used mutations to model LUAD is the *Kras* mutation. The first model to recapitulate the endogenous level of expression of mutated *Kras* was a GEMM with a latent *Kras* allele that activates via a spontaneous recombination event and predominantly gives rise to lung cancer (Johnson et al., 2001). This quickly gave way to GEMMs with an inducible allele of *Kras* carrying a G12D mutation, which is the most frequent *KRAS* mutation seen in patients (Jackson et al., 2001; Meuwissen et al., 2001). In this model, expression of this *Kras-G12D* allele is prevented by the presence of a STOP cassette, which can be excised at the flanking loxP sites using the Cre recombinase. The Cre recombinase can be easily introduced using intratracheal delivery of adenoviral or lentiviral particles encoding for Cre recombinase (DuPage et al., 2009). Cre mediated excision of the STOP cassette leads to activation of the *Kras-G12D* allele and a subset of the affected cells give rise to mostly adenoma, which can progress at low frequency and with long latency to adenocarcinoma. This model is made more aggressive by combining mutant *Tp53* alleles with the *Kras* mutation (Jackson et al., 2005), both of which are relevant mutations in patients (Collisson et al., 2014). The resulting *Kras* and *Tp53* mutant (KP) tumors progress much faster than *Kras* (K) only tumors, and recapitulate advanced human LUAD. Tumors of all

grades and lymph node metastases are seen in the majority of animals, with few animals progressing to distant metastases.

The development of GEMMs that recapitulate advanced human LUAD faithfully created the ability to study the various stages of tumor progression, and to dissect pathways important for tumor development, progression, and dissemination. One early insight from the KP GEMM was that, even though tumorigenesis is driven by mutant *Kras*, there is still amplification in MAPK signaling during the adenoma to adenocarcinoma transition (Feldser et al., 2010). This is consistent with the previous findings that activation of *Kras-G12D* alone in different epithelial tissue types leads to proliferation but not detectable phosphorylated ERK (Tuveson et al., 2004), and that K only GEMMs rarely progress to adenocarcinoma (Jackson et al., 2001; Meuwissen et al., 2001). Moreover, activation of the ARF/TP53 pathway and downstream cell cycle arrest or apoptosis is limited to the amplified MAPK signaling setting (Feldser et al., 2010).

KP GEMMs have also been vital in describing changes associated with metastases. Using an extracellular matrix (ECM) array, researchers identified a panel of ECM molecules (Fibronectin, Laminin, Galectin-3, and Galectin-8) that are enriched in metastases but not present in primary tumors (Reticker-Flynn et al., 2012). This study also identified SPP1/osteopontin as the ECM molecule that is upregulated in high grade tumor cells and metastases (Reticker-Flynn et al., 2012). Another key insight from KP GEMM of LUAD has been the identification of markers for TPCs. Careful orthotopic serial transplantation experiments have revealed markers such as SCA1, CD24, NOTCH, and ITGB4 as key markers that enrich for functional TPCs (Curtis et al., 2010; Lau et al., 2014; Y. Zheng et al., 2013). These insights have added to the repertoire of functional markers and therapeutic target candidates for LUAD.

To understand the genetic alterations important for tumor progression, with the hope of expanding on the mutational analysis from patient samples, a lot of effort was put into characterizing the mutational profile of KP LUAD (McFadden et al., 2016). However, to the researchers' surprise, the mutational burden in murine KP primary tumors and cell lines was much lower than that seen in patient samples. This highlights one of the limitations of the GEMM to model LUAD: there is less pressure on tumor cells to acquire and select for advantageous mutations, since the tumorigenesis is driven by a powerful oncogene and loss of a major tumor suppressor. Perhaps unsurprisingly, KP LUAD have little to no lymphocyte infiltration, possibly owing to the low mutational burden and lack of neoantigens (Cheung et al., 2008; DuPage et al., 2011). This highlights another major limitation of the GEMM.

Dedifferentiation as a route to KP LUAD progression

Since not all tumors in the KP GEMM LUAD model acquire the ability to metastasize, this provides the perfect opportunity to assess differences in malignant and non-malignant tumor that otherwise have the same driver mutations. Researchers were able to identify NKX2.1/*TFF-1* as a suppressor of malignant progression in this model (Winslow et al., 2011). This is consistent with the previous finding that *NKX2.1* expression correlates with better prognosis in lung cancer patients (Berghmans et al., 2006). However, this finding was somewhat controversial, because frequent amplification of *NKX2*.1 is seen in patient samples, and oncogenic functions have been established for *Nkx2*.1 in cell lines (Kendall et al., 2007; Kwei et al., 2008). Results from KP GEMM clearly established that *Nkx2.1* controls the differentiation status and metastatic potential of lung cancer cells. *Nkx2.1* does this is, in part, by repressing the chromatin regulator,

HMGA2, which is normally restricted to the embryonic lineage. HMGA2 has been previously shown to be a marker of poor prognosis, and *in vitro* data suggests a pro-tumorigenic role (Y. S. Lee & Dutta, 2007; Sarhadi et al., 2006). Collectively, these data suggest that LUAD progresses from NKX2.1 positive to a NKX2.1 negative and HMGA2 positive state. By extension of this logic, perhaps the presence of *Nkx2*.1 amplification in human tumors reflects the fact that it promotes the early stages, but not progression, of human LUAD.

Further insights into the role of *Nkx2.1* in LUAD came from the study where *Nkx2.1* was deleted in KP tumors (Snyder et al., 2013). This led to expression of a latent gastric transcriptional program that is marked by expression of HNF4a, ONECUT2 and other gut lineage genes (Snyder et al., 2013). This latent gastric lineage is repressed by NKX2.1 through sequestration of FOXA1/2 and AP1 transcription factors at pulmonary genes (Maeda et al., 2012; Snyder et al., 2013). Loss of HNF4a on top of NKX2.1 loss leads to upregulation of HMGA2, suggesting a linear pathway for tumor progression in the KP LUAD model. Additional factors, such as FOXA2 and CDX2, have also been found to cooperate with NKX2.1 in inhibiting malignant progression of LUAD, further revealing regulatory nodes involved in metastasis (Li et al., 2015).

The KP LUAD model appropriately recapitulates different stages of LUAD progression, without the need for additional driver mutations to drive those transitions. This strongly suggests that epigenetic changes are responsible for promoting tumor progression. The NKX2.1/HMGA2 axis, combined with previous studies showing a correlation between dedifferentiated status and worse survival in lung cancer patients, raises the possibility that epigenetic changes might enable acquisition of a more dedifferentiated status as a mechanism of tumor progression (Liu, Kho, Kohane, & Sun, 2006). This is also consistent with the presence of recurring mutations in

epigenetic modulators in human LUAD patients (Collisson et al., 2014). Thus, this LUAD. model provides a perfect setting to examine the role of the *Bmi1* epigenetic regulator in tumor development and progression.

Bmi1 in lung cancer

The correlation of *Bmi1* expression levels and NSCLC prognosis has been studied since 2001. First, in NSCLC mostly of squamous subtype, *BMI1* mRNA levels in patient tumors was shown to inversely correlate with *Ink4a/Arf* levels (Vonlanthen et al., 2001). Subsequent independent studies, including a meta study, showed that high *BMI1* mRNA levels in LUAD is correlated with worse overall survival, larger tumor size, poor differentiation, and distant metastasis (Meng et al., 2012; Zhang et al., 2017; Zhang et al., 2014). To assess the putative oncogenic role of *BMI1* *in vitro*, multiple studies assessed the phenotypic effect of shRNA mediated knockdown of *BMI1* in human cell lines, A549 (*Ink4a* mutant) and SPC-A1 (*Ink4a* WT), and found that *BMI1* knockdown was tumor suppressive regardless of the *Ink4a* status (Meng et al., 2012; Q. Yu et al., 2007; X. Zheng et al., 2014). Potential mechanisms for this tumor suppression were touted to be downregulation of AKT pathway, upregulation of cell cycle inhibitors p21/p27, and/or cell cycle arrest before the S phase.

The importance of *Bmi1* in lung cancer has been recapitulated in GEMMs of LUAD driven by different genetic alterations. In a study conducted in our lab and others, *Bmi1* deficiency was shown to be tumor suppressive by launching *Kras* driven tumors in germline *Bmi1* KO animals (Figure 1.5; Dovey et al., 2008). The *Bmi1* KO animals had both reduced tumor number and impaired progression compared to the WT controls, suggesting a role for *Bmi1* at tumor

initiation. This phenotype could be partially rescued upon loss of *Arf*, corroborating previous conclusions that *Bmi1* imparts its oncogenic function through repression of *Ink4a/Arf* locus (Jacobs et al., 1999b). Notably, the tumor suppressive phenotype of *Bmi1* KO also correlates with bronchioalveolar stem cells (BASCs) exhaustion, in either the presence or absence of oncogenic stimuli. This phenotype is partially rescued by deletion of *Arf*, or deletion of *p57* (Dovey et al., 2008; Zacharek et al., 2011). A similar tumor suppressive effect of *Bmi1* KO was also observed in a LUAD model driven by loss of *Cebpa* (Yong et al., 2016), and C-RAF driven model of NSCLC (Becker et al., 2009), suggesting a driver mutation agnostic function of *Bmi1*. These findings fit with the prevailing view that *Bmi1* is required to enable stem cells and TPCs function, primarily via suppression of *Ink4a/Arf*.

Since BMI1 has been implicated in repression of *Ink4a/Arf* locus during development *in utero*, it is possible that the central role of *Ink4a/Arf* in these *Bmi1* deficient GEMM reflects the lack of proper silencing of *Ink4a/Arf* locus during development, as shown by high expression levels of *Ink4a/Arf* in *Bmi1* deficient postnatal stem cells compared to WT controls (Dovey et al., 2008). To confirm that *Bmi1* deletion can be a good therapeutic approach, it is essential to assess this in animals that have developed in normal *Bmi1* WT condition with appropriate *Ink4a/Arf* silencing.

Previous work from our lab tested the consequences of tumor specific deletion of *Bmi1* at the time of tumor initiation in adult animals in both K only, and KP, LUAD models (Figure 1.5; Karl et al., unpublished data). These studies described a profound tumor suppressive phenotype in early stage tumors that resulted in significant extension of survival in both models upon deletion of *Bmi1*. Notably, *Bmi1* was not required for tumor initiation, as both *Bmi1* KO and WT

mice had similar number of tumors. In contrast, the tumors originating in *Bmi1* KO state were significantly smaller compared to WT tumors and this difference was driven by slower proliferation rate in *Bmi1* KO tumors. Interestingly, this proliferation defect was evident at the grade 2 stage but not at grade 3, suggesting that tumor cells eventually adapt to *Bmi1* loss. Of note, there was no evidence of differential *Ink4a/Arf* expression between *Bmi1* KO and WT tumors, antithetical to previous literature.

Gene expression analysis of individual tumors revealed a cell cycle defect in grade 2 *Bmi1* KO tumors but not grade 3, consistent with the observed grade-specific proliferation defects. In addition, *Bmi1* KO tumors also had upregulation of lineage inappropriate genes irrespective of grade, consistent with previously identified function of *Bmi1* to repress non-lineage genes (Yuan et al., 2011). Some of the *Bmi1* KO tumors were positive for HMGA2, a marker of tumor progression in the KP LUAD model (Snyder et al., 2013; Winslow et al., 2011). Surprisingly, staining of serial sections of tissue with NKX2.1 revealed that some of these HMGA2 expressing tumor cells were also positive for NKX2.1. Since these two markers are mutually exclusive with each other during tumor progression, the double positivity of these tumor cells suggested that this might result from alterations in gene reguation upon *Bmi1* loss rather than being a sign of tumor progression. However, since HMGA2 positive tumors accounted for a small fraction of total tumors, this could not account for the apparent adaptation phenotype we observe. This study was unable to establish the mechanisms that underlie the tumor suppressive phenotype upon *Bmi1* loss, or the subsequent adaptation to *Bmi1* loss.

Remaining questions

Since most cancer patients are diagnosed when the cancer is already aggressive, assessing the impact of *Bmi1* deficiency on KP LUAD by deleting *Bmi1* in either the germline or at the time of initiation of tumor does not mimic the clinical treatment paradigm. This provides strong rationale for assessing the role of *Bmi1* in already established, aggressive tumors. Additionally, there is evidence in the literature that BMI1 expression is high in the early stages of primary tumors, but is downregulated in matched lymph node metastases (Xiong et al., 2015), suggesting that *Bmi1* may play different roles during tumor initiation versus tumor progression. Since most of the studies in GEMMs has focused on *Bmi1*'s role during tumor initiation, we feel there is a huge need to address its role in existing tumors.

In this thesis, I address the role of *Bmi1* during tumor progression in KP LUAD, where the tumor bearing animals have developed *in utero* with normal *Bmi1* expression, and the tumors progressed through early stages of tumorigenesis with normal *Bmi1* expression as well (Figure 1.5). My data show that once the tumors reach higher grades, *Bmi1* functions as a tumor suppressor by limiting the dedifferentiation of tumor cells, which is necessary for malignant progression. Thus, *Bmi1* plays a context dependent role in tumorigenesis, and targeting *Bmi1* in LUAD might have an unwanted effect in aggressive cancer.

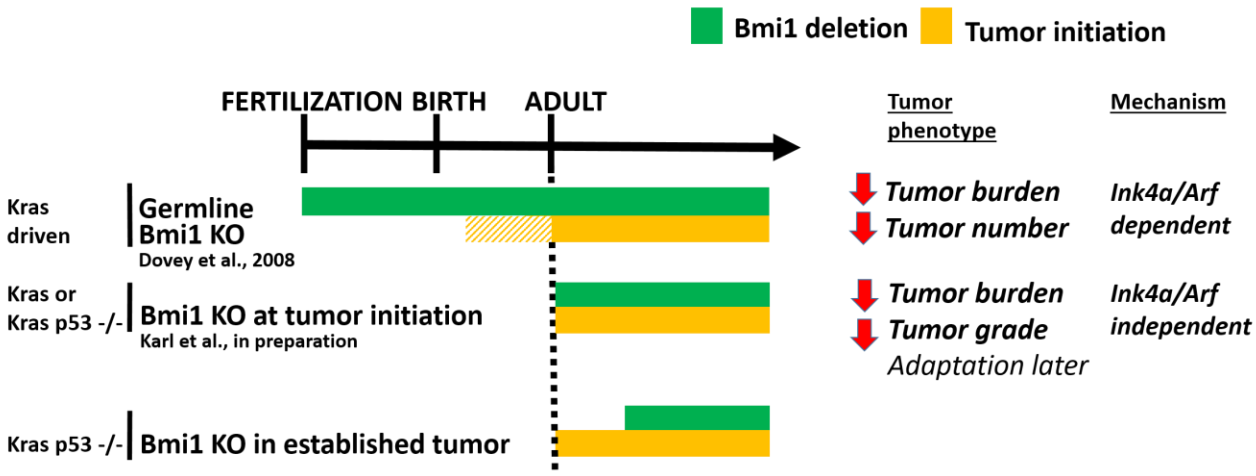


Figure 1.5. Schematic summarizing the work done in our lab to assess the impact of Bmi1 deletion in GEMMs of LUAD. Deletion of Bmi1 in germline leads to development of animal in utero in the absence of BMI1. Launching Kras driven LUAD in this context shows that Bmi1 deficiency causes tumor suppression, which is mediated by Ink4a/Arf derepression. In adult animals that developed with normal Bmi1 levels, deletion of Bmi1 at the time of tumor initiation causes tumor suppression of early tumor grades without differential expression of Ink4a/Arf. This tumor suppressive phenotype subsides by the time tumors reach grade 3. Our current model assesses the impact of Bmi1 deletion in established high grade KP LUAD tumors.

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CHAPTER 2: BMI1 ACTS AS A TUMOR SUPPRESSOR IN HIGH GRADE LUNG ADENOCARCINOMA

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I primarily drove the science discussed in this thesis and performed majority of the experimental design, animal husbandry, animal breeding, sample prep and collection, immunohistochemistry (IHC), quantification of histology slides and IHC, and downstream analysis from RNA sequencing experiments. Paul S Danielian helped with some of the animal breeding and sample collection. Elaine YS Kuo and Lynn Liu helped with some of the animal husbandry and immunohistochemistry. Peter Westcott performed the grading of HnE slides using Aiforia software. Charlie Whittaker performed DeSeq2 analysis and initial GSEA analysis. Vincent Butty performed single cell sequencing analysis. Jacqueline A Lees mentored on experimental design and anlaysis.

Abstract

Bmi1 has been proposed as a therapeutic target in cancer given its oncogenic role in variety of cancer. Previous studies in genetically engineered mouse models (GEMMs) for numerous tumor types, including lung adenocarcinoma (LUAD), have demonstrated that the germline deletion of *Bmi1* has tumor suppressive effects that are largely dependent on its ability to repress the *Ink4a/Arf* locus. We recently showed that deletion of *Bmi1* at the time of tumor initiation in otherwise normal mice also suppresses LUAD but this effect is not mediated by *Ink4a/Arf* derepression. However, neither of these studies mimic the treatment paradigm in clinic, in which patients typically present with advanced stage tumors at the start of treatment. In this study, we use an LUAD model, driven by oncogenic *Kras* activation and *Tp53* deletion, which allows us to subsequently trigger *Bmi1* deletion in established high grade tumors. Our data show that deletion of *Bmi1* is not tumor suppressive in this context. Instead, it enables acquisition of a gastric lineage program associated with dedifferentiation, which is a well-established driver of LUAD progression. Moreover, the *Bmi1* deficient tumors show an increase in the fraction of cells displaying established markers of tumor progression, metastasis, and tumor propagating cells. Overall, these findings reveal a context-dependent role of *Bmi1* in tumor progression and caution against BMI1 as a therapeutic target in LUAD.

Introduction

Cancer has remained a huge unmet clinical need despite decades of effort towards understanding the biology and exploiting vulnerabilities as therapies. The majority of cancer related deaths result from the distant dissemination of cancer cells, leading to metastases, and acquired resistance to therapies, leading to relapse (reviewed in Siegel et al., 2019). It has been postulated that the culprit behind these two phenomenon are a subpopulation of cancer cells that are at the apex of a hierarchy within the tumor, and have the potential to divide indefinitely (Desai et al., 2019). These cells have been termed cancer stem cells or tumor propagating cells (TPCs). Given that tumors tend to dedifferentiate towards an embryonic like state as they progress (Ben-Porath et al., 2008), and TPCs are similar to stem cells in hierarchy, considerable attention has been given to pathways and processes important for the self-renewal properties of stem cells as candidate therapeutic targets. The role of epigenetics in the maintenance of stem cell self-renewal, and also regulation of proper differentiation, has been well established (Atlasi & Stunnenberg, 2017; Avgustinova & Benitah, 2016). Thus, a lot of effort has been dedicated towards exploiting epigenetics to target cancer cells, and particularly TPCs (Brien et al., 2016).

One such epigenetic modulator is BMI1, which is a component of polycomb repressive complex 1 (PRC1). Early experiments showed that germline deletion of *Bmi1* caused defects in the self-renewal capacity of stem cells of various tissue compartments (Molofsky et al., 2003; Park et al., 2003; van der Lugt et al., 1994), and this phenotype was mostly accounted for by upregulation of the *Cdkn2a (INK4a*/*ARF*) locus, which encodes the INK4A and ARF tumor

suppressors (Jacobs et al., 1999). INK4A is an inhibitor of cell cycle in the pRB pathway, while ARF leads to p53 activation and thereby induces cell cycle arrest or apoptosis (Ivanchuk et al., 2001; Sharpless & DePinho, 1999). These potent tumor suppressive roles of INK4A and ARF led to the hypothesis that cancer cells could employ upregulation or amplification of *Bmi1* as a mechanism to repress *Ink4a/Arf.* Amplification of *BMI1* is rare in tumors, but higher expression of *BMI1* correlates with increased grade of in many human cancer types (Mohty et al., 2007; van Kemenade et al., 2001). Moreover, high *BMI1* expression was found to be a marker of poor prognosis in various tumors types (Farivar et al., 2013; Mohty et al., 2007; Qin et al., 2009). However, contrary to the findings in mice, *BMI1* and *INK4a/ARF* expression do not show a simple inverse relationship in patient samples (Beà et al., 2001).

To validate the oncogenic function of *Bmi1*, researchers have analyzed both cancer cell lines and mouse models. Much of the work in cancer cell lines revealed a tumor suppressive phenotype upon *Bmi1* deletion or knockdown, and concurrent upregulation of *Ink4a/Arf*, confirming *Bmi1*'s oncogenic function (H. Chen et al., 2011; Godlewski et al., 2008; Jagani et al., 2010; Xu et al., 2011). Similarly, genetically engineered mouse models (GEMMs) also support the notion that *Bmi1* is oncogenic: germline deletion of *Bmi1* impaired tumor initiation in a pancreatic model (Bednar et al., 2015); blocked tumor progression in an intestinal tumor model (Maynard et al., 2013), and impaired metastasis in a melanoma model (Ferretti et al., 2016). Given *Bmi1*'s role in normal adult stem cells, it's contribution has also been directly assessed in TPCs. The germline deletion of *Bmi1* loss resulted in loss of the undifferentiated state and transplant potential in a mouse model of leukemia (Lessard & Sauvageau, 2003). Similarly,

knockdown of *BMI1* resulted in lower TPC frequency, as measured in limited dilution assays in both cancer cell lines and primary samples from colorectal cancer patients (Kreso et al., 2014b).

Even though most of the literature is consistent with the role of *Bmi1* in tumor promotion through its ability to repress *Ink4a/Arf* locus, there are evidence for alternate mechanisms of BMI1 action. In a murine model of pancreatic cancer, loss of *Bmi1* is tumor suppressive. However, this phenotype is independent of *Ink4a/Arf* and instead appears to result from dysregulation of reactive oxygen species (Bednar et al., 2015). Another study showed that *Bmi1* deficient immortalized granulocyte/macrophage progenitors (GMPs) have low replating efficiency in colony forming assays that are independent of *Ink4a/Arf* status (Yuan et al., 2011). This defect is phenocopied by overexpression of *Tbx15*, a GMP-lineage inappropriate transcription factor that is upregulated in the *Bmi1* deficient immortalized GMPs (Yuan et al., 2011). These, and other studies, argue that *Bmi1*'s role of repressing *Ink4a/Arf* is not the sole mechanism by which it influences cancer biology.

Our lab has been studying the role of *Bmi1* in tumor initiation and progression for over a decade. One of the models we use is a murine lung adenocarcinoma (LUAD) model driven by oncogenic *Kras* expression either alone (K model) or in combination with loss of *Tp53* (KP model), which are two of the most mutated genes in lung cancer (Jackson et al., 2001; Jackson et al., 2005; Collisson et al., 2014). This mouse model has been instrumental in understanding the various stages of LUAD progression. One of the early insights was that after tumor initiation by endogenous levels of oncogenic *Kras*, the ability of tumors to transition from adenoma to adenocarcinoma requires amplification of MAPK signaling, which subsequently leads to upregulation of ARF (Feldser et al., 2010). Additional analyses showed that NKX2.1, a master

transcription factor of lung development (Herriges & Morrisey, 2014), and a hallmark of early stage tumors, acts as a barrier for tumor progression and dedifferentiation of tumor cells (Snyder et al., 2013; Winslow et al., 2011). Notably, loss of NKX2.1 results in activation of a gut lineage program that is marked by HNF4a, which subsequently leads to a more dedifferentiated, embryonic like state that is marked by HMGA2 (Snyder et al., 2013). Other studies established SCA1, CD24, NOTCH, and ITGB4 as key markers of functional TPCs (Curtis et al., 2010; Lau et al., 2014; Zheng et al., 2013). Collectively, these observations construct a tumor progression timeline for LUAD, which informs our analyses of *Bmi1*'s role in tumor initiation and progression.

Early work from our lab revealed that induction of *Kras* driven LUAD in *Bmi1* germline deficient animals limited tumor progression to early stages (hyperlasia and grade 1 tumors), and this could be partially rescued by loss of *Arf* (Dovey et al., 2008). This finding was encouraging for the notion that BMI1 might be a good therapeutic target for LUAD. However, since BMI1 is required for the appropriate silencing of *Ink4a/Arf* during development, the germline absence of BMI1 in this model meant that *Ink4a/Arf* was highly expressed in the adult lung stem cells and initiating tumor cells before oncogenic *Kras* was activated (Dovey et al., 2008). Clearly, this situation is not a physiologically relevant model of LUAD patients, who developed with normal levels of *Bmi1* and thus appropriate embryonic silencing of *Ink4a/Arf*. As a first step to assessing the effect of *Bmi1* loss in the adult context, we used a conditional *Bmi1* allele to delete *Bmi1* at the same time as induction of either K or KP LUAD models (Karl et al., in preparation). In both contexts, *Bmi1* loss impaired proliferation and tumor progression, resulting in a significant extension of lifespan. Notably, in contrast to the context of germline *Bmi1* deficiency, there was

no detectable upregulation of *Ink4a/Arf*. Instead, there was clear evidence of cell cycle progression defects, including impaired S phase progression and induction of G2M checkpoint markers, and also upregulation of lung-lineage inappropriate genes. Moreover, once the *Bmi1* KO tumors reached grade 3, they no longer displayed a proliferation defect, suggesting some form of adaptation to *Bmi1* loss.

While raising questions about the ability to mobilize *Ink4a* and/or *Arf* as the mechanism of tumor suppression, this model continues to support the notion that BMI1 might be a good therapeutic candidate for cancer. However, the model still had limitations, because we do not get to treat cancer patients at the time of tumor initiation. Thus, to better mimic the clinical treatment paradigm, we have now generated a mouse model that allows us to induce LUAD that are wildtype for *Bmi1* and subsequently trigger *Bmi1* deletion in the resulting high grade tumors. In this context, we find that *Bmi1* deletion acts to promote the activation of gene expression signatures associated with dedifferentiation and LUAD progression. This argues that *Bmi1* functions in a context dependent manner and behaves as a tumor suppressor in late stage tumors. Based on these findings, we believe that *Bmi1* should not be considered a therapeutic target in KP LUAD.

Results

The goal of this study is to better mimic the treatment paradigm in the clinic by assessing the effect of *Bmi1* deletion in established murine LUAD tumors. To achieve this, we needed to uncouple the mutational events necessary for tumor initiation from that required for deletion of *Bmi1*. Thus, we opted to combine the FlpO-frt and tamoxifen inducible Cre-loxP systems to induce spatially controlled, but temporally separate, genetic alteration events. First, we used FlpO responsive alleles of oncogenic *Kras* (*Kras-frt-STOP-frt-G12D*) with *Tp53* (*Tp53*$^{frt/frt}$), to model LUAD (Figure 2.1A), which we refer to as KP LUAD. Intratracheal delivery of adenoviral particles carrying the FlpO recombinase gene driven by the CMV promoter (ad-FlpO) yields activation of the mutated *Kras* allele and homozygous deletion of *Tp53* in sporadic lung cells, many of which undergo malignant transformation and give rise to LUAD tumors with variable latency, resulting in a wide spectrum of tumor grades at later time points. In addition to these cancer driver alleles, we included a tamoxifen inducible Cre, CreER^{T2}, whose expression is controlled by the Rosa26 promoter and also a FlpO responsive element (R26-frt-STOP-frt-CreER). Thus, during tumor initiation by ad-FlpO, we also trigger expression of CreER gene in the nascent tumor cells. We note that tissue resident macrophages also take up the virus particles, resulting in the same genetic alterations in these cells as well (Zheng et al., 2013; our unpubslihed observations). Otherwise, the expression of CreER is tumor cell-specific.

In addition to the FlpO responsive alleles, our mouse model includes either Cre-responsive, conditional mutant alleles of *Bmi1* (*Bmi1*$^{fl/fl}$) or control *Bmi1*$^{+/+}$ alleles, as well as a Cre responsive allele of TdTomato under the control of the Rosa26 promoter (*R26-lox-STOP-lox-*

TdTomato; Figure 2.1A). Upon exposure to tamoxifen (in our case by intraperitoneal injection), we can trigger *Bmi1* deletion in the $Bmi1^{fl/fl}$ animals and expression of TdTomato in both $Bmi1^{fl/fl}$ and $Bmi1^{+/+}$ animals to mark tumor cells that saw Cre activity. Importantly, we validated the efficiency of this system in pilot experiments, showing that almost all of the tumor cells within established tumors have TdTomato expression (and thus, Cre activity) after injection of tamoxifen. Moreover, this achieved near complete deletion *of Bmi1* in the $Bmi1^{fl/fl}$ animals (Figure 2.1B), as further confirmed by our subsequent RNA sequencing data (described below). For simplicity, we will refer to the tumors as *Bmi1* KO or WT.

Since we aimed to delete *Bmi1* in advanced tumors and have subsequent time to assess the impact, we first had to establish the temporal dynamics of tumor progression in our mouse model to determine when to delete *Bmi1* and how long we could monitor the mice before they became moribund. In the KP LUAD model, lifespan is affected by the titer and infecting capacity of the virus, the persistence of exogenous genes introduced, and the type of recombinase enzyme used and their processivity. These variables affect the number of cells that get the appropriate genetic alterations needed for tumor initiation, and only a subset of these cells will actually give rise to tumors, resulting in some animal to animal variation even within a given cohort. When numerous tumors are launched, morbidity is caused by reduced airway space from tumor growth. Thus, the time to morbidity is dictated by both the number of tumors and their rate of progression. After careful titering, we established that mice infected with 1X10^7pfu (plaque-forming units) of ad-FlpO have a high tumor number that causes a moribund state around average of 20 weeks post tumor launch (data not shown). When we increased the virus titer to 2.5X10^7pfu, the mice develop more tumors and develop morbidity

at earlier time points and with smaller tumors (data not shown). Our analyses showed that more than half of tumors had reached grade 3, or higher, by 9 weeks (2.5X10^7pfu) or 13 weeks (1X10^7pfu) post viral infection.

To allow us to assess effect of *Bmi1* deletion across a larger spectrum of tumor progression stages and achieve different temporal dynamics of tumor progression, we used both viral titers to launch tumors in large cohorts of mice, which we will refer to as cohort A (1X10^7pfu) and cohort B (2.5X10^7). To mimic the treatment paradigm in clinic, where patients are often diagnosed with highly progressed disease, we deleted *Bmi1* via tamoxifen treatment after more than half of tumors had reached grade 3 or higher (Figure 2.1C). We then collected tumor samples 3 days (D3), 14 days (D14), and 45 days (D45) post tamoxifen to capture both the immediate and longer term effects of *Bmi1* deletion. All analyses were conducted on 3 or more mice for each genotype and cohort (denoted by day and cohort e.g. D3A in the figures). This represents an average of approximately 50 tumors per mouse, yielding a large overall sample size.

We began by conducting histological analyses of the tumors (Figure 2.2). At D3, we expected to see little to no consequences of *Bmi1* deletion, because our cell-based studies (data not shown) and tumor analyses (described below) show that transcriptional changes post clearance of BMI1 from chromatin takes more than 3 days. Accordingly, in the D3 samples we saw no significant difference in the tumor number, tumor burden, tumor size distribution, or tumor grade distribution between *Bmi1* KO and WT from either cohort (Figure 2.2 A-F). We also screened for apoptosis using cleaved caspase 3, and observed very little in either KO or WT samples, with no significance differences between these two genotypes(data not shown). In

addition, we assessed proliferation, by detection of BrdU (injected 60 minutes before sacrifice). We did observe significant differences between genotypes, but the KO was higher in one cohort (A), and lower in other (cohort B) (Figure 2.2E). Given this variation, and the fact that Bmi1 is not cleared from the chromatin by this timepoint, we suspect that this reflects the biological noise of the proliferation assay.

We then extended our analyses to the D14 and D45 samples. At D14 there was no significant difference in tumor number, tumor size distribution, tumor grade distribution, apoptosis or proliferation between *Bmi1* KO and WT in either of these cohorts (Figure 2.2 A-F). We did observe a significant difference in tumor burden but only in one of the two cohorts (D14B) and with Bmi1 KO having higher burden than WT (Figure 2.2B). Notably, tumor number also trended higher in D14B, although this did not reach statistical significance (Figure 2.2A), offering a possible benign explanation for the higher tumor burden in D14B KO versus WT. At D45, we also saw disappointingly little impact of Bmi1 loss. At this time point, there was no significant difference in tumor number, burden or grade distribution, or in apoptosis between KO and WT in either of the cohorts (Figure 2.2 A-F). We did observe a significant difference in tumor size, only in cohort A and not cohort B, and again the KO was higher than the WT (Figure 2.2C). By plotting tumor size against the cumulative sum of tumor size, we determined that large tumors (above 10mm^2 in size) accounted for a higher proportion of the overall tumor burden in the D45A KO (40%), compared to the D45A WT (17%; Figure 2.2D). Interestingly, we observed a similar trend (but not statistical significance) towards a higher contribution of larger tumors for *Bmi1* KO in the other timepoint/cohorts, D14A, D14B and D45B (Figure 2.2D). The other significant difference we observed at D45 was a lower level of BrdU incorporation in *Bmi1* KO,

compared to, WT for both the A and B cohorts (Figure 2.2E). This was the only histological result that was encouraging of a tumor suppressive effect of *Bmi1* loss, but unfortunately it did not translate into any detectable reduction in tumor size, tumor burden or tumor progression. Indeed, the only significant histological differences observed (tumor burden at D14B and tumor size in D45A) were both higher in the KO than the WT. Thus, at least in the timeframe of our analyses, Bmi1 loss in existing tumors LUAD yielded no detectable reduction of tumor bulk.

Given the proposed role of *Bmi1* in maintaining the self-renewal capacity of tumor promoting cells (TPCs), we wondered whether *Bmi1* loss was specifically impairing the TPCs rather than the bulk tumor cells. To address this, we performed a transplant experiment. Specifically, we waited 52 days post tamoxifen treatment and *Bmi1* deletion in cohort A mice, and then used FACS to isolate CD45-, CD31-, Ter119-, TdTomato+ cells from WT and KO lungs (n=2 mice per genotype). These markers should effectively separate the tumor cells from any contaminating stromal cells, including any TdTomato positive hematopoietic cells. Because we are sorting from whole lung, the isolated cells include TPCs from numerous independently arising KO and WT tumors of mixed sizes and grades. We then orthotopically transplanted 5000 KO and WT tumor cells into lungs of multiple immunocompromised mice using intratracheal intubation (8 recipients per sample). After 17 weeks, we quantified the tumor number and burden and observed no significant difference between the WT and KO (Figure 2.3). Thus, at least in the context and timeframe of this experiment, *Bmi1* deletion had no measurable effect on the tumorigenic capacity of the LUAD TPCs.

Given these observations, we wondered whether Bmi1 loss was yielding any detectable changes in gene expression. To address this, we first used the FACS based protocol described

above to isolate tumor cells from the lungs of mice from cohort B (2.5X10^7 viral titer) at the D3, D14 and D45 time points (4 mice per time point and genotype) and performed bulk mRNA sequencing. We then identified significant differentially expressed genes (judged as FDR < 0.05; Fold change > 2) using DeSeq2 analysis (Figure 2.4A). In the D3 samples, there were no significant differences between WT and KO tumors. This is consistent with the notion that it takes time for *Bmi1* to be depleted from the chromatin and for expression changes to ensue. Our analyses did detect genes with significant differential expression at both D14 and D45 (Figure 2.4A). Notably, despite Bmi1's documented role in repressing *Ink4a/Arf* locus, there was no significant difference in the level or either *Ink4a* or *Arf* mRNA between WT and KO tumors at either timepoint (data not shown). We then compared the significant differentially expressed genes at D14 and D45 post-deletion (Figure 2.4B). Interestingly, we found that fewer genes showed significant differences at D45, versus the earlier time point, and there was actually little overlap between the two (Figure 2.4B). Given the selective pressure that occurs in tumors, we hypothesize that *Bmi1* loss leads to upregulation of multiple programs, that are then selected for, or against, as the tumor grows. The programs that are potentially selected for would be the ones enriched at D45.

To identify differentially regulated pathways between WT and KO tumors, we performed gene set enrichment analysis (GSEA) against the C2 gene set collection in the molecular signatures database (MSigDb). Since *Bmi1* is an epigenetic repressor, we looked specifically at gene sets that are enriched in KO over WT. We then focused on ones enriched at both D14 and D45, to identify programs that are selected for in the tumors. Only 5 gene sets fulfilled these criteria (Figure 2.4C). Remarkably, one of these was *Hnf4a* targets, a hallmark of the gut lineage

signature that characterizes the dedifferentiation of KP LUAD tumor cells to the more embryonic state (Figure 2.5A; Snyder et al., 2013). Snyder and coworkers identified this gut lineage signature by establishing that deletion of the lung master transcription factor, *Nkx2.1*, acts to promote LUAD progression, and they defined a core set of genes that defined the lung, lung/gut, and gut states characteristic of this transition. Using these core gene sets, we performed unsupervised hierarchical clustering on our WT and KO samples. This clustering clearly separated all four of the D45 *Bmi1* KOs from the rest of the samples at the first branch point of the dendogram, and showed that the D45 *Bmi1* KOs were highly enriched for lung/gut and gut markers (Figure 2.5B). Moreover, two of the four D14 *Bmi1* KO samples showed a similar expression pattern to the D45 *Bmi1* KOs, although they did not segregate with the D45 *Bmi1* KOs. To extend our analysis beyond the core lung, lung/gut, and gut gene sets, we generated larger, high confidence gene sets, comprised of genes identified by Synder and coworkers as being upregulated or downregulated in the *Nkx2.1* deficient tumors with a log fold difference > 2 and p-value < 0.01. We appended these gene sets to the MSigDb C2 collection and ran GSEA on our D45 samples. Strikingly, the *Bmi1* KOs were significantly enriched for genes that are upregulated by *Nkx2.1* deletion while *Bmi1* WT were significantly enriched for genes downregulated by *Nkx2.1* deletion (Figure 2.5C). Thus, taken together, our data indicate that *Bmi1* loss within existing tumors promotes acquisition of gene expression changes, including expression of the embryonic gut lineage program, that are indicative of LUAD tumor progression (Figure 2.5D). This finding is in stark contrast to our original hope that *Bmi1* loss would result in tumor suppression.

The gene expression changes we observed in our *Bmi1* KO tumors could arise in two distinct ways. *Bmi1* loss might simply promote the gene-expression transitions previously associated with LUAD progression, NKX2.1 to HNF4A and ultimately to HMGA2. Alternatively, *Bmi1* loss could cause gene expression changes that do not follow the normal LUAD progression program, such as the co-expression of NKX2.1 and HMGA2 that occurs when *Bmi1* is deleted at the time of tumor initiation (Karl et al., in preparation). To distinguish between these two possibilities, we conducted immunohistochemical staining for NKX2.1, HNF4A and HMGA2 in D45 *Bmi1* KO tumors (Figure 2.6, and data not shown). These markers were all detected within *Bmi1* KO tumors and their expression patterns closely mirrored those of *Bmi1* WT tumors. Specifically, NKX2.1 and HMGA2 staining were mutually exclusive, with HMGA2 being restricted to higher grade invasive regions, and HNF4A was mostly co-expressed with NKX2.1 or, at low frequency, with HMGA2. These data argue that *Bmi1* deletion in existing tumors promotes gene expression changes that are characteristic of the normal LUAD progression events.

To further explore the nature of this dedifferentiation, and also the frequency of cells with TPC markers, we also performed single cell RNA sequencing. For this, we used WT and KO mice from cohort A (1X10^7 viral titer) at a further extended time point of 52 days. Tumor cells from 4 mice per genotype were isolated by FACS, exactly as described above for the bulk sequencing, and prepared for sequencing using the Seqwell method. The sequencing reads were processed with the Seurat 3.0 package and the structure within our dataset defined using UMAP based plots (Figure 2.7A). Most of the cells clustered in one large cloud, with cluster 7 and 10 branching away from the main body. Cluster 7 represented cells in S-phase, based on the specificity of Ki67 expression, while cluster 10 contained ciliated cells in lung epithelium (data

not shown). Notably, none of the clusters identified by UMAP were significantly enriched for either KO or WT tumor cells (Figure 2.7B). This is consistent with our conclusion that *Bmi1* loss primarily keeps tumor cells within the spectrum of states seen in normal LUAD, at least at this timepoint of 52 days post-deletion.

To determine whether the single cell RNA sequencing appropriately segregated the tumor cells by virtue of their differentiated states, we examined expression of *Sftpa1* (a prominent pulmonary marker) and *Onecut2* (a key gut program marker). As expected, expression of these two genes was mutually exclusive, with the gut marker being especially enriched in cluster 3, and to some extent in cluster 2 (Figure 2.7C-D). Having established the ability of the UMAP clustering to appropriately segregated these dedifferentiated states, we then assessed the difference between WT and KO tumor cells. For this, we called a cell positive for a gene if it had more than 3 reads, and we generated a list of cell frequency for each gene. We then calculated the differentially expressed genes, based on the number of positive cells, and performed GSEA to identify upregulated programs. Consistent with our bulk sequencing studies, a higher proportion of KO than WT cells were positive for the Synder gut signature (Figure 2.8A). Moreover, consistent with the known correlation between dedifferentiation and metastatic potential, a second gene set (Winslow), which we based on an identified metastatic signature for KP LUAD model (Winslow et al, 2011), was also detected in a higher proportion of KO cells than WT cells (Figure 2.8B). GSEA also identified the epithelial-to-mesenchymal transition (EMT), and Naba core matriosome gene sets as being enriched in the KO tumor cells (Figure 2.8C-D). Importantly, both of these gene sets are also associated with tumor progression and metastasis, bolstering the idea that KO tumors are more advanced than WT controls.

To further explore this notion, we also compared the expression of two other markers of KP LUAD tumor progression, HMGA2 (Snyder et al., 2013), and SPP1, which was identified as the only ECM marker of metastasis that was autonomously expressed in KP LUAD tumor cells (Reticker-Flynn et al., 2012). Both of these markers were also detected in more KO than WT cells (Figure 2.9A-B). We also looked at markers of TPCs for KP LUAD, *Sca-1* alone (Curtis et al., 2010; Lau et al., 2014), *Sca-1* and *CD24* double positive (Rowbotham et al., 2018), and *CD24*, *Notch* (1, 2, or 3), and *Itgb4* triple positive (Zheng et al., 2013), identified by serial transplant experiments. *Sca-1/Ly6A* alone, was not enriched in KO tumor cells compared to WT (data not shown). When combined with *CD24* expression, more KO cells were double positive for these markers compared to WT cells, even though both genotype had a high frequency of cells positive for these markers (Figure 2.9C). In contrast, we identified rare cells that were positive for all of the other 3 markers (*CD24, ITGB4, Notch 1/2/3*), and found that these were twice as abundant in KO compared to WT samples (Figure 2.9D). Additionally, the KO state also increased the frequency of cells with one or two of these markers (Figure 2.9D). Collectively, these data show that *Bmi1* deletion acts to promote gene signatures associated with KP LUAD tumor progression and TPC potential. This is in direct opposition to our original hypothesis that *Bmi1* deletion would lead to tumor suppression, potentially by impairing TPC function.

Discussion

Our data show that *Bmi1*'s role in tumor biology is context dependent, and it acts as a tumor suppressor by acting as a barrier to dedifferentiation in advanced KP LUAD tumors. This is in sharp contrast to previous literature that has pegged *Bmi1* as an oncogene and as a good therapeutic candidate in multiple cancer types. The key difference between our study and majority of the other studies is the context in which *Bmi1* has been deleted. Prior research on *Bmi1* in GEMMs of cancer has been conducted using germline deletion or tissue specific deletion of *Bmi1 in utero*. As has been previously established, animals that develop in the context of *Bmi1* deficiency have high levels of *Ink4a/Arf* in adult tissue where it is normally absent (Dovey et al., 2008). Concurrent ablation of *Ink4a/Arf* in these mice leads to rescue of the tumor suppressive phenotype suggesting that this can account for some of the tumor suppressive effect of *Bmi1* deletion (Maynard et al., 2013). Our unpublished work has shown that deletion of *Bmi1* during tumor initiation in mice that developed in normal *Bmi1* context *in utero* is still tumor suppressive in early stage tumors, but this phenotype is independent of *Ink4a/Arf* deregulation suggesting alternative mechanisms might be in play (Karl et al., unpublished). Intriguingly, the tumor suppressive phenotype is alleviated in grade 3 or higher tumors arguing for either acquired resistance or *Bmi1* independence at grade 3 stage. These experiments have set up the stage for context dependent phenotype upon *Bmi1* deletion and beg the question of whether established tumors still care about *Bmi1* status.

This thesis work adds to the previous studies and assesses the phenotype of *Bmi1* deletion in already established tumors. In contrast to previous literature, including work from our lab, we

do not observe any tumor suppressive phenotype upon *Bmi1* loss in the 45 days of monitoring post *Bmi1* deletion. Surprisingly, gene expression data reveals that *Bmi1* KO tumors are enriched for markers of dedifferentiation and tumor progression such as gut lineage markers, HMGA2, TPC markers, EMT markers, and previously described metastasis markers in KP LUAD (Winslow et al., 2011). Derepression of progression markers, most of which are non-pulmonary state markers, is consistent with previously observed phenotype that deletion of *Bmi1* results in deregulation of lineage-inappropriate genes (Yuan et al., 2011; Karl et al., unpublished). However, it is unclear if these markers are direct targets of BMI1.

Previous observations of deregulation of genes upon *Bmi1* loss would argue that the progression markers we observe might simply be deregulation of genes rather than associated with actual progression. However, our immunohistochemistry results show that expression of progression markers follow similar pattern as normal tumor progression, suggesting that we are indeed observing tumor progression in our system. The strongest evidence toward this is restriction of HMGA2 expression in highly invasive grade 4 tumors and its mutual exclusivity with NKX2.1 expression, as has been described previously for KP LUAD progression (Snyder et al., 2013; Winslow et al., 2011). One plausible explanation towards gain of tumor progression markers could be that many programs are deregulated upon Bmi1 loss but only a subset of these programs are selected for and maintained in the population. This explanation is supported by the observation that many genes are differentially expressed at 14 days post *Bmi1* deletion (as judged by logFC>1, FDR<0.05), however, only a small number of the genes are differentially expressed at 45 days post *Bmi1* deletion. Importantly, tumor progression markers such as gut lineage signature is highly enriched at both 14 days' and 45 days' time point.

Despite the observation of multiple markers of tumor progression and TPCs, we did not observe any histological evidence of increased tumor progression or metastasis in *Bmi1* KO tumors compared to controls. Antithetical to higher frequency of TPCs in KO tumors from single cell sequencing analysis, we did not observe higher tumor number or burden upon transplant of tumor cells into recipient mice. All of these evidence beg for longer monitoring of tumors post *Bmi1* deletion. It is plausible that gene expression changes precedes any phenotypic changes, and we might be capturing the early changes that haven't manifested into actual phenotypic changes. Currently, experiments are underway for longer monitoring post *Bmi1* deletion for histological analyses. To address the TPC frequency, experiments are also underway using limited dilution assay, which is a more precise measure of TPC frequency.

Even though our result goes against two decades of research in the *Bmi1* field, recent studies in other epigenetic targets show similar phenotype as our studies. This thesis work combined with previous unpublished data from our lab (Karl et al., unpublished) argue that *Bmi1* deletion is tumor suppressive in early stage KP LUAD tumors, but later stage tumors either do not care about *Bmi1* status or are pushed faster along the tumor progression cascade. Similarly, studies deleting *Eed*, a PRC2 component, or *G9a*, a H3K9 histone methyltransferase, have shown that deletion of these epigenetic regulators is tumor suppressive early on, but upon long term monitoring, the tumors readily become more aggressive and bear previously established markers of tumor progression (Avgustinova et al., 2018; Serresi et al., 2016). In addition, deletion of G9a has also been shown to increase TPCs in KP LUAD model (Rowbotham et al., 2018). These recent studies argue that longer monitoring post deletion of epigenetic regulators yield phenotypes previously masked by more acute phenotype.

Our thesis work highlights the context dependent tumor suppressive role of *Bmi1* in KP LUAD model. But it is essential to examine role of *Bmi1* in LUAD models driven by other genetic drivers, as well as other cancer types. Our efforts in colorectal cancer model is a step towards addressing this. Additionally, while we attempted to mimic the treatment paradigm in clinic, a better mimicry would have been to delete *Bmi1* in metastases, as more than half of patients are diagnosed at that stage (SEER, 2019). However, the KP LUAD model at a high viral titer has a low and highly variable frequency of metastasis with a long latency period. Further studies for *Bmi1* deletion are warranted in animals where a small number of tumors are launched with low viral titer, thus allowing mice to survive long enough for the tumors to metastasize.

So far, our work cautions any further studies looking at BMI1 as a therapeutic candidate in KP LUAD, and potentially other cancer types. Inhibitors against BMI1 are currently being developed in clinical trials (Infante et al., 2017; Kreso et al., 2014). Our results would argue that more preclinical studies are needed to understand the longer term effect of *Bmi1* inhibition before further pursuing the inhibitors in clinical trials.

Figures

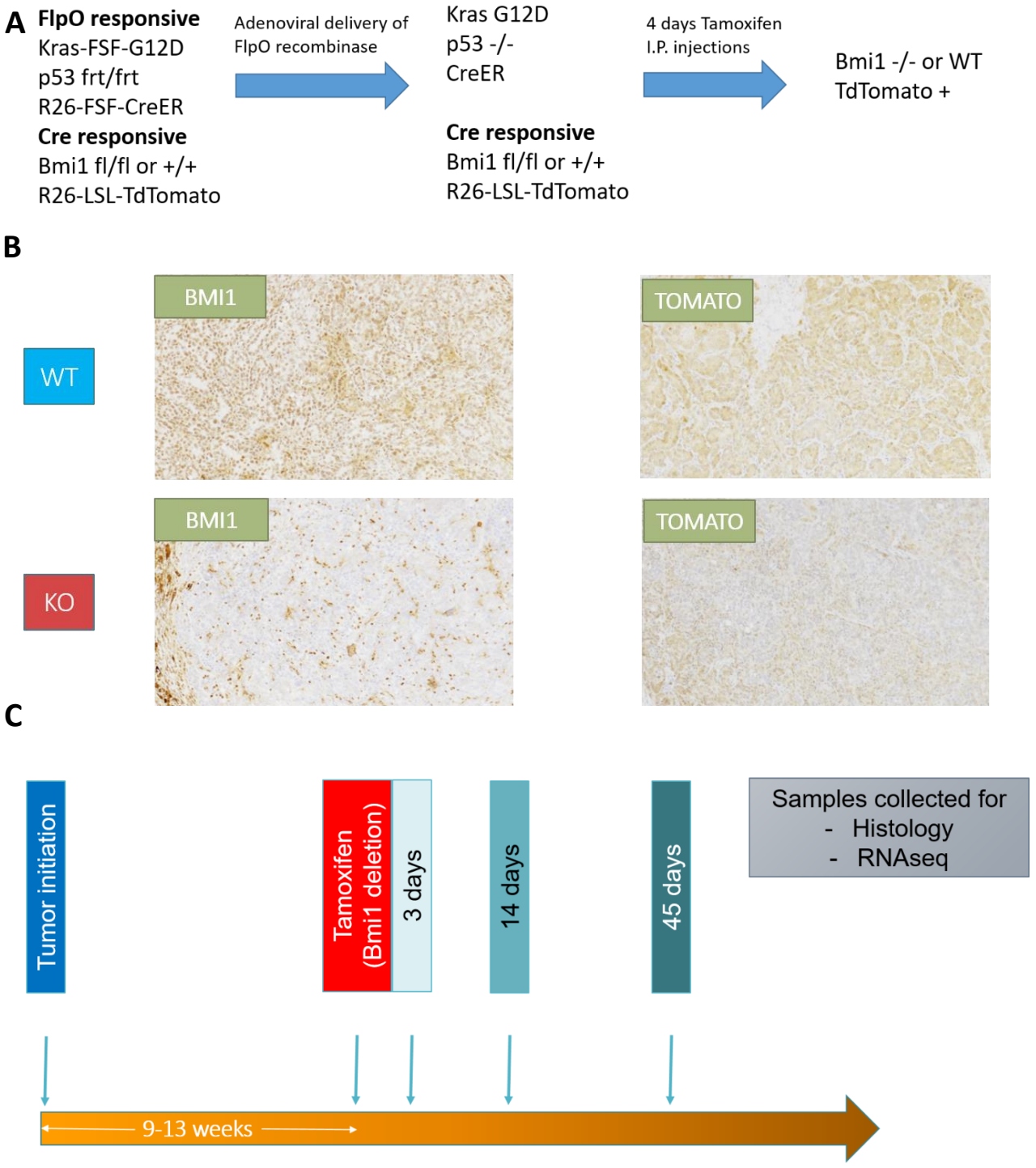


Figure 2.1 *Schematic of the mouse model used for this study.* ***A****. Mice with the indicated genotypes were infected with ad-FlpO to launch tumors, and treated with tamoxifen at later time point to delete Bmi1.* ***B.*** *IHC confirms the expression of TdTomato in both genotypes and deletion of Bmi1 specifically in* $Bmi^{fl/fl}$ *tumors after tamoxifen treatment.* ***C.*** *Schematic representing the timeline of tumor launch, tamoxifen treatment, and sample collections.*

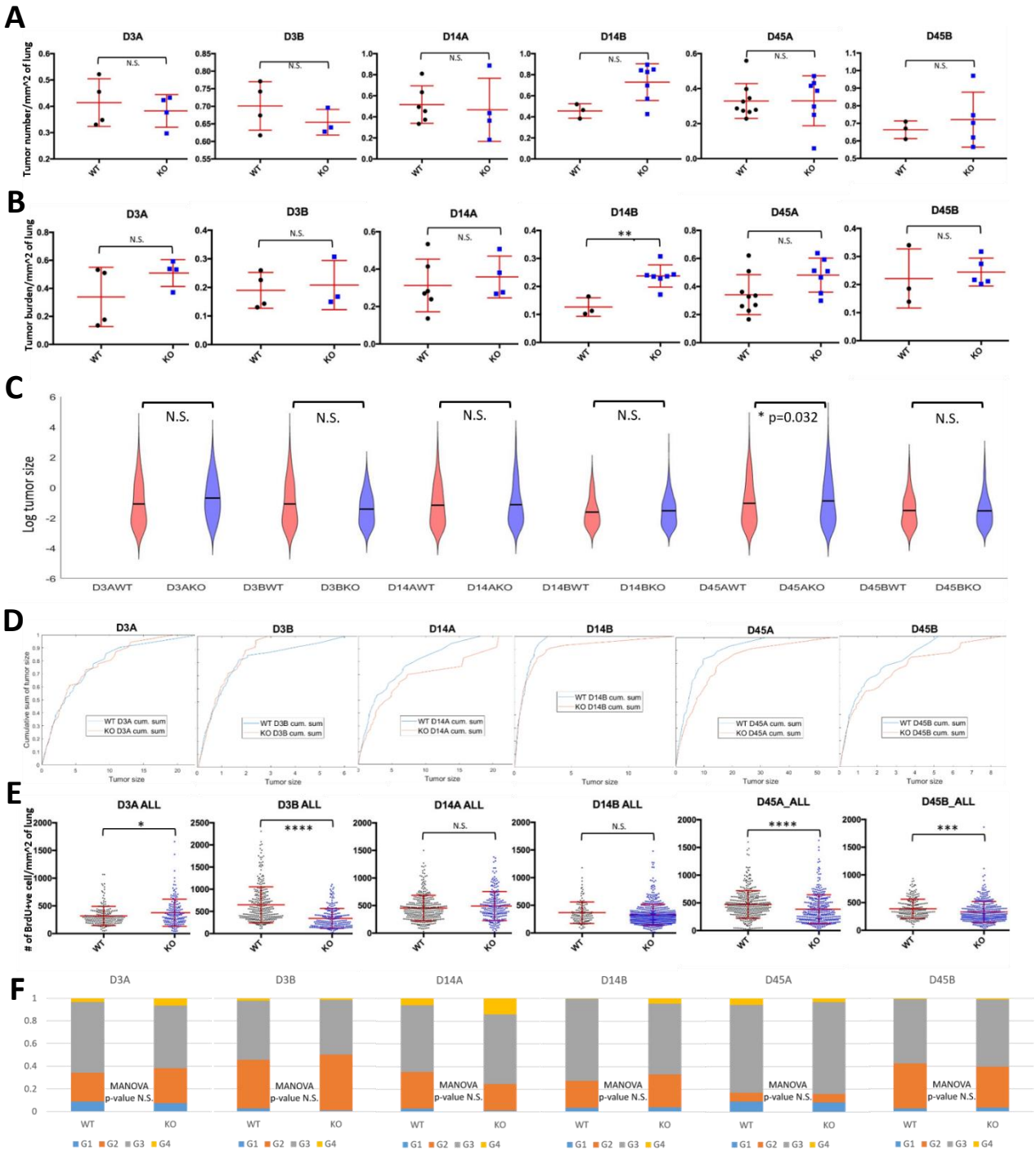


Figure 2.2. *Data from histological analyses of Bmi1 WT and KO tumors from two different cohorts (A and B) at the indicated times after Bmi1 deletion reveal no overt evidence that Bmi1 loss is tumor suppressive. Figures are plotted for tumor number (**A**), tumor burden (**B**), tumor size distribution (**C**), size distribution vs cumulative sum of tumor size (**D**), BrdU incorporation rate (**E**), and tumor grade distribution (**F**). Statistical significant was determined using the: Mann-Whitney test for **A** and **B**, KS-2 sample test for **C**, unpaired student t-test for **E**, and MANOVA for **F**. The only statistically significant results are KO having higher tumor burden at D14B (**B**) and larger tumors at D45A (**C**), and WT having a higher mean proliferation rate at D45A and D45B (**E**).*

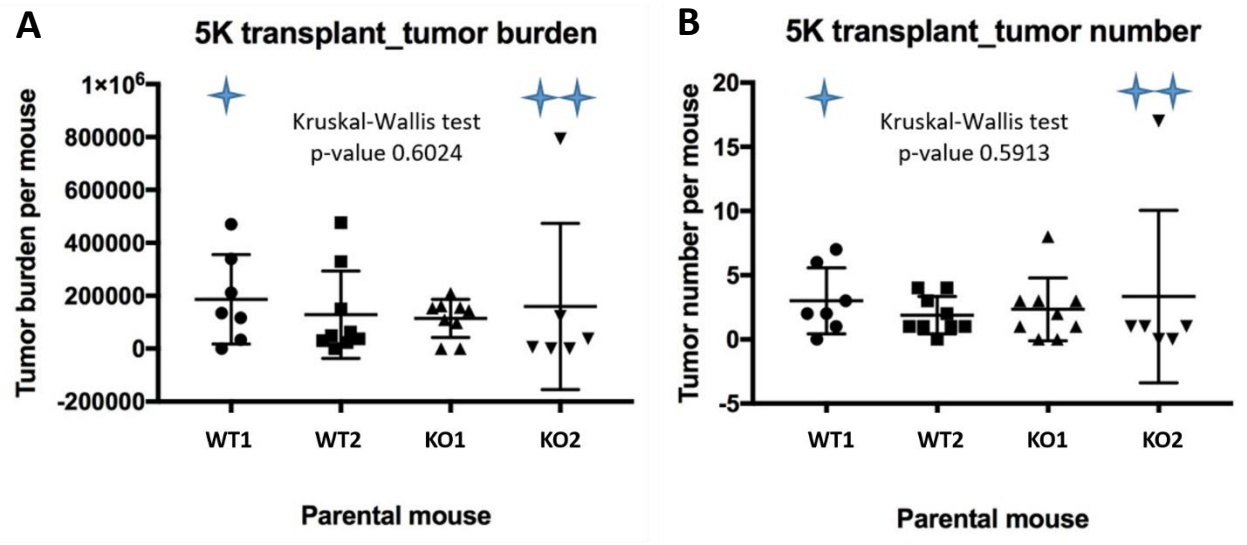
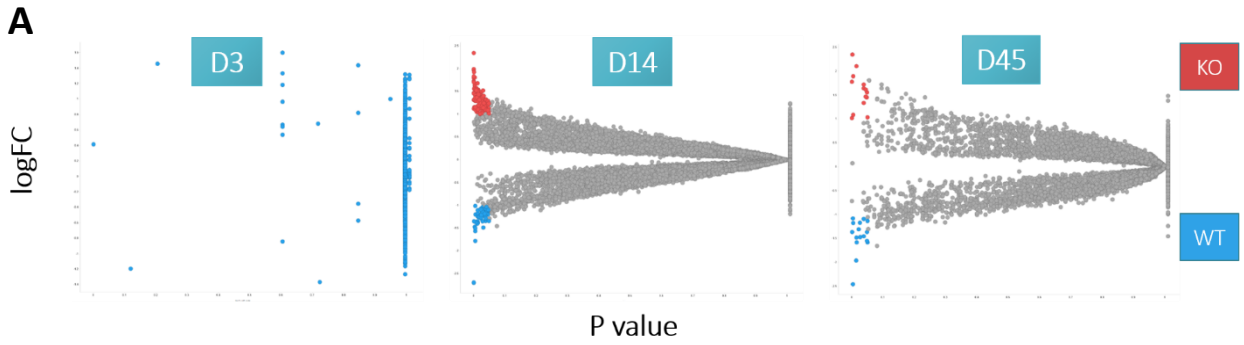
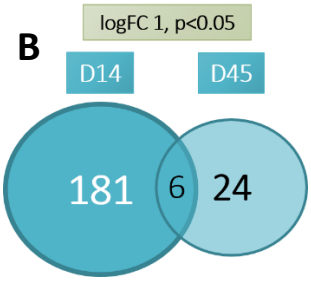


Figure 2.3. *5000 tumor cells were isolated from primary mouse tumors and transplanted into immunocompromised mice. After 17 weeks of transplant, tumor burden (**A**) and tumor number (**B**) were measured. The blue stars indicate mice that were sacrificed before the 17 weeks' time point because they became moribund. These animals were not included in the quantification of total tumor burden or tumor number because samples could not be collected. The data reveal no difference between WT and KO tumor cells in tumor forming capability.*





C

NAME	Group	ko3.wt3.FDR q-val	ko14.wt14.FDR q-val	ko45.wt45.FDR q-val
WINTER_HYPOXIA_UP	c2	0.20758092	0.004041865	0.005403686
VECCHI_GASTRIC_CANCER_ADVANCED_VS_EARLY_DN	c2	0.5979636	0.004649216	0.017540552
VANDESLUIS_COMMD1_TARGETS_GROUP_4_UP	c2	0.94045275	0.013711575	0.03412702
SUMI_HNF4A_TARGETS	c2	0.9210548	0.022022203	0.004862754
WAMUNYOKOLI_OVARIAN_CANCER_LMP_UP	c2	0.034739967	0.045367174	0.004751117

Figure 2.4. *Comparison of the gene expression patterns of Bmi1 WT and KO tumors at the indicated time points. DeSeq2 was used to establish differentially expressed genes as shown by volcano plot of FDR p-value vs log fold change (**A**). Differentially expressed genes (logFC>1, FDR<0.05) are highlighted in red for higher in Bmi1 KO and blue for higher in WT. There are no differentially expressed genes at D3. D14 shows the highest number of differentially expressed genes and that number decreases by D45. The overlap of differentially expressed genes between D14 and D45 are shown by the venn diagram (**B**). Preranked genes from DeSeq2 analysis was used for GSEA analysis. The table represents all gene sets enriched in both D14 and D45 in Bmi1 KO tumors from C2 gene sets collection of mSigDb (**C**). Only 5 gene sets made the criteria of FDR<0.05 and enriched at both time points, and Hnf4a targets was one of these hits.*

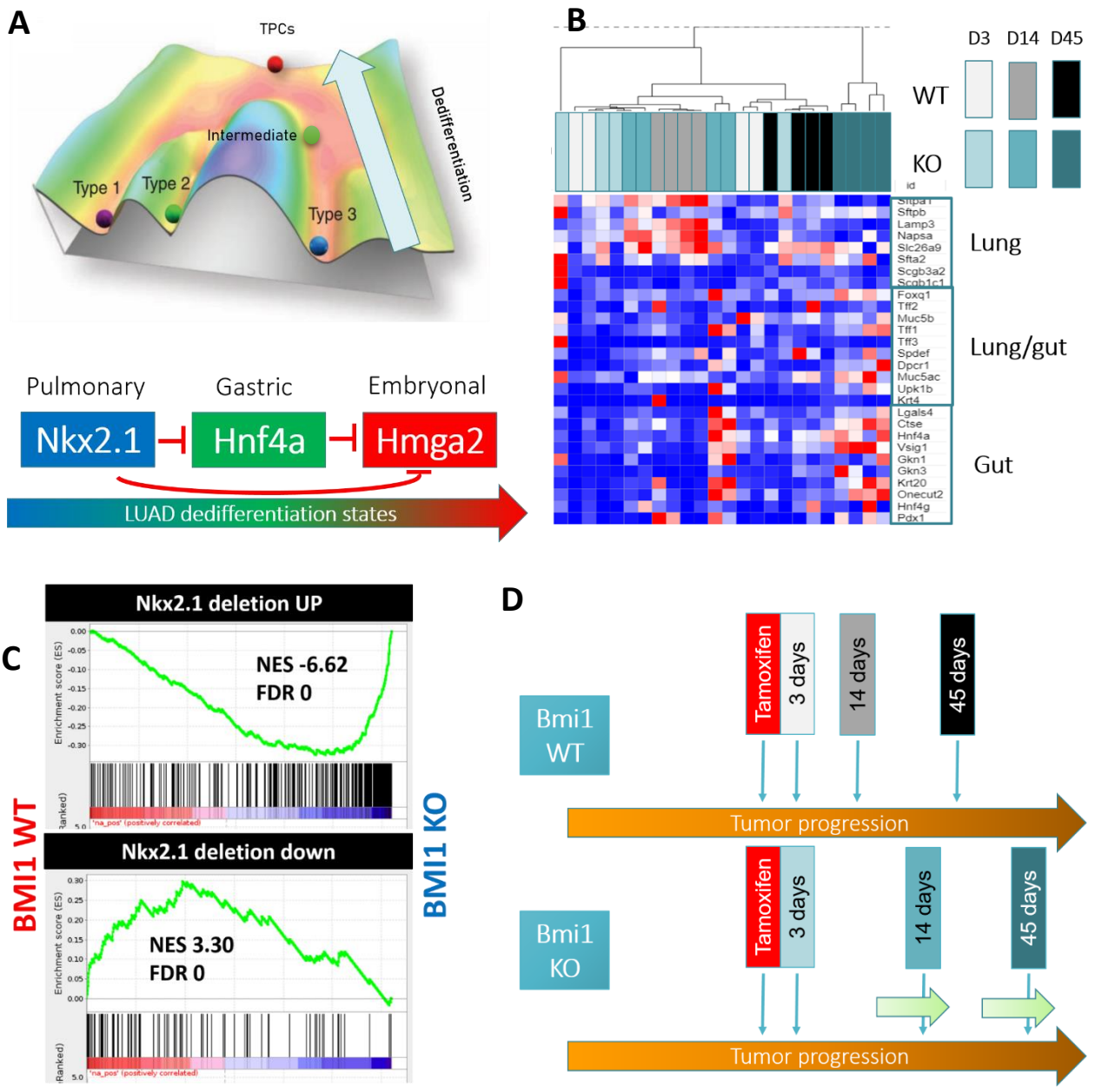


Figure 2.5. *Dedifferentiation is a hallmark of tumor progression in KP LUAD. During tumor progression, cancer cells dedifferentiate to acquire characteristics of stem cells to become TPCs, as depicted by the Waddington plot* (adapted from Furusawa & Kaneko, 2012) ***(A)****. For KP LUAD, this dedifferentiation involves transitions from the pulmonary state, marked by Nkx2.1, to the gastric state, marked by Hnf4a, and eventually an embryonic-like state, marked by Hmga2* (adapted from Snyder et al., 2013) ***(A)****. Given the enrichment of Hnf4a targets gene sets in GSEA, unsupervised hierarchical clustering was performed on gene expression from WT and KO tumors using core genes sets for the lung, lung/gut, and gut lineages from Snyder et al., 2013.* ***(B)****. D45 Bmi1 KO tumors segregated away from the other samples and were enriched for lung/gut and gut lineage markers. A high confidence gene list (FDR < 0.01, logFC > 2) generated from gene expression analysis in Snyder et al., 2013 was used as a gene set in C2 collection for GSEA analysis. D45 Bmi1 KO tumors enrich for genes upregulated upon deletion of Nkx2.1, while Bmi1 WT tumors enrich for genes downregulated* ***(C)****. Schematic representing acceleration of tumor progression in Bmi1 KO tumors compared to Bmi1 WT tumors at the indicated time points* ***(D)****.*

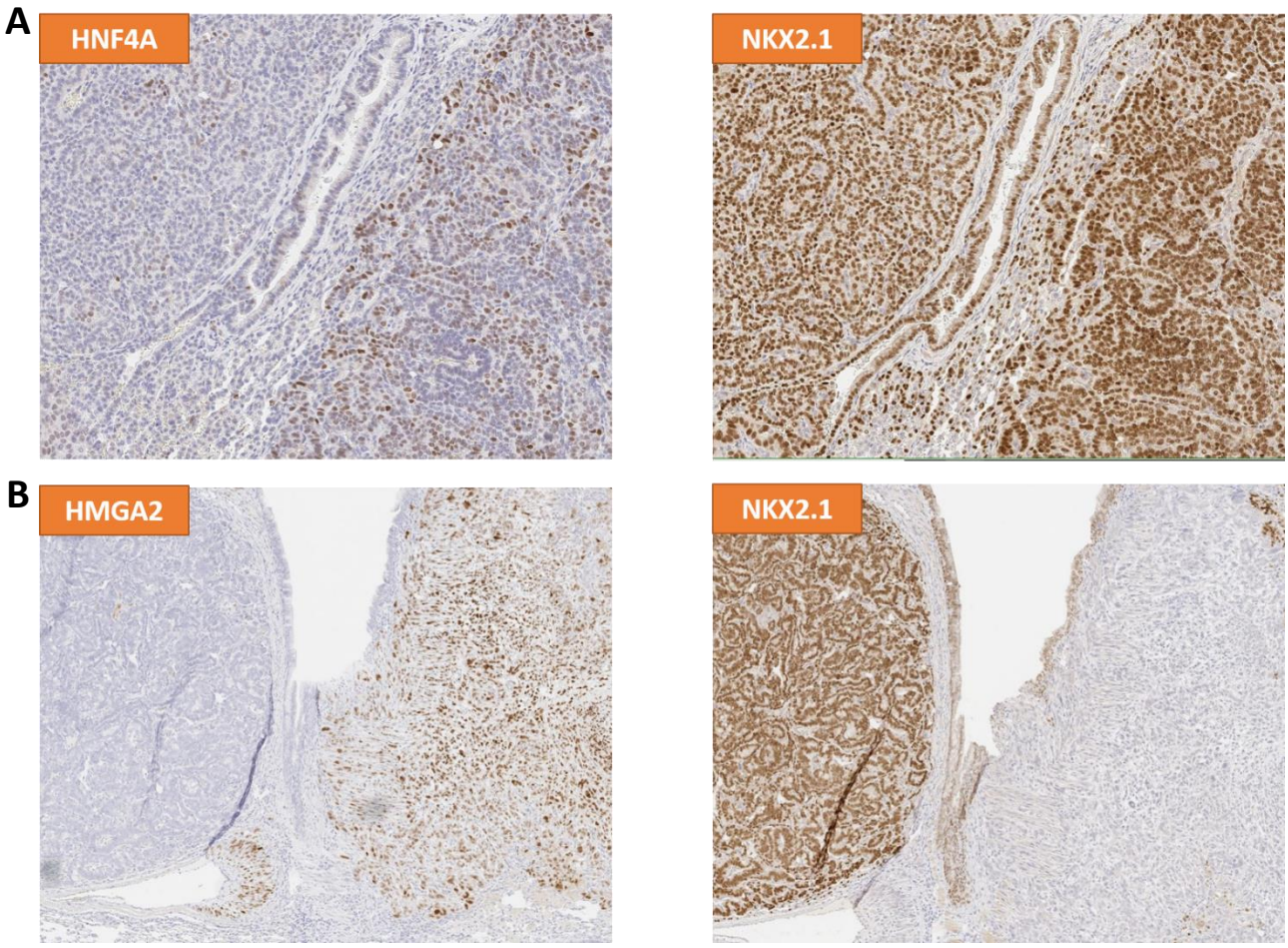


Figure 2.6. *Immunohistochemistry staining of D45 Bmi1 KO tumors. Staining of adjacent sections with HNFAA and NKX2.1 reveals overlapping expression, with NKX2.1 being ubiquitous and HNF4A being sparse (**A**). Staining of HMGA2 and NKX2.1 in adjacent sections reveals mutually exclusive expression, with HMGA2 expression being limited to high grade, invasive tumor regions (**B**).*

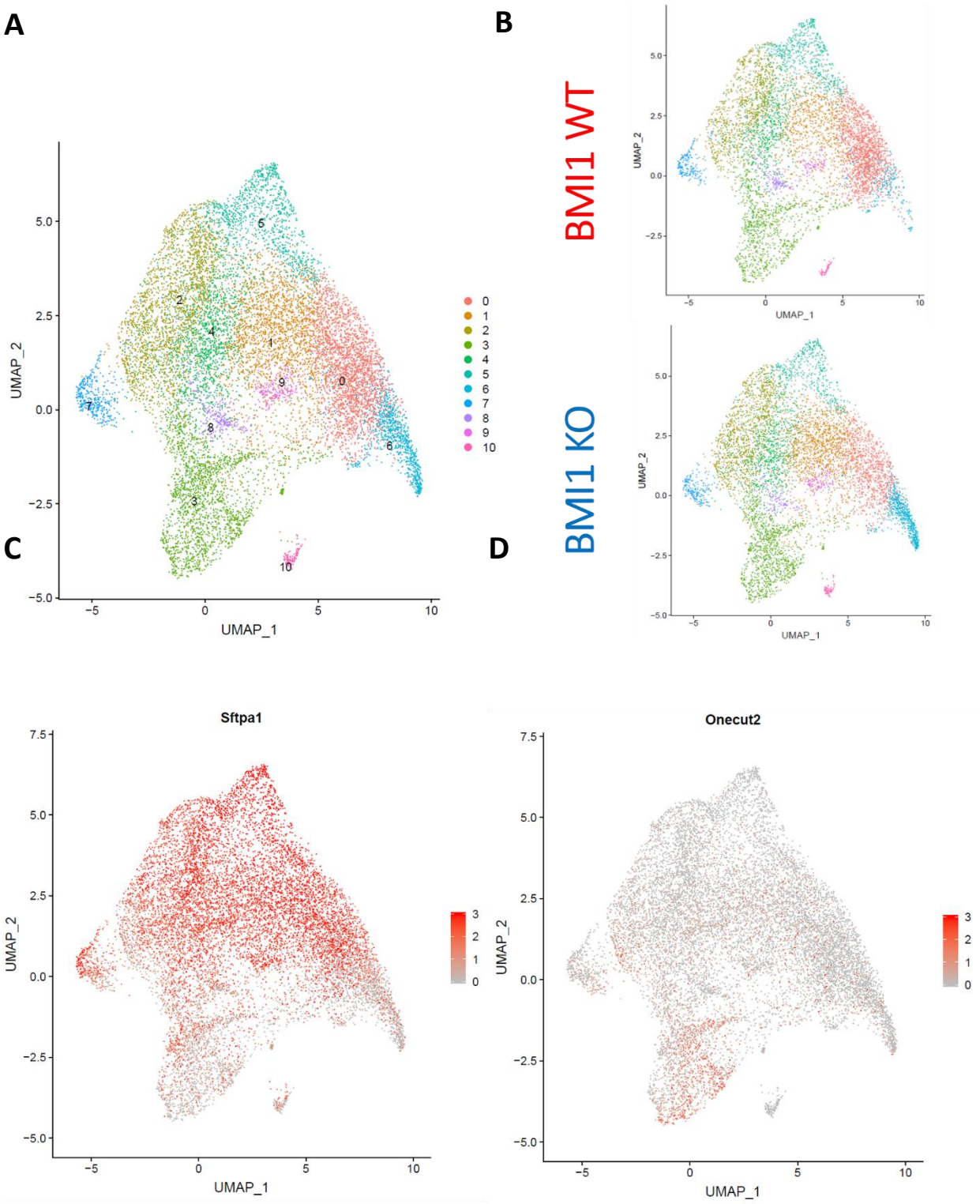


Figure 2.7. *UMAPs depicting the single cell RNA sequencing results. Mixing of all 8 samples (4 of each genotype) results in a UMAP with 10 clusters (**A**), none of which significantly enrich for tumors cells from either genotype (**B**). Overlay of expression pattern of Sftpa1, a pulmonary marker (**C**), and Onecut2, a gut lineage marker (**D**), shows that UMAP is able to distinguish between the two differentiation programs.*

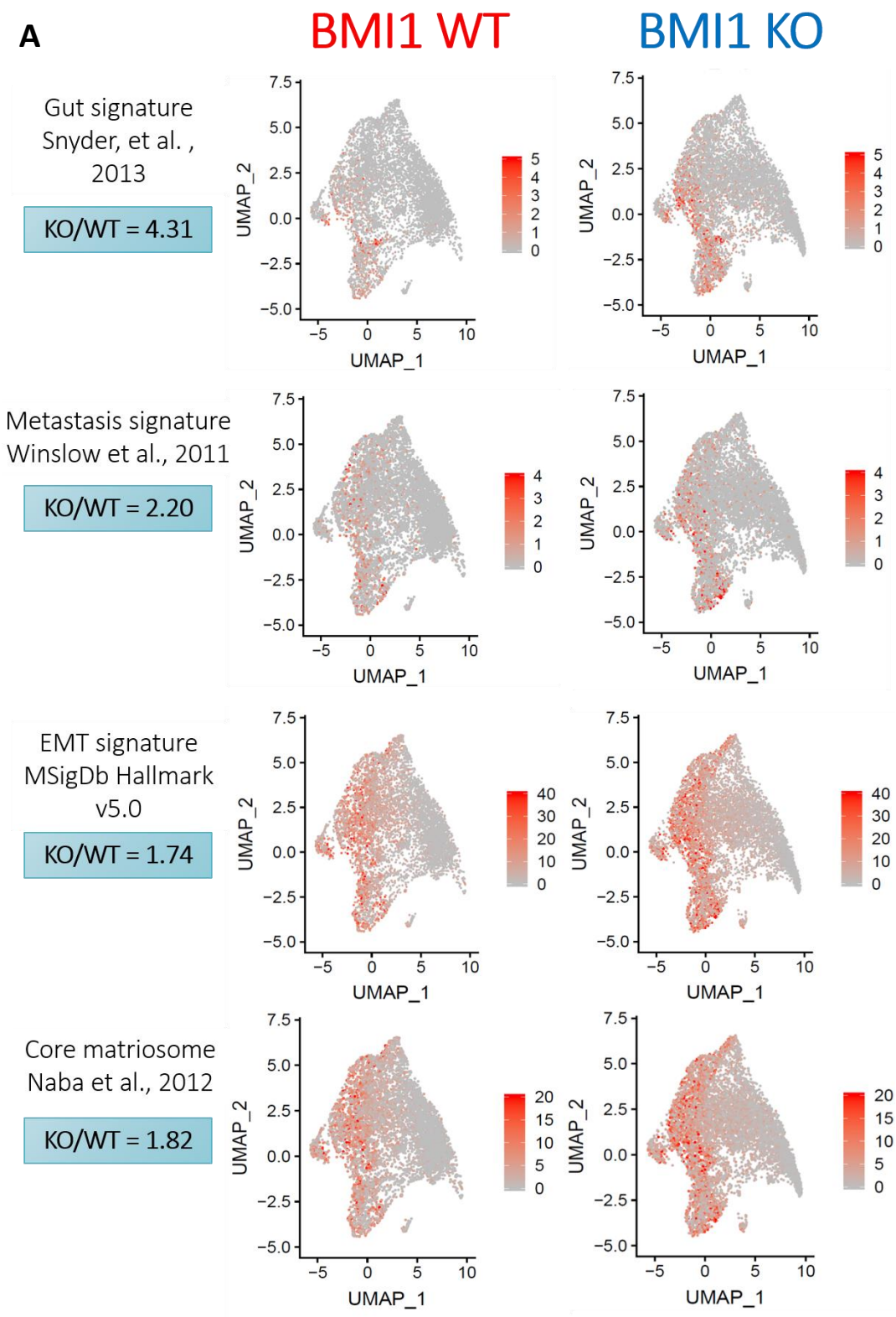


Figure 2.8. *Depiction of WT and KO cells expressing: the gut lineage signature defined in Snyder et al., 2013 (**A**), the metastatic signature defined in Winslow et al., 2011 (**B**), the EMT signature from the hallmark collection in mSigDb (**C**), and the Naba core matriosome signature from the C2 collection in mSigDb (**D**). The ratio of KO/WT cell number shows that a higher fraction of KO cells express these signatures that WT cells.*

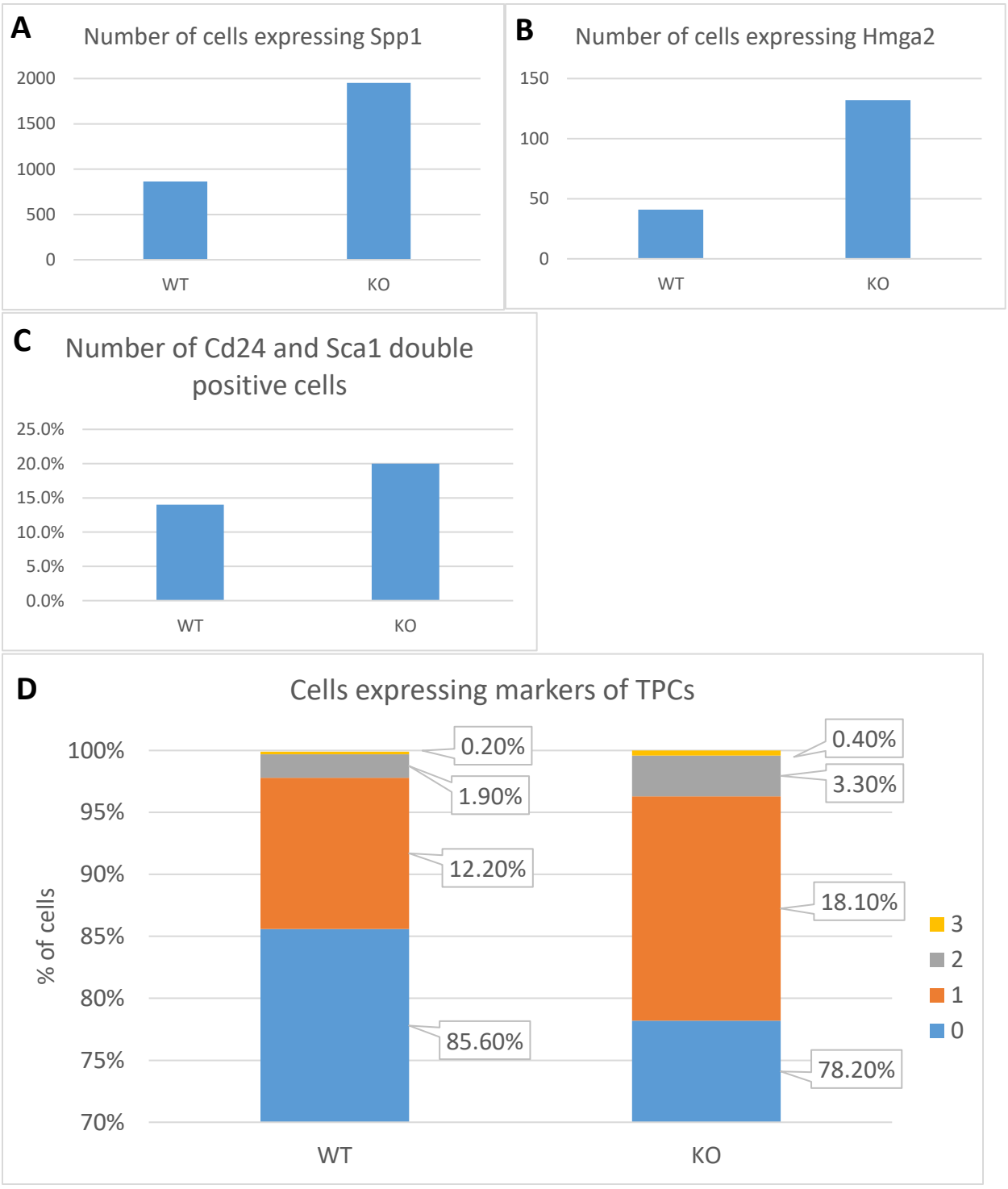


Figure 2.9. *Cell frequency per genotype for each gene plotted for key tumor progression markers: Spp1 (**A**), Hmga2 (**B**), double positive for CD24 and Sca1 (**C**), and 1, 2 or all 3 of CD24, Itgb4, and Notch (1, 2, or 3) (**D**). Legend for **D** reads as: 3 = cells positive for all 3 markers, 2 = cells positive for any 2 markers, 1 = cells positive for any 1 marker, and 0 = cells not positive for any markers. In each marker set, Bmi1 KO tumors have a higher frequency of cells expressing these markers compared to WT tumors.*

Materials and Methods

Mice, and tumor initiation and *Bmi1* deletion

Mice harboring $Kras^{Frt\text{-}STOP\text{-}Frt\ G12D}$ (Young et al., 2011), $p53^{frt/frt}$ (Lee et al., 2012), Rosa26-Frt-STOP-FRT-CreERT2 (Schönhuber et al., 2014), $Bmi1^{fl/fl}$ (M. a Maynard et al., 2013), and Rosa26-Lox-STOP-Lox-TdTomato (Madisen et al., 2010) have been previously described. Mice were infected with adenovirus carrying FlpO recombinase (ad-FlpO) (Vector Biolabs) as described in Dupage et al., 2009, with either 1X10^7 plaque forming units (pfu) or 2.5X10^7pfu. Tamoxifen (Sigma Aldrich) was dissolved in filter sterilized corn oil (Sigma Aldrich) at 10mg/ml, and mice were dosed at 10ul/g for four days via intraperitoneal injection. Animal studies were approved by Committee for Animal Care at MIT.

FACS preparation and sorting

Mice were sacrificed, and lungs were perfused with PBS. Lungs were then chopped using sterilized razor blade and incubated at 37 C for 1 hour in 2mg/ml collagenase/dispase (Sigma Aldrich, 10269638001) and 0.025mg/ml DNase (Sigma Aldrich) in DMEM (Thermo Fisher). The cells were then filtered through 70um filter and red blood cells were lysed using ACK lysis buffer (Thermo Fisher). Following a wash, the cells were incubated with APC conjugated antibodies against CD45 (eBioscience 17-0451-82), CD31 (BioLegend 102510), and Ter119 (BD biosciences 557909) for 20 minutes in ice. After a spin and a wash, the cells were resuspended in PBS+10% FBS with DAPI for FACS sorting. Cells were sorted using BD FACS Aria3 for DAPI negative, APC negative, and TdTomato positive population.

Tumor transplantation

FACS sorted tumor cells were spun and resuspended in serum free optimum (Thermo Fisher) with 10mM EDTA at a count of 5000 cells/70ul. Cells were transplanted into immunocompromised mice using the same technique as intratracheal delivery of adenovirus, as described in Dupage et al., 2009. The only alteration in protocol was an additional 35ul wash after 70ul of cells.

Histology and Immunohistochemistry

BrdU was injected intraperitoneally at 30mg/kg 1 hour before sacrificing the animals. After sacrifice, lungs were perfused with PBS and image was taken for TdTomato expression when applicable. The tissue was then fixed in 10% formalin overnight. Tissue was dehydrated and embedded in paraffin wax to be cut at 4 micron sections. Immunohistochemistry (IHC) was performed using standard protocol with following antibodies: RFP (1:1000, Rockland antibodies 600-401-379), BMI1 (1:200, Millipore F6(05-637), HNF4A (1:1000, Cell Signalling 31135), HMGA2 (1:2000, BioCheck Inc 59170AP), NKX2.1 (1:500, Abcam ab76013), Cleaved Caspase 3 (1:200, Cell Signalling 9661L), BrdU (1:500, Abcam ab6326), and p19 (1:100, Novus 5-C3-1). Thermo Autostainer 360 machine was used for IHC of all antibodies except p19. Images were scanned using Leica Aperio AT2 digital scanner, or captured using Nikon Eclipse microscope with a DS RI2 camera.

Histological grading and quantification

After haematoxylin and eosin staining and digital scanning, deep learning neural network based software, Aiforia, was used to quantify tumor burden and tumor grade distribution based on

trained and pathologist verified algorithm developed by Wescott et al. Tumor size, tumor number, and tumor proliferation rate was quantified using open source QuPath software (Bankhead et al., 2017). An algorithm to detect tumors was trained using a subset of tumors from our samples. A manual quality assessment was made post classification of the tumors by the software. Tumor proliferation rate was measured by calculating number of BrdU positive nuclei per mm^2 of tumor area. Graphical software PRISM and functions in MATLAB were used to generate graphs and perform statistical tests. Violin plots were generated by independent package from file exchange for MATLAB (Hoffmann, 2015).

RNA sequencing and analysis

10000 tumor cells were isolated using the FACS sorting method described above and sorted directly into Trizol (Invitrogen) in an Eppendorf tube coated with FBS. Chloroform extraction was performed on the mix and the aqueous phase was collected for RNA. RNA was then extracted using the RNeasy Micro Kit (Qiagen). The quality of RNA was analyzed using AATI FEMTO Pulse analyzer. Samples were then prepared for sequencing using Kapa mRNA Hyperprep, and sequenced using HiSeq2000 (Illumina).

Differentially expressed genes were analyzed using DeSeq2 package (Love et al., 2014). Preranked gene list using output from DeSeq2 was used to run gene set enrichment analysis (Subramanian et al., 2005). Additional gene sets curated were appended to the C2 gene sets in the molecular signature database v6.2. Unsupervised heirarchial clustering was performed using Morpheus software from Broad Institute (Broad Institute, n.d.).

Single cell RNA sequencing and analysis

Tumor cells were isolated using the FACS sorting method described above and prepared for single cell RNA sequencing using the Seq-Well pipeline described by Gierahn et al., 2017. The cDNA was then sequenced using NextSeq (Illumina). The reads were analyzed using Seurat 3.0 package (Stuart et al., 2018) and visualized using UMAP (McInnes et al., 2018).

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CHAPTER 3: DISCUSSION AND FUTURE DIRECTIONS

Review of Results

In this thesis, I examined the role of *Bmi1*, a component of polycomb repressive complex 1 (PRC1), in tumor progression using a genetically engineered mouse model (GEMM) of lung adenocarcinoma driven by oncogenic *Kras* and loss of *Tp53* (KP LUAD). Many of the prior research in GEMMs established an oncogenic function for *Bmi1*, irrespective of the tumor type being studied (reviewed in Sauvageau & Sauvageau, 2010; Siddique & Saleem, 2012). Most of these studies attributed *Bmi1*'s oncogenic function to repression of *Ink4a/Arf* locus. However, these studies were conducted using mice that had developed *in utero* in the absence of BMI1, resulting in improper silencing of *Ink4a/Arf* tumor suppressor locus, in the tissue being studied. In hindsight, it is unsurprising that this pre-existing deregulation of *Ink4a/Arf* would act to suppress tumors at initiation and early stage of progression.

Our lab has been interested in uncoupling the role of BMI1 during development *in utero* from its role in adult cells. To this end, our lab has focused on preclinical validation of BMI1 as a therapeutic target in cancer models where the animals develop in the context of wildtype (WT) *Bmi1*. Unpublished work from our lab showed that deletion of *Bmi1* at the time of tumor initiation is still tumor suppressive but this was independent of derepression of *Ink4a/Arf* (Karl et al. In preparation). Notably, this tumor suppressive phenotype was alleviated when the tumors reached grade 3 stage of tumor progression. This begged the question of whether this observed adaptation to *Bmi1* loss is an acquired resistance mechanism or a universal phenotype of grade 3 or higher tumors. In other words, we wondered whether established tumors that have progressed to grade 3 or higher would care about deletion of *Bmi1.* This is

relevant in modeling the treatment of lung adenocarcinoma patients in the clinic because patients are often diagnosed at the later stages of tumor progression.

The data in this thesis show that deletion of *Bmi1* in established tumors is not tumor suppressive and there is no derepression of *Ink4a/Arf* locus. Specifically, we observed no induction of apoptosis, reduction in tumor burden, reduction in tumor number, reduction in tumor size, or suppression of tumor progression 45 days after deletion of *Bmi1*. In contrast, *Bmi1* knockout (KO) tumors tended to be larger, and yield a higher total tumor burden, compared to *Bmi1* WT control tumors. Interestingly, the *Bmi1* KO tumors did show reduced mean proliferation rate, compared to WT tumors, which stands juxtaposed to the *Bmi1* KO tumors being larger in size.

Our sequencing of RNA from bulk tumors showed that *Bmi1* KO tumors were significantly enriched for dedifferentiation markers associated with a gastric lineage, which correlates with tumor progression in the KP LUAD model (Snyder et al., 2013). Notably, we observed that many genes were significantly derepressed (logFC > 1, FDR < 0.05) 14 days post *Bmi1* deletion but far fewer genes were significantly different at day 45. Moreover, there was little overlap between these two subsets. We presume that the changes at day 14 reflect the early results of *Bmi1* deletion, including the derepression of *Bmi1* repressed loci and also changes in downstream, indirect targets. As there is significant selection pressure in tumors to lose cells with disadvantageous programs, and select for those with advantageous programs, we speculate that the changes at at day 45 reflect this selection pressure in play. Importantly, although relatively few genes show significant derepression (logFC > 1, FDR < 0.05) at day 45, gene set enrichment analysis, which looks at the overall trend of all genes rather than these individual

genes, clearly identified the gastric lineage program as being activated in *Bmi1* KO tumors. It is currently unclear whether the genes defining the gastric lineage program are direct targets of *Bmi1* or not. It would be interesting to address this question.

Our single cell sequencing of *Bmi1* KO and WT tumors further highlighted tumor progression markers acquisition in *Bmi1* KO tumor cells. In addition to gastric lineage markers, *Bmi1* KO tumors are also enriched for HMGA2, markers associated with metastasis in KP LUAD model, epithelial-to-mesenchymal transition (EMT), and genes of extracellular matrix, all of which have been associated with tumor progression in the KP LUAD model (Rowbotham et al., 2018; Serresi et al., 2016; Snyder et al., 2013; Winslow et al., 2011). Despite clear upregulation of markers of tumor progression, we did not observe any obvious histological changes in the tumors at 45 days post *Bmi1* deletion that pointed to increased tumor progression, such as increase in frequency of grade 4 tumors, or dissemination of tumor cells to lymph nodes or distant organs. This begs for longer term monitoring experiments to assess whether these gene expression changes have any phenotypic consequences.

In addition to markers of tumor progression, the single cell sequencing also strongly suggested that the *Bmi1* KO tumors had higher frequency of tumor propagating cells (TPCs) based on two different TPC marker systems. The first system is defined by expression of *Sca1/Ly6a* (Curtis et al., 2010; Lau et al., 2014) in combination with *Cd24* (Rowbotham et al., 2018). Although *Bmi1* KO and WT tumors contain a similar frequency of *Sca1* expressing cells, the *Sca1* and *Cd24* double positive cells were detected at higher frequency in the *Bmi1* KO tumors. The second system involves *Cd24*, *Itgb4*, and *Notch (1//2/3)* cell surface markers (Zheng et al., 2013), and we find that *Bmi1* KO tumors have higher frequency of cells expressing all three, a combination

of any two, or just one of the markers, compared to the WT controls. Given that single cell sequencing experiments suffer from low gene coverage, these experiments cannot reveal the absolute population frequency of TPCs in the tumor. Thus, it is essential to confirm this result with FACs based analysis that measure the expression of markers at the protein level instead of transcript level. Obviously, TPCs are ultimately defined by functional activity. Our preliminary analyses of tumor propagating activity, by transplanting tumor cells from autochthonous tumors into recipient mice, showed no significant difference between *Bmi1* KO and WT. Experiments that will truly quantify TPC frequency are described below in the Future Directions section.

Overall, our data shows that deletion of *Bmi1* in established KP LUAD tumors does not have any overt tumor suppressive phenotype but rather allows for acquisition of markers of tumor progression. This is in sharp contrast to previous studies of *Bmi1* in cancer demonstrating its oncogenic role, and the observations that *Bmi1* expression level is often correlated with worse prognosis (Zhang et al., 2017; Zhang et al., 2014). However it is important to note that these clinical prognosis studies combine patients of different tumor progression stages, and oncogenic drivers. Since our model only captures LUAD driven by oncogenic *Kras* and loss of *Tp53*, and also points to the tumor progression stage specific role of *Bmi1*, it is important to compare *Bmi1* expression level in similar context in patients. Our attempt to parse out *Bmi1*'s role in tumor genotype specific and tumor stage specific manner was thwarted by the fact that only 4% of lung adenocarcinoma patients in TCGA (n=22) have high expression of *Bmi1* as defined by z-score > 2. This sample number is insufficient to assess correlation with tumor genotype and stage. However, a previous study showed that *Bmi1* expression is lower in lymph

node metastasis of LUAD compared to primary tumors (Xiong et al., 2015), which is consistent with our finding that *Bmi1* loss enables acquisition of pro-metastatic gene expression signatures. Clearly, further analyses of the relationship between *Bmi1* levels and tumor stage in clinical samples are warranted to determine prognostic value for *Bmi1* expression.

A cautionary note on epigenetic targets in cancer

This body of work highlights a cautionary note on the different roles of *Bmi1* during development versus in adult stem cells, and in the context of normal tissue homeostasis versus tumor progression. Even though *Bmi1* is overexpressed in multitude of human cancer types (reviewed in Koppens & van Lohuizen, 2016), work from our lab, including this thesis, shows that *Bmi1*'s role in KP LUAD is context dependent, and that loss of *Bmi1* can induce tumor promoting programs. In fact, *Bmi1*'s role as a tumor suppressor had been described previously in a myelofibrosis mouse model (Oguro et al., 2012), but this study has gone largely unnoticed because of the mountain of evidence arguing for the oncogenic function of *Bmi1*. Intereresting, loss of *Bmi1* was tumor promoting in myelofibrosis because of upregulation of *Hmga2*, which they showed is a direct target of BMI1 (Oguro et al., 2012). Obviously, this has direct parallels with our LUAD model, where *Hmga2* induction is a hallmark of tumor progression and is upregulated in the *Bmi1* KO tumors.

The context dependent role of epigenetic regulators in cancer is even more pronounced if we look at examples beyond PRC1. Components of polycomb repressive complex 2 (PRC2) have been shown to be mutated or overexpressed, depending on the human tumor type, but this complex is generally thought to be oncogenic in function (reviewed in Koppens & van Lohuizen,

2016). However, a recent study on *Eed*, a PRC2 component, showed that deletion of *Eed* at the time of initiation in the KP LUAD model led to more aggressive tumors and reduced survival (Serresi et al., 2016). Moreover, the *Eed* KO tumors showed a switch to a more gastric-like lineage with mucinous characteristics, and *Hmga2* was upregulated concurrently with *Nkx2.1* downregulation at higher frequency compared to controls. This switch was driven by chromatin changes at developmental and pro-oncogenic loci that led to the observed gene activation. *Eed* loss also led to acquisition of a more EMT-like state, and tumors cells invaded frequently into mediastinal lymph nodes. These phenotypes are similar to our findings when *Bmi1* was deleted after tumor establishment, but not at initiation. We saw similar gene expression changes upon *Bmi1* KO in established tumors without accompanying changes in the tumor histology. It is possible that 45 days of monitoring was insufficient for phenotypic changes to manifest in our study and suggests the need for longer monitoring periods, as were employed in the *Eed* KO study.

The importance of studying the long term effects of epigenetic repressor deletion in cancer is further highlighted by recent studies of *G9a*, a H3K9 methyltransferase, which is thought to be a good therapeutic target for cancer. In a model of squamous cell carcinoma (SCC), deletion of G9a at tumor initiation is tumor suppressive via stress induced activation of TP53 pathway (Avgustinova et al., 2018). However, after long latency period, there is an adaptation to *G9a* loss, mostly via the acquisition of loss of function mutations in *Tp53*, which leads to highly malignant and poorly differentiated SCC compared to WT tumors in the same timeframe. *G9a* deletion in established tumors also led to initial regression in more than half of the tumors. However, further monitoring showed that the remaining *G9a* deleted tumors, and also regressed tumors that

relapsed, readily underwent malignant conversion to aggressive SCC and displayed a more dedifferentiated phenotype. The phenotypes here are similar to the consequences of *Bmi1* KO at tumor initiation where we observe early tumor suppressive effects that eventually subside.

Notably, knockdown of G9a in KP LUAD model showed no signs of tumor suppression, even at early stages of tumor progression (Rowbotham et al., 2018). Rather, it improved tumor initiation, as demonstrated by increased numbers of tumors, and enhanced tumor progression, as measured by an increase in the frequency of grade 4 tumors and thoracic pleura and lymph node metastases. In addition, knockdown of G9a in KP LUAD also increased the frequency of TPCs. The differential effects of G9a loss in SCC and KP LUAD highlight context dependency, and the necessity of conducting preclinical testing in appropriate contexts before making general conclusions about the function of the gene in cancer and/or testing inhibitors in clinical trials. Similar caution needs to be applied to testing BMI1 inhibitors in clinical trials, and our results begs for more preclinical testing before moving forward with the BMI1 inhibitor that has recently been tested in a phase I clinical trial (Infante et al., 2017). In concert with our own findings, these studies reinforce the notion that long term studies, in various contexts, are required to fully appreciate the consequences of deleting epigenetic regulators that control global changes in chromatin and gene expression.

The effect of *Bmi1* loss is likely specific to the stage of dedifferentiation

There has been a lot of interest in targeting TPCs in cancer as they are thought to be responsible for both metastasis and relapse after therapy (reviewed in Desai et al., 2019). This has led to considerable efforts to understand both the origin and the biology of TPCs. Given

that many tumor types tend to lose differentiation markers associated with the tissue of origin and gain dedifferentiation markers associated with embryonic stem cells (ESCs) during disease progression, many parallels can be drawn between dedifferentiation towards TPCs and reprogramming of cells towards inducible pluripotent stem cells (IPSCs) (reviewed in Friedmann-Morvinski & Verma, 2014). Analogous to IPSC reprogramming being facilitated by expression of the Yamanaka factors (*Oct3/4, Sox2, c-Myc,* and *Klf4*) (Takahashi & Yamanaka, 2006), a reprogramming and dedifferentiation cascade is induced as an early step of cancer initiation by the driver oncogene, such as *Kras* (Ischenko et al., 2013). This cascade then progresses with loss of differentiated markers and acquisition of more embryonic-like state to give rise to TPCs (Friedmann-Morvinski & Verma, 2014).

Antithetical to the previously described role of *Bmi1* in maintaining TPC frequency, deletion of *Bmi1* in established tumors in this thesis work led to increase in frequency of cells harboring expression of previously described markers of TPCs in the KP LUAD model. This was, however, not observed in tumors where *Bmi1* was deleted at initiation. Collectively, these findings suggest that *Bmi1* is required to enable dedifferentiation and tumor progression at early stages of tumor initiation but, at later stages of tumor progression, it becomes a barrier for dedifferentiation and reprogramming towards TPCs. This is highly plausible, given the similarity between TPCs and ESCs, because *Bmi1* is not expressed in ESCs and upregulation of *Bmi1* during embryonic development is important to repress the pluripotency network (Kloet et al., 2016; Morey et al., 2012).

Similar context dependent roles have been elucidated for other epigenetic regulators in IPSC reprogramming. Knockdown of components of PRC1, PRC2, or the H3K9 methyltransferases

acted to impair reprogramming efficiency of fibroblasts into IPSCs (Onder et al., 2012). In contrast, knockdown of *Setdb1*, a H3K9 methyltransferase, was shown to increase the reprogramming efficiency from pre-IPSCs (cells showing pluripotent properties without activation of the core pluripotency network) to fully reprogrammed IPSCs (J. Chen et al., 2013). Thus, the cell state within the dedifferentiation spectrum provides the context that dictates the role of epigenetic regulators in reprogramming, with epigenetic repressors likely promoting reprogramming in earlier stages, and acting as a barrier in later stages. These results could be extrapolated to cancer dedifferentiation and thus progression. *Bmi1* loss could be tumor suppressive during early stages of tumor initiation because *Bmi1* is required for early reprogramming and dedifferentiation. In later stages of cancer progression, similar to the context of IPSC reprogramming, *Bmi1* could act as a barrier to dedifferentiation towards TPCs. We can imagine that cells fall within a spectrum of dedifferentiated cell states in already established tumors, and that loss of *Bmi1* allows some of them to push through the dedifferentiation cascade more efficiently than WT. In achieving this end, it is possible that loss of *Bmi1* in established tumors acts in a stochastic manner to derepress a variety of differentiation programs, similar to the consequences of *Bmi1* KO at tumor initiation, but there is a stronger selection pressure for cells that acquire dedifferentiated states, such as the gastric lineage program and TPC signatures, that enable tumor progression.

Lung to Gut Specification

There is extensive evidence that dedifferentiation plays a critical role in in cancer progression, including for LUAD (Li et al., 2015; Snyder et al., 2013; Winslow et al., 2011). Prior study in KP

LUAD established that NKX2.1 physically interacts with FOXA1/2, and deletion of *Nkx2.1* leads FOXA1/2 to move from pulmonary genes to gastric genes, including *Hnf4a* (Snyder 2013). This suggests that NKX2.1 is directly responsible for recruiting FOXA1/2 to pulmonary lineage genes and inhibiting expression of gastric lineage genes. When *Foxa1/2* are deleted simultaneously with *Nkx2.1*, the cells assume a more squamous phenotype (Camolotto et al., 2018). This suggests that the cells preserve the ability to transdifferentiate but their lineage identities are maintained by key transcription factors. As WT KP LUAD progresses, coexpression of *Hnf4a* with *Nkx2.1* emerges (our own observations). In patients, *Nkx2.1* expression is lost in a subpopulation of patients, and this is correlated with worse outcomes and non-pulmonary cell state (Barletta et al., 2009; Cardnell et al., 2015). These events are thought to reflect dedifferentiation of the tumor cells to more progenitor-like programs. Notably, the *Nkx2.1* low, gastric program high phenotype is largely observed in LUAD patients with oncogenic *Kras* (Guo et al., 2017). This raises the possibility that this dedifferentiation program is specifically enabled by oncogenic *Kras*, and that other forms of LUAD might take different routes to tumor progression.

The mechanism by which lung to gut dedifferentiation is triggered, or *Hnf4a* and *Nkx2.1* are co-expressed, is unclear. Apart from FOXA1/2 moving to gastric lineage genes in *Nkx2.1* KO tumors, precise mechanism as to why this occurs during normal tumor progression hasn't been fully established. One molecular subtype of LUAD with high expression of *Hnf4a* and no detectable *Nkx2.1* is invasive mucinous adenocarcinoma (IMA), and induction of *Foxa3* or *Spdef* along with mutant *Kras* is sufficient to drive similar mucinous tumors in GEMMs (Guo et al., 2017), suggesting this might be one route to dedifferentiation. Additionally, *Eed* KO during

tumor initiation leads to gastric dedifferentiation and subsequent acquisition of HMGA2 in KP LUAD tumors (Serresi et al., 2016). *Eed* KO leads to EMT, and chromatin level and gene expression changes driven by TEAD 1, downstream of TGFb and Hippo pathway. However, it is unclear whether TEAD1 directly regulates EMT and/or gastric dedifferentiation.

In our study, *Bmi1* loss yields spectrum of *Nkx2.1* single positive, *Hnf4a* and *Nkx2.1* double positive, *Hnf4a* and *Hmga2* double positive, and *Hmga2* single positive tumors, as occurs in normal progression in KP LUAD. Although we cannot rule out that transdifferentiation is occurring, these data suggest that *Bmi1* loss is acting to promote the typical dedifferentiation program. Obviously, questions remain about how *Bmi1* loss promotes these gene expression changes and whether it extends to tumors beyond KP mutant LUAD, as discussed in the Future Directions section below. We observe upregulation of *Foxa3* at 14 days post *Bmi1* deletion (logFC = 0.98, FDR = 0.0856), which could potentially be driving this transition. However, *Foxa3* is no longer differentially expressed at 45 days post *Bmi1* deletion, possibly owing to WT tumors starting along the dedifferentiation cascade as well.

Future directions

Our data clearly show that there is an increase in frequency of cells expressing markers of dedifferentiation along the gastric lineage, as well as markers associated with tumor progression and metastasis, such as *Hmga2*, *Spp1*, epithelial-to-mesenchymal (EMT) signature, and core extracellular matrix genes. However, we do not observe a corresponding increase in invading grade 4 tumors, or lymph node metastasis, or secondary tumors upon transplantation into recipient mice. There is strong reason to believe that any phenotypic changes would lag

behind the observed gene expression changes. In the studies described above, where deletion of epigenetic repressors led to more aggressive tumors, there was a long latency period between the deletion of genes and emergence of aggressive phenotype. This suggests that we need to extend our monitoring period beyond just 45 days post *Bmi1* deletion. We have an experiment ongoing in which we deleted *Bmi1* at a slightly earlier time point to allow 11 weeks of monitoring post *Bmi1* deletion. Additional experiments need to be conducted in which we lower the viral titer to decrease the number of tumors launched, thereby allowing the mice to live longer before they become moribund, and allowing longer monitoring post *Bmi1* deletion. Previous work from our lab and others has shown that KP LUAD model can progress to distant liver metastasis under these conditions.

Our ongoing experiment, and the approach of inducing tumors with lower viral titer, will allow us to determine whether the increase in markers of tumor progression occurring in *Bmi1* deficient tumors can result in phenotypic changes. We want to determine whether there is an increase in dissemination to lymph nodes, nearby sites such as heart and rib cages, and also in distant organs such as liver. We can also monitor circulating tumor cells from the blood as a measure for tumor cell dissemination. Our expectation is that *Bmi1* KO tumor cells would be more likely to disseminate compared to WT tumor cells. This experiment also allows us to examine tumor burden and histology after a longer latency period post *Bmi1* deletion. It would be intriguing to assess whether we see increased grade 4 frequency, mucinous tumors, and more HMGA2 positive tumors, similar to the phenotype observed upon *Eed* deletion in KP LUAD (Serresi et al., 2016).

Our single cell sequencing data also suggests that there are more TPCs in *Bmi1* KO tumors as measured by expression of CD24, ITGB4, and Notch (1/2/3), and also SCA1 and CD24. We are planning on using our ongoing experiment to perform FACS analysis of tumor cells to check for TPC populations based on analysis of these two marker combinations. A more direct assessment of TPC frequency is to perform *in vivo* limiting dilution transplant assay (O'Brien et al., 2010). We are planning on performing this assay with our mice from ongoing experiment to examine whether there is a true increase in TPC frequency upon *Bmi1* loss.

It is also essential that we understand the mechanistic bases for how loss of *Bmi1* is not tumor suppressive in late stage tumors, but rather promotes acquisition of markers of tumor progression in this context. Augmenting the RNA sequencing analyses with ATAC-seq to understand the chromatin level changes, and CHIP-seq for histone marks to understand the changes in state of repressing and activating marks, would be highly informative. Currently, we do not know the precise molecular mechanism(s) by which *Bmi1* loss leads to dedifferentiation, or the identity of the transcription factors or epigenetic regulators involved. Motif analysis at active and open promoters and enhancers could give us insight into the players responsible for the dedifferentiation. We also do not know whether the genes upregulated and enriched for in *Bmi1* KO tumors are direct targets of *Bmi1*. Chromatin level interrogation would address these questions.

Even though we do not see a tumor suppressive phenotype upon deletion of *Bmi1*, we know that complete loss of PRC1 is tumor suppressive but also highly toxic to the animals (Chiacchiera et al., 2016). Canonical PRC1 can contain two PCGFs: PCGF2 (MEL18), or PCGF4 (BMI1), while non-canonical PRC1 can contain any of the 6 PCGF proteins (Gao et al., 2012;

Kloet et al., 2016). It is plausible that one of the other PCGFs is functionally redundant to BMI1 when it is deleted, either at tumor initiation or in established tumors. It is also possible that in the absence of BMI1, another PCGF might sequester the PRC1 complex towards different sets of targets. A prime candidate for this hypothesis is MEL18. It is important to assess whether double KO of *Bmi1* and *Mel18* has tumor suppressive, or tumor promoting phenotypes and whether there is a therapeutic window. *In vitro* experiments are currently underway, and it would also be possible to address this question in the context of established autochthonous tumors by crossing a *Mel18*$^{fl/fl}$ allele into our mouse model.

Lastly, it is important to assess whether the phenotype we see in our KP LUAD model could hold true in other contexts. As we note above, it is plausible that *Bmi1* deletion phenotype we observe is genotype and/or tumor type specific, and *Bmi1* loss could still be tumor suppressive in other contexts. Our colorectal cancer model development is a step towards addressing this question, and the preliminary results suggests that *Bmi1* deletion is not tumor suppressive. Specifically, we do not observe any effect upon deletion of *Bmi1* in transformed colon organoids, or orthotopic transplant tumors. Similar to KP LUAD model, we have not monitored the transplanted tumors for long after *Bmi1* deletion, and based on our observations and others, it might be key to understand any effect upon *Bmi1* loss. It is also important to assess *Bmi1*'s role in autochthonous colorectal cancer tumors as the role of *Bmi1* could be highly context dependent, as shown in our KP LUAD model. Since both of the tumors have common lineage ancestors, it would be important to test this in other settings as well. The KP model can be used to develop sarcoma or pancreatic cancer, which would mean our mice already have the right allele combinations to address the importance of *Bmi1* in these tumors types. Additionally,

it would be important to address the role of *Bmi1* in LUAD driven by different oncogenes to examine whether tumor genotype is a determinant of *Bmi1* loss phenotype.

Limitations of our mouse model

The main goal of this thesis was to examine the role of *Bmi1* in LUAD in a more clinically relevant mouse model. There are obvious issues with previously used germline KO models of *Bmi1* as described above. However, it is important to note that the model we used in this thesis does not completely mimic the treatment paradigm in the clinic, and thus also has shortcomings. The primary limitation is that it is cell autonomous, assessing the consequences of loss of *Bmi1* specifically in tumor cells. However, small molecule inhibitors against BMI1 would have couple of key differences. First, the inhibitors would target BMI1 in all tissue types, including the stromal cells and immune cells, and not just tumor cells. This is especially important given that *Bmi1* has been shown to play a role in maintaining stem cell function via its in role in microenvironment (Oguro et al., 2006), and in maintaining normal hematopoietic differentiation, and especially white blood cell production, at least when absent in utero (Oguro et al., 2010). A way to model this would be to combine a CreER allele that is ubiquitously expressed with our *Kras-frt-STOP-frt-G12D*; *Tp53*$^{frt/frt}$; *Bmi1*$^{fl/fl}$ LUAD model, resulting in *Bmi1* deletion in whole animals upon tamoxifen treatment. The second difference is that any small molecule inhibitor would likely yield partially inhibition of BMI1 function rather than completely abolishing the BMI1 protein. There might be dose dependent phenotypes, which we are not modelling. Also, in the complete absence of BMI1, other PCGFs might direct PRC1 towards different targets, thus, altering PRC1 function.

Concluding remarks

Given the mountain of evidence, from more than a decade worth of research, supporting an oncogenic function of *Bmi1*, there is a lot of interest in targeting BMI1 in cancer. This has even led to development of inhibitors against BMI1 (Infante et al., 2017; Kreso et al., 2014a), which are already being used in clinical trials. Although there is some reason to question the specificity of the current BMI1 inhibitors (our unpublished observations), we believe that there is reason to be concerned about the use of BMI1 inhibitors in patients in general. Our results reveal a context dependent role for *Bmi1*, at least in the KP LUAD model, and argue for a tumor suppressor role in late stage disease. Based on these findings, it seems critical that more preclinical testing needs to be done before moving forward with the BMI1 inhibitors. Our results and other recently published literature on targeting epigenetic regulators in GEMMs also beg the cancer field that the biology of potential therapeutic targets be assessed in autochthonous mouse models that provide stronger preclinical modelling of human cancer.

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APPENDIX: BMI1 DELETION IS NOT TUMOR SUPPRESSIVE IN COLORECTAL CANCER

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Abstract

Bmi1 is a known regulator of stem cell function, and it has also been shown to have oncogenic role in cancer. Previous studies have described the tumor suppressive effects of germline *Bmi1* deletion in various cancer models, including colorectal cancer (CRC). However, no study has examined the role of *Bmi1* in tumor progression when the animals developed *in utero* with normal *Bmi1* levels. In this study, we describe a novel model of CRC that was developed to assess the consequences of *Bmi1* loss in autochthonous CRC tumors. Our preliminary data strongly suggest that *Bmi1* deletion is not tumor suppressive in our tumor organoid and orthotopic transplant models. This is consistent with our findings in lung adenocarcinoma, suggesting that *Bmi1* loss will be an ineffective cancer treatment in at least two cancer types.

Introduction

According to the Cancer Facts & Figures data published in 2019, colorectal cancer (CRC) is the 3rd leading cause of estimated new cases and estimated deaths in the United States (Siegel et al., 2019). Given the huge push for screening for polyps using colonoscopy, more than 3/4th of the cases are diagnosed at local tumor stage or with regional spreading to lymph nodes (SEER, 2019a). These early diagnosis cases have a significantly better 5-year survival rate compared to diagnosis at distant metastatic stages (Siegel et al., 2019). However, there is still a huge unmet clinical need for the 1/4th of patients that are diagnosed at metastatic stages.

Given the role of tumor propagating cells (TPCs) in cancer spreading and metastasis (reviewed in Desai et al., 2019), there has been a lot of interest in identifying TPCs in CRC and exploiting their vulnerabilities as therapies. TPCs has been identified in patient samples using a combination of cell surface markers and functional assays such as xenotransplantation or tumor sphere formation *in vitro* (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Vermeulen et al., 2008). Similarly, TPCs have also been identified using lineage tracing experiments in genetically engineered mouse models (GEMMs) of CRC (Schepers et al., 2012). These data, combined with well-established stem cell organization and dynamics in colonic crypts (Humphries & Wright, 2008), has made CRC an excellent model to assess TPC biology.

Given our lab's interest in understanding the role of *Bmi1* in tumor biology and TPCs broadly, we have extended our studies from lung cancer to CRC. CRC GEMMS are very well established given the thorough understanding of the genetic progression of colorectal cancer in patients (Fearon & Vogelstein, 1990). Most are driven by loss of *Apc*, a tumor suppressor lost in about

90% of patients, in combination with known cooperating mutations, such as oncogenic *Kras* and/or loss of *Tp53* (reviewed in Romano et al., 2018). One of the biggest drawback of early CRC models was that the tumors developed primarily in small intestine, which is not the predominant site of disease in patients. This has recently been rectified using more colonic epithelium specific promoters, such as promoter of carbonic anhydrase I or *Cdx2* or *Ts4*, to drive the Cre recombinase that led to genetic alterations necessary for tumor initiation (reviewed in Romano et al., 2018).

Using an early, *Apc*-driven model of CRC, our lab showed that germline absence of *Bmi1* or tissue specific deletion of *Bmi1 in utero* is tumor suppressive, resulting in impaired CRC initiation and progression of early tumor stages (M. A. Maynard et al., 2014). Similar to many other *Bmi1* studies, this phenotype was largely due to *Ink4a/Arf* derepression, and loss of *Arf* largely rescued the tumor suppressive effect. Other studies have addressed the role of *BMI1* in the TPCs of colorectal cancer patient samples, showing that knockdown of *BMI1* decreased the TPC frequency as measured by limited dilution xenotransplantation assay (Kreso et al., 2014b).

Similar to the study in KP LUAD model, we are interested in assessing the effect of *Bmi1* deletion in established tumors in GEMMs of CRC. Given the requirement of alleles to uncouple genetic events needed to launch tumors from deletion of *Bmi1*, we were unable to use any of the existing CRC models. This led us to develop a new autochthonous and transplant model of CRC in collaboration with Yilmaz and Jacks laboratories. Using these models, we show that deletion of *Bmi1* is not tumor suppressive, antithetical to previously described literatures.

Results

The goal of our study is to establish the role of *Bmi1* in tumor progression in CRC, and assess whether deletion of *Bmi1* has any impact on established tumors. This required us to uncouple the mutational events necessary for tumor initiation and subsequent deletion of *Bmi1*, in a similar manner to the KP LUAD studies described in Chapter 2. Since we wanted to mimic the treatment paradigm in the clinic, we decided to use a model that progresses easily to invasive adenocarcinoma stage, with some metastasis: CRC driven by loss of *Apc*, activation of oncogenic *Kras*, and deletion of *Tp53* (AKP) (Romano et al., 2018). Unfortunately, there is no *Apc* conditional allele that is flanked by frt sites to allow deletion by FlpO recombinase. Thus, we decided that we would use viruses to deliver the FlpO recombinase, along with components to enable either CRISPR mediated deletion of *Apc* or hairpin mediated knockdown of *Apc*, into the KP Bmi1$^{fl/fl}$ GEMM described in Chapter 2.

The easiest way to stably deliver these components is to use a lentiviral vector. In a collaboration with the Yilmaz and Jacks laboratories, we established a colonoscopy assisted injection technique that delivered the payload underneath the colonic epithelium in the submucosal layer (Figure 3.1A) (Roper et al., 2017). By injecting into *Rosa26 lox-STOP-lox TdTomato* mice, it was shown that injection of a lentivirus carrying the Cre recombinase resulted in expression of TdTomato in stem cells at the base of the colonic crypt (Figure 3.1B). Furthermore, injection of same virus in *Apc*$^{fl/fl}$ mice yielded adenoma polyps, as detected by colonoscopy and characterized by histology (Figure 3.1C). These experiments validated the system as a new mouse model of CRC.

Once this injection technique was established, we then attempted to deliver lentivirus with sgApc-Cas9-FlpO into our KP *Bmifl*^{cflc} and KP *Bmi1*$^{+/+}$ LUAD GEMM (Figure 2.1). However, this viral vector was very large, preventing production of high titer of virus, and we were unable to generate any tumors. To circumvent this viral titer problem, we collaborated with the Langer lab to general lipid nanoparticles carrying mRNA for Cas9 and sgRNA against *Apc*. However, this technique did not yield deletion of *Apc*, most likely due to fast clearance/degradation of sgRNA. We then sought to knockdown *Apc* to achieve tumor initiation. However, KP mice receiving injections of lentivirus carrying shApc and FlpO developed aggressive colorectal sarcoma, driven by oncogenic *Kras* and deletion of *Tp53* in the stromal cells in the submucosa, and became moribund before CRC was observed.

Based on our inability to generate autochthonous CRC, we decided to use orthotopic transplantation route to assess *Bmi1*'s role. We generated colon organoids using the colonic tissues isolated from our KP *Bmi1*$^{fl/fl}$ and KP *Bmi1*$^{+/+}$ mice using previously described methods (Figure 3.2A) (Miyoshi & Stappenbeck, 2013; Sato et al., 2011). These organoids were then infected with lentivirus carrying either sgApc-Cas9-FlpO or shAPC-FlpO to generate tumor organoids with *Apc* deletion/knockdown, oncogenic *Kras*, and deletion of *Tp53* (AKP organoids). We then deleted *Bmi1* and activated TdTomato by plating the organoids in presence of 4-hydroxy-tamoxifen (4OHT). Organoids with *Bmi1*$^{fl/fl}$ alleles that did not get (4OHT) served as a paired cell line control, while organoids with *Bmi1*$^{+/+}$ (WT) alleles served as a control for any potential effect of 4OHT treatment.

To assess the effect of *Bmi1* deletion, we performed a serial replating assay. Tumor cells were FACs sorted for GFP that was introduced via a second lentiviral infection, and 1000 tumors cells

were plated at each replating. We measured tumor sizes at day 5 and day 7 post plating to give the organoids time to grow. We did not see a significant difference between *Bmi1* KO organoids and the controls at any of the time point for any of the replating, as tested by Mann Whitney test except for day 5 time point at replating # 3 (Figure 3.2B). However, this difference was seen between organoids treated with 4OHT and controls for both *Bmi1* KO and WT. Thus, this does not appear to be a *Bmi1* specific effect. We also calculated the growth rate for each of the conditions by calculating surface area change between day 5 and day 7. Again, no significant effect was observed upon deletion of *Bmi1* (Figure 3.2C).

To assess the effect of *Bmi1* deletion *in vivo*, we orthotopically transplanted organoids into immunocompromised mice using colonoscopy assisted injection technique (Roper et al., 2017). The tumors were allowed to grow for 2.5 weeks before the mice were treated with tamoxifen to delete *Bmi1* (Figure 3.3A). The mice were then monitored for 15 days after last dose of tamoxifen before sacrifice. Using colonoscopy, we were able to detect tumors in each case before tamoxifen injection, and the tumors were confirmed to be TdTomato positive post tamoxifen injection (Figure 3.3B). Moreover, we confirmed the deletion of *Bmi1* using immunohistochemistry of the tumor sections (Figure 3.3C). We could not directly compare tumor sizes with this techniques, because the injection method doesn't reliably deliver the same number of organoids each injection, and we couldn't measure tumor size before tamoxifen injection based on colonoscopy. Moreover, since CRC tumors are graded based on invasiveness, and since the organoids are transplanted submucosally, we cannot compare grade as a marker of tumor progression. However, assessment of proliferation rates of tumors, based

on analysis of BrdU incorporation after a 1 hour pulse, showed no significant difference between *Bmi1* deleted tumors and any of the controls (Figure 3.3D).

Given the issues of sarcoma formation in the autochthonous tumor model, and inability to monitor tumor progression based on invasiveness in the orthotopic transplant model, we are currently exploring alternatives to the colonoscopy assisted injection technique. In particular, we are testing a variant of the organoid transplantation method in which the colonic epithelium is damaged by dextran sodium sulfate (DSS) treatment prior to organoid delivery via enema (O'Rourke et al., 2017). In this method, the organoids engraft within the epithelial layer, allowing subsequent monitoring of tumor invasiveness phenotype. We are also attempting to extend this technique to generate autochthonous tumors by injecting lentivirus into the lumen instead of organoids during enema. We hypothesize that disruption of the epithelium by DSS might enable access to stem cells at the base of the crypts, which we found to be limited when using an enema of virus without DSS treatment.

Summary

Taken together, our preliminary studies suggest deletion of *Bmi1* is not tumor suppressive in CRC. Our *in vitro* replating experiments showed that deletion of *Bmi1* did not exhaust tumor propagating potential and it did not alter the growth rate of tumor organoids. The *in vivo* transplantation studies support these findings, showing that deletion of *Bmi1* in established tumors did not alter the proliferation rate. Further refinement of the model is warranted to assess the effect of *Bmi1* on tumor progression as measured by tumor invasiveness and metastasis. More work is also needed to establish autochthonous model of CRC in which we can assess *Bmi1*'s role in tumor progression and maintenance.

Figures

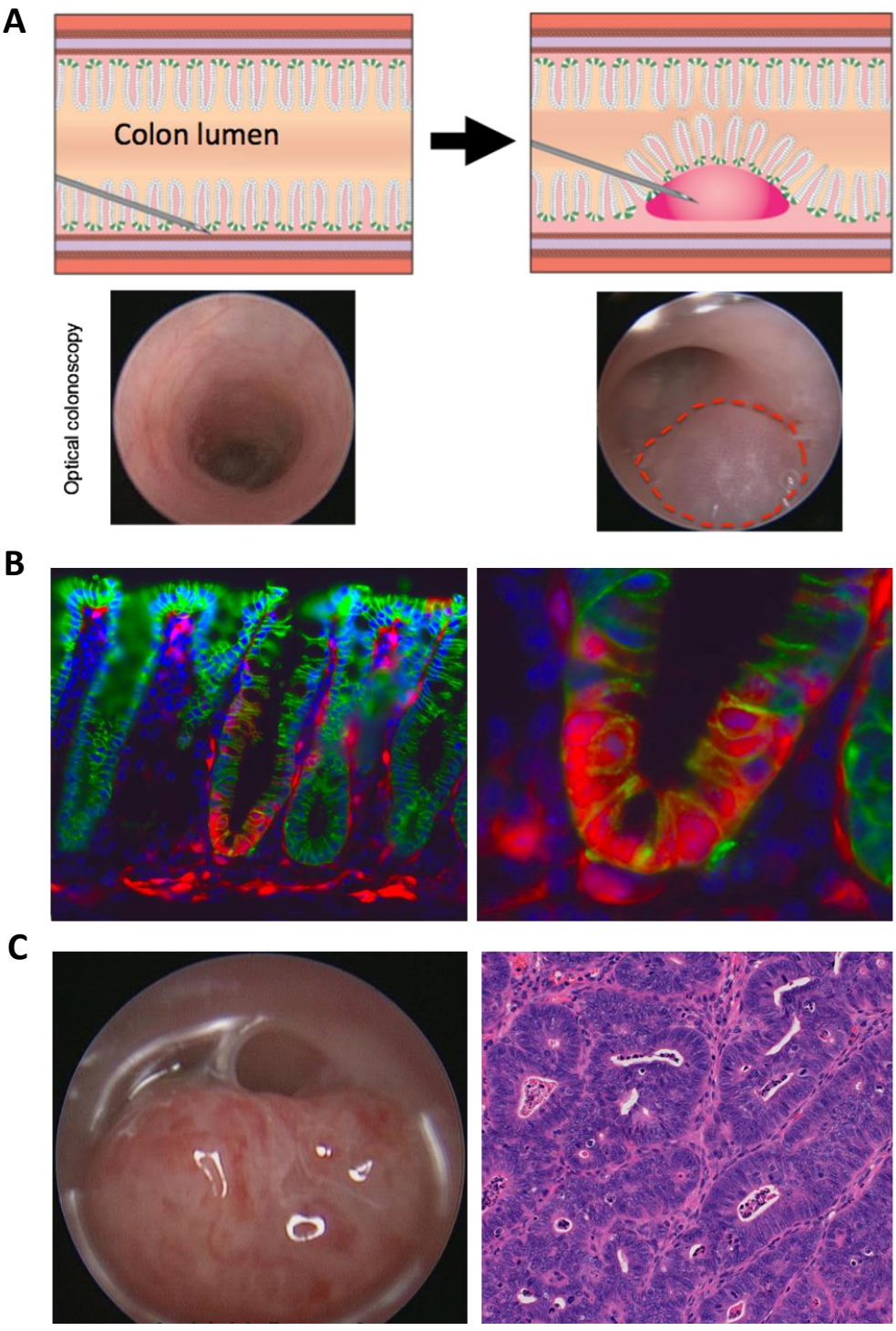

Figure 3.1. Coloscopy guided injection technique. The injection is performed by guiding the needle underneath the colonic epithelium and injecting in the submucosal layer (A). Infection of R26-lox-STOP-lox-TdTomato mice with PGK-Cre lentivirus yields TdTomato expression in stem cells at the base of the crypts, along with stromal cells (B). Injection of Apc fl/fl mice with same virus yields adenoma polyp as diagnosed by colonoscopy and histology (C).

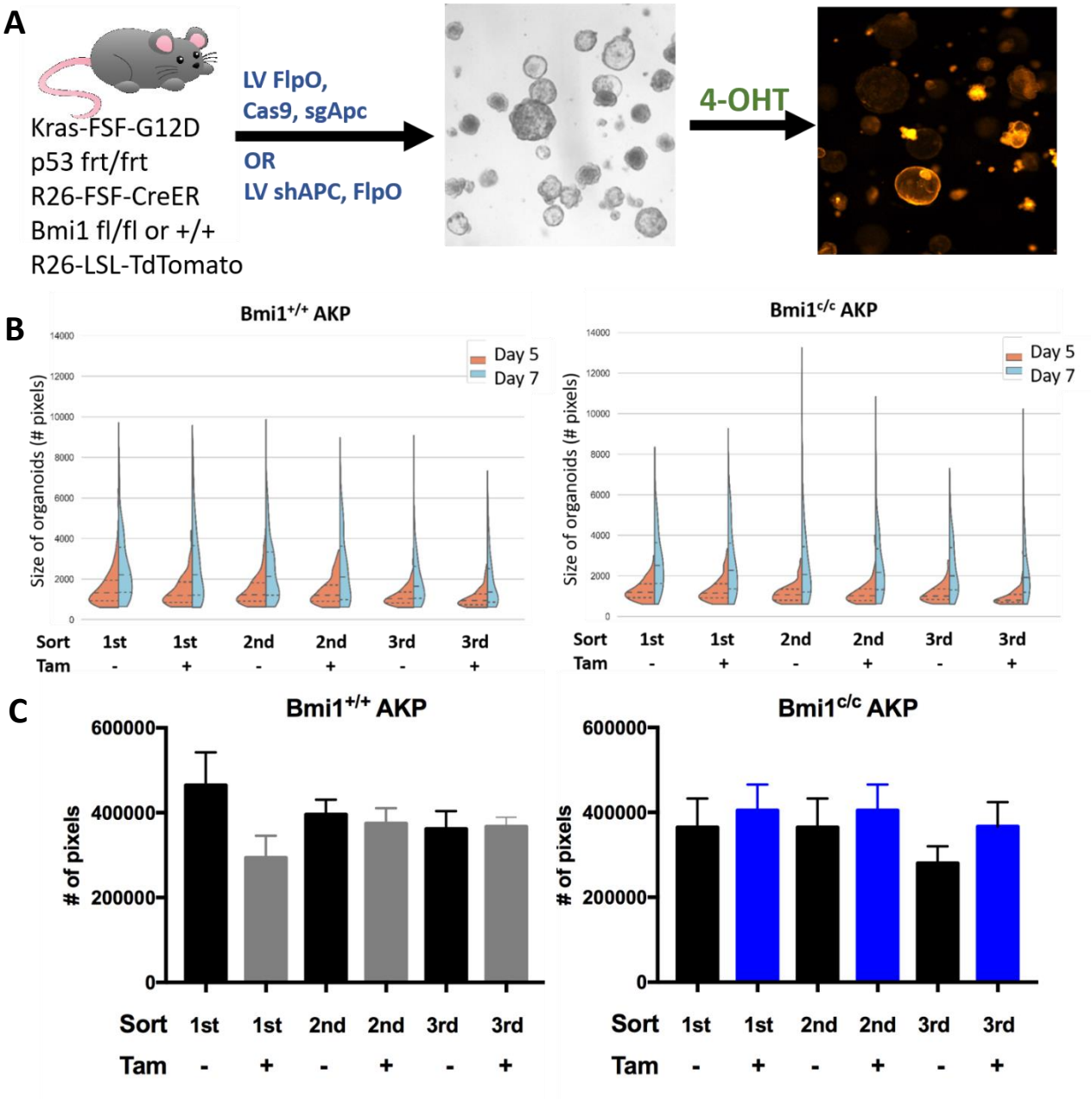

Figure 3.2. In vitro colon organoid assay. A. Colon crypts are isolated from mice with given genotype and grown as organoids in culture. The organoids are transformed by lentiviral infection to generate tumor organoids with Apc deletion/knockdown, oncogenic Kras activation, and loss of Tp53 (AKP). AKP organoids can then be treated with 4-hydroxy-tamoxifen (4OHT) to delete Bmi1 and activate TdTomato expression. B. Tumor organoids were monitored in a replating assay for 3 passages, and organoid sizes quantified at day 5 and day 7. Comparison of size distribution by unpaired t-test revealed no significant difference in any of the comparisons except for day 5 passage 3 between + and – tamoxifen, in both genotypes. C. Tumor organoid growth rate is calculated by subtracting surface area of organoids between day 7 and day 5. No significant difference was observed upon deletion of Bmi1.

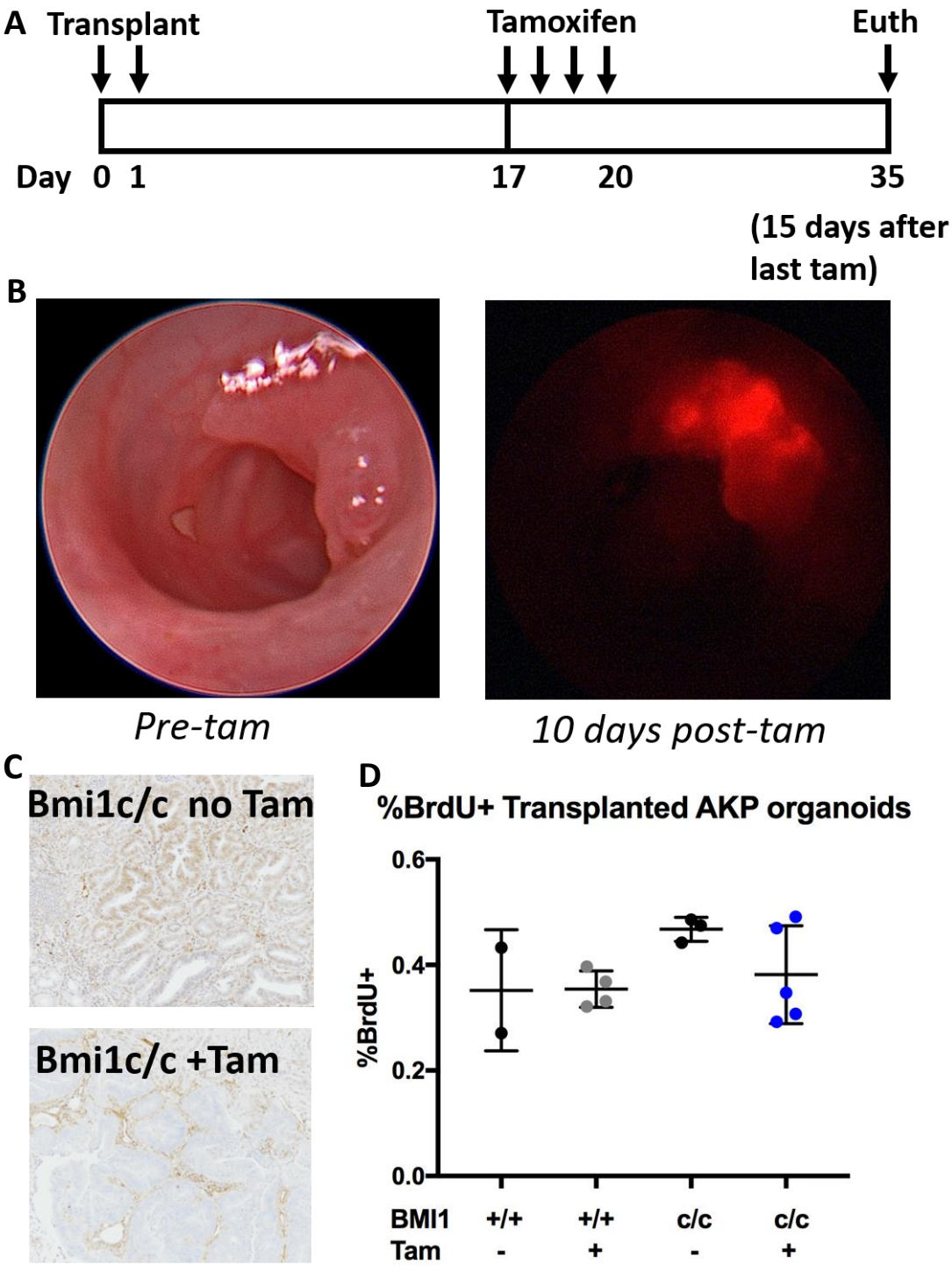

Figure 3.3. In vivo orthotopic transplantation of colon organoids into immunocompromised mice. A. Schematic representing transplantation, tamoxifen treatment, and mice sacrifice time points. B. Tumors were detected by optical colonoscopy prior to tamoxifen injection. Fluorescent colonoscopy detected TdTomato positive tumors post tamoxifen injection. C. IHC confirms deletion of Bmi1 in tumor cells with tamoxifen treatment. D. BrdU incorporation rate is measured between each genotype with or without tamoxifen treatment. No significant difference observed upon deletion of Bmi1 as quantified by Mann-Whitney test.

Materials and Methods

Lentiviral production

Lentiviral backbone pLL3.3 was a gift from Tyler Jacks (MIT). This backbone was modified using Gibson cloning to include transgenes and hairpin of interest (Gibson et al., 2009). Lentiviral particles were generated by transfecting 293FT cells with viral vector, packaging plasmid, psPAX2, and envelope plasmid, pMD2.G (Dull et al., 1998) using TransIT-LT1 (Mirus). Supernatant was collected at 2 days and 3 days post transfection, and concentrated by ultracentrifugation at 25000rpm. Viral vector was determined by infecting 3TZ cells. CRISPR guide against *Apc* used was ggcactcaaaacgcttttga.

Mice

Mice harboring $Kras^{Frt\text{-}STOP\text{-}Frt\ G12D}$ (Young et al., 2011), $p53^{frt/frt}$ (Lee et al., 2012), Rosa26-Frt-STOP-FRT-CreERT2 (Schönhuber et al., 2014), $Bmi1^{fl/fl}$ (M. a Maynard et al., 2013), and Rosa26-Lox-STOP-Lox-TdTomato (Madisen et al., 2010) have been previously described. Animal studies were approved by Committee for Animal Care at MIT.

Colonoscopy assisted injection

This injection technique has been described in Roper et al., 2017. Briefly, mice are anesthetized, and colon is flushed using DI water. Colonoscopy was performed using equipment from Karl Storz: Image 1 H3-Z Spies HD Camera System (part TH100), Image 1 HUB CCU (parts TC200, TC300), 175 Watt D-Light Cold Light Source (part 20133701-1), AIDA HD capture system, and 0" Hopkins Telescope (part 64301AA), and emission 554 nm filter. Colonoscopy was used to guide

a needle (Hamilton Inc, 7803-05) into the colon lumen, and virus or organoid was injected into the submucosal layer of the colon wall.

DSS enema model

Transplant of organoids using DSS enema technique has been described in O'Rourke et al., 2017. Briefly, mice were treated with 3% dextran sulfate sodium (MP Biomedical) for five days in their drinking water. Their body weight was measured daily and stool was examined for change in consistency. The animals were allowed to recover for 2 days, after which they were given an enema of either organoids for transplantation or virus for infection. The anal verge was sealed temporarily with Vetbond Tissue Adhesive (3M) to prevent the enema being expelled immediately.

Crypts isolation

Colonic crypts isolation has been previously described in Sato et al., 2011. Briefly, mice are sacrificed and colon is isolated. After washing the colon, it is opened along the lumen and cut into small pieces. These pieces are incubated with 5mM EDTA in PBS while rocking at 4 C for an hour. After incubation, colonic pieces are washed and manually titrated using a pipette to dissociate crypts. The supernatant is then spun down to retrieve crypts for plating.

Organoid culture

Colonic organoid culture has been previously described in Sato et al., 2011. Briefly, about 500 crypts are mixed with growth factor free matrigel matrix (Corning 356239) such that the end solution is roughly 70% matrigel. 50ul of matrigel crypt mix is plated as a bubble in 24 well plate

and incubated in 37 C incubator for 15 mins by inverting the plate. After 15 mins, 700ul of media is added to cover the matrigel plug.

Previously described WRN conditioned media (Miyoshi & Stappenbeck, 2013) supplemented with ROCK1 inhibitor, Y-27632 (ApexBio A3008) is used for colonic organoids prior to transformation. After transformation by deleting *Apc* (plus activation of oncogenic *Kras* and deletion of *Tp53*), minimal media (Advanced DMEM supplemented with B-27 Supplement (Thermo Fisher) is used.

Organoid cultures are passaged by manual dissociating the matrigel plug, followed by brief incubation with TrypLE Express (Thermo Fisher) at 37 C, and further manual titration to break the organoids. The resulting mix is then passaged at 1:5 ratio by mixing with fresh matrigel.

The same method is used to prepare organoids as a single cell suspension for FACS sorting. The mix is filtered through 40um filter to get rid of clumps.

Organoid quantification

To quantify the number of organoids and their sizes, pictures are taken under a dissecting microscope at a consistent magnification. The pictures are then processed using ImageSegmenter app in MATLAB to generate filled circles in place of organoids. This is then used to count and calculate area.

Transplant

Organoids are prepared in the same manner as for passage. Organoids are then resuspended such that 1 24 well worth of organoid is in 100ul volume of serum free Optimem (Thermo

Fisher) with 10% matrigel (Corning). The organoids are then injected into the submucosal layer using colonoscopy assisted injection technique described above. Animals are then monitored routinely using colonoscopy. Tamoxifen (Sigma Aldrich) was dissolved in filter sterilized corn oil (Sigma Aldrich) at 10mg/ml, and mice were dosed at 10ul/g for four days via intraperitoneal injection.

Histology and Immunohistochemistry

BrdU was intraperitoneally injected at 30mg/kg, 60 minutes before sacrificing the animals. After sacrifice, the colon was isolated, washed, and opened along the lumen. The tissue was stretched along the length and fixed in 10% formalin overnight. After fixation, tissue was dehydrated in ethanol, embedded in paraffin wax, and sections were cut at 4 micron. Standard protocol was used for immunohistochemistry with following antibodies: RFP (1:1000, Rockland antibodies 600-401-379), BMI1 (1:200, Millipore F6(05-637), Cleaved Caspase 3 (1:200, Cell Signalling 9661L), BrdU (1:500, Abcam ab6326), and p19 (1:100, Novus 5-C3-1). Thermo Autostainer 360 machine was used for bulk staining of slides for all antibodies except p19. Images were scanned using Leica Aperio AT2 digital scanner, or captured using Nikon Eclipse microscope with a DS RI2 camera.

Tumor proliferation rate determined by BrdU incorporation was quantified using QuPath, an open source software (Bankhead et al., 2017). An algorithm to distinguish tumor cells from stroma was trained using a subset of our samples. Tumor proliferation rate was measured by calculating number of BrdU positive nuclei per mm^2 of tumor area. Graphical software PRISM was used to generate graphs and perform statistical tests.

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