Genetic screens \textit{in vivo} using the CRISPR/Cas9 system

by

Donald Christian Ellis

B.S., Biophysics
Duke University 2014

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Biology

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 2017

© 2017 Massachusetts Institute of Technology. All rights reserved.
Genetic screens *in vivo* using the CRISPR/Cas9 system

by

Donald Christian Ellis

Submitted to the Department of Biology on January 23, 2017 in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

ABSTRACT

An unmet and paramount need in the field of cancer research is to rapidly translate basic biological findings to clinically relevant therapeutics for cancer patients. Recent technological advances have generated many innovative applications to cancer biology and in a short time have yielded a wealth of information about putative vulnerabilities across a range of cancers. The proposed work involves the development of a technique to quickly probe potential cancer-specific vulnerabilities *in vivo* adopting methods used in genetic screens. By harnessing the information obtained from large datasets *in vitro* and the utility of cutting-edge endogenous mouse models, the general aim of this work is to create a method that shortens the gap between findings in the lab to viable treatment options for cancer patients.

Thesis Supervisor: Tyler Jacks
Title: Director, Koch Institute for Integrative Cancer Research
Table of contents

Abstract ................................................................................................................................. 2
Acknowledgements .............................................................................................................. 4
Introduction ......................................................................................................................... 5
1. Literature review ........................................................................................................... 6
1.1 Modeling cancer in mice .............................................................................................. 6
1.2 Types of mouse cancer models ................................................................................... 14
1.3 Lentiviral vectors in the post-CRISPR era ................................................................. 25
2. CRISPR screens in the KP model of NSCLC ............................................................... 34
2.1 Rationale ..................................................................................................................... 34
2.2 Methodology .............................................................................................................. 35
2.3 Technical considerations ............................................................................................ 36
2.4 Results ......................................................................................................................... 48
3. Discussion ....................................................................................................................... 66
3.1 Lentivirus silencing in vivo and implications ............................................................ 67
3.2 Significance .................................................................................................................. 74
Materials and methods ...................................................................................................... 77
Abbreviations .................................................................................................................... 79
Vector maps .......................................................................................................................... 80
References ........................................................................................................................... 82
Acknowledgements

I am deeply grateful for the opportunity to be mentored by my supervisor Tyler Jacks, who has been a source of substantial support in every aspect. I have benefited enormously from his advice and guidance. I was constantly challenged intellectually by Tyler and I owe much of the substantial progress I have achieved to the many insights I gained from our conversations. My time in Tyler’s lab has been a great privilege to me.

The continuous support from my family has helped me tremendously during my time at MIT. I am thankful especially for my mother, who has supported me throughout all of my endeavors and tribulations. Spending time with my family has always been a grounding experience, helping to remind me about the world that exists just outside the lab window.

I am thankful for the love, engagement, and feedback during this report from my girlfriend Eider. I also thank Bobby and Tim for their advice and suggestions as I moved through my projects.

I appreciate the friendship and intellectual exploration I have shared with my lab-mate Will, though we overlapped for a short time only. Lakshmi, a former undergraduate student, has contributed substantially to much of my research and was a pleasure to work with. I am grateful for the many laughs and conversations I have had with Judy, who I will miss dearly. Lastly, I appreciate the fruitful discussions and assistance from all of the Jacks lab members who make the environment such a wonderful place to work.
Introduction

Rather than a summary of all the areas I have explored, my thesis focuses on a single research topic into which I have delved most deeply: genetic screens in vivo and the role of lentivirus silencing. This thesis is divided into three main sections.

The first section is a literature review; this review encompasses the history of modeling cancer in mice and how insights were first gained from these models, a description of the types of mouse models commonly employed by cancer biologists, and the role that lentiviral vectors can play in maximizing the information acquired from mouse models. The purpose of the literature review is to place the proposed project into context from a historical point of view and to highlight its merits and potential pitfalls.

The second section is a description of the work proposed and the initial results obtained. The rationale for this project is to develop a technique that allows for the rapid interrogation of cancer-specific vulnerabilities in an in vivo system that accurate recapitulates the human disease. The specific aim of the project is to characterize putative KRAS synthetic lethal partners using the CRISPR/Cas9 system an autochthonous lung adenocarcinoma genetically engineered mouse model. An important theme emerging from this section is the phenomenon of lentivirus silencing, which has posed a major barrier to the successful completion of this work.

The final section includes a deeper discussion into the origins and consequences of lentivirus silencing in vivo and the potential significance of the proposed work. Possible interpretations of the specific causes of silencing and how it fits into the literature will be explored, as well as potential solutions that arise from analysis of lentivirus biology itself. The preclinical value intrinsic to the successful completion of the work will be discussed and I will highlight its theoretical potential to impact both basic studies in cancer biology and translational research.
1. Literature review

1.1 Modeling cancer in mice

The history of cancer research is a colorful one. Cancer has a deceptively simple definition: a disease involving the abnormal growth of cells capable of spreading to other parts of the body. Over a century of attempts to unravel the complex mechanisms that drive cellular transformation has led to many groundbreaking discoveries into the nature of cancer. Many great scientists proposed inventive, controversial, and ultimately unsupported theories about cancer. Each new piece of evidence offered a wide range of interpretations, leading to heated debates within the scientific community and many dogmatic attitudes in cancer research. Controversies persisted for a better part of the 20th century: the current theory of the genetic basis for cancer was not widely accepted until the 1980s. From a modern perspective, it may appear surprising that it took the scientific community so long to grasp the fundamentals of cancer (we made it to the moon in 1969, after all). The field of cancer research was not lacking in brilliant minds; rather, the difficulty of the task mirrored the protean complexity of cancer itself.

Despite its elusive nature, cancer is in fact still subject to the laws of nature and predictions can be tested by the scientific method. To summarize the scientific method: an observation is made, a hypothesis is generated to explain the observation, and experiments are designed and carried out to test the hypothesis. The observations made from the experiments begin the cycle anew, and such is science. Although the concept is simple, the history of cancer research reveals a fascinating yet subtle deviation from the canonical scientific method due to its unique requirement for animal modeling. Of course the idea of using model organisms to test hypotheses was not unique or novel at the time, but its requirement in cancer is distinct. For example, developmental biology involves the process of a single cell becoming a complex multicellular organism. This process is a set of pre-programmed steps that are fundamental to the normal development of the organism. Experimentally, empirical observations of an organism’s development establish the concept of a normal, or wild-type, organismal phenotype. In genetics and developmental biology, the mechanisms governing phenotypic abnormalities are deduced by comparing the mutant organism against the wild-type. Biochemistry and molecular biology explore the molecular pathways that
occur in living organisms *in vitro*, but all experiments are performed in a setting governed by previously established chemical laws. Cause and effect relationships are inferred using experiments in which the controlled variables are well-defined and measurable.

The main reason that the scientific method in cancer is so distinct is that cancer can only be fully understood in the context of an animal, yet is fundamentally different from the animal. The distinction arises because the “controlled variable” is the animal, whose complexity allows cancer to exist while shaping its form. Many theories that take only the transformed cell and its transformative agent into consideration were attractive because they disentangled the cell from its context in the animal. Indeed, Occam’s razor would have pointed to a foreign invader or an imbalance of chemicals as the likely culprit for uncontrolled cell growth. In the following historical summary, I will describe some of the breakthroughs that led to our current understanding of cancer and the pivotal role that animal models played in the process.

**A historical summary of animal models and cancer**

Metabolic theories for the origin of cancer were highly prominent throughout the century. Nobel Laureate Otto Warburg is widely recognized for his observation that cancer cells display an abnormal ratio between the metabolic processes of glycolysis and respiration, commonly referred to as the “Warburg Effect.”¹ A less well-known fact is that he believed that this metabolic alteration actually caused cancer, rather than being a property of cancer. Undaunted by the lack of evidence supporting the metabolic theory of cancer, he maintained this position to his death and actively discouraged research into other theories.² Despite his great contributions to science, his theory of cancer was ultimately disproven from *in vivo* data. Today, the field of cancer metabolism has benefited greatly from his early observations that different metabolic processes occur between cancer and normal cells.³ The reason I begin with this example is to illustrate the fundamental requirement for animal models both today and in the history of cancer research; at the time, the nature of his work simply was not amenable to *in vivo* modeling.

A very notable example of a highly reproducible and influential animal model of cancer began with Peyton Rous at the turn of the 19th century. Rous, a doctor who ran the cancer research lab at the Rockefeller Institute (now known as Rockefeller University), was approached by a woman
who was concerned about the poor health of her chicken. This fowl had a large lump on its breast and she asked Rous to take a look at it for her. Rous happily took the chicken and proceeded to etherize the chicken, chop up the mass growing from the breast of the tumor, and then implant some of the tissue onto the same chicken’s other breast as well as onto the breasts of two other chickens of the same brood. When the chicken died about month later, he observed in the autopsy a smaller but somewhat similar mass at the site where he transplanted the tumor. One of the other two chickens developed tumors similar to the tumor found in the original chicken. 4

Given that the two new chickens came from the same brood, they presumably belonged to the same flock of the woman who had given Rous her first sick chicken. At the very least, he must have inquired as to where she acquired the chicken. Whether or not the woman had any idea of what Rous had in store for her first chicken, Rous’ explanation for needing two more chickens of the same brood and their consequent causes of death was probably somewhat awkward. Indeed, since no tumor transfers seemed to work in any store-bought chickens, he continued his experiments in the same purebred chicken line.

The theory that viruses caused cancer gained major credibility in 1911 from Rous’ groundbreaking work in which he dissociated chicken tumor tissue and created a filtered cell-free extract that was able to give rise to cancer when injected into the breast of another chicken. 5 Despite these observations, the theory gained little traction and contemporaries felt the work was irrelevant to humans and a pathological oddity. 6 The concept that cancer was caused by an infectious agent was practically ruled out at the time, in part due to the accumulation of so much negative data from research inspired by recent discoveries of the contagious origins of many diseases. 7 Moreover, a large number of epidemiological studies linked cancer to environmental causes, which did not seem to fit with the virus theory.

The first documented association between cancer and the workplace, dubbed “Chimney sweep’s cancer,” was reported in 1775. The English surgeon Percival Pott noted that chimney sweeps, young boys in the UK who were employed as child labor to clear chimneys of soot and ash, developed this particular type of cancer at an unusually high rate. 8 Given the well-established association between skin cancer and coal tar workers by the early 1900s, scientist Yamagiwa
simulated this occupational hazard in rabbits by repeatedly applying coal tar on their ears. Yamagiwa published the first experimental induction of cancer in 1918 when he showed that 7 of his rabbits developed skin cancer at the site of application, 2 of which were metastatic, spreading to distant organs. Many frustrated scientists who attempted to repeat these experiments failed, placing Yamagiwa’s work into question. William Woglom noted that the likely cause of many failed replications were simply due to lack of patience. Some cancers took over a year to emerge, and he used 137 rabbits. A true testament to the power of dedication, he and his assistant painted tar on the ears of 137 rabbits every 2 or 3 days for months without any evidence of tumor formation.

While theories of the metabolic, environmental, and viral origins abounded, many years passed before the genetic basis of cancer was elucidated. Physicist Niels Arley, who headed the department of geophysics research at Copenhagen University, first proposed the theory of oncogenes in 1950. Oncogenes were postulated to be normal genes in an organism that had undergone some change that conferred the cell its tumorigenic properties. His contemporaries considered the theory to be absurd. Despite Peyton Rous’ substantial contribution to the field of cancer research, he pointed out in his 1966 Nobel Prize acceptance speech:

“A favorite explanation has been that oncogenes cause alterations in the genes of the cells of the body, somatic mutations as these are termed. But numerous facts, when taken together, decisively exclude this supposition.”

In the following years, based on numerous facts, when taken together, the theory of an oncogenic basis for cancer became practically irrefutable.

Rosalind Franklin’s X-ray diffraction images of deoxyribonucleic acid (DNA), which revealed unprecedented insight into its double helical structure, formed the basis of the postulation of the exact chemical structure of DNA by Watson and Crick in 1953. The field of biology was revolutionized by further work that confirmed DNA formed a chemical code comprised of discrete units, or genes, that contained the instructions required to produce proteins, more complex molecules that can carry out specific functions in cells. The concept of the genome as a collection
of genes and an understanding of its chemical nature provided a molecular basis to bolster the theory of oncogenes. A slight alteration in the genetic information of the cell could lead to an alteration in the proteins it produced. A proto-oncogene, a normal gene with the potential to become an oncogene through some alteration, could then produce an oncoprotein that is similar to the normal protein but confers upon the cell oncogenic properties. Epidemiological studies noting hereditary predispositions to certain types of cancer lead to the theory that the alterations in putative tumor suppressor genes, or cellular genes that normally act to prevent aberrant growth, played a role in cancer development.\textsuperscript{15}

Peter Duesberg and Peter Vogt demonstrated that Rous sarcoma virus (RSV) contained sequences of RNA that were not present in similar, but transformation-defective, viruses. The fact that RSV had a larger molecular weight than similar non-transforming viruses was discovered in 1970 using gel electrophoresis, which involves using an electric field to cause the unidirectional migration of negatively charged nucleic acids through a porous gel, whose purpose is to alter each molecule’s migration rate by decoupling molecular size from its size to charge ratio, which is roughly constant for any length of nucleic acid.\textsuperscript{16} This work provided an initial clue that there was some unique transforming region of RSV that may not necessarily be viral in origin. In 1976 Michael Bishop and Harold Varmus developed a subtraction hybridization procedure to generate a radiolabeled complementary DNA (cDNA) probe that bound to this putative oncogenic region of RSV, dubbed \textit{v-src}. Using this probe, they found that it hybridized to uninfected chicken cell DNA, other avian genomes, and even other uninfected vertebrates.\textsuperscript{17,18} A few years later, Bishop and Varmus, along with Joan Brugge and Raymond Erikson, demonstrated that the protein product of \textit{v-src} was similar in structure and function to a normal protein product found in uninfected cells.\textsuperscript{19,20}

These bodies of work demonstrated the presence of genes in healthy organisms that were similar to the oncogenes carried by viruses, but their function and precise relationship to the virus itself still remained unclear. In 1976, the same year, Temin hypothesized that RNA-directed DNA synthesis of a virus could lead to viral integration into the genome. From a virologist’s point of view, one could propose a scenario in which copies of an ancient, inactive oncogenic provirus were present and common in some ancient species. Throughout evolution, the provirus could have persisted and contained sufficient homology to RSV that the cDNA probe could hybridize to the
genome of uninfected cells. Such a hypothesis is not so implausible, particular given that it is now known that roughly 8% of the human genome is composed of viral DNA.\textsuperscript{21}

The successful cloning of the first oncogene from human bladder carcinoma cell lines was published in 1982 from the groups of Robert Weinberg, Michael Wiglerand and Mariano Barbacid.\textsuperscript{22,23,24} The same year these oncogenes were shown to share homology with the viral $\text{ras}$ oncogenes found in the by Harvey and Kirsten to cause rat sarcomas (hence the name for the human proto-oncogenes $\text{HRAS}$ and $\text{KRAS}$) and the precise change in amino acid sequence that transformed the proto-oncogene into an oncogene was discovered.\textsuperscript{25,26,27} These discoveries lead to an explosion in oncogene research. In the coming years different cellular oncogenes were discovered, their functions in growth and transformation became more apparent, and the concept that viral oncogenes were derived from normal components of the cellular growth machinery became largely irrefutable.\textsuperscript{28} In the same decade, the theory of tumor suppressor genes, or genes whose loss or alteration in function lead to unrestrained cell growth, gained increasing support experimentally.\textsuperscript{29} The combination of observations that genetic alterations caused gain or loss of function in proto-oncogenes and tumor suppressors paved a path to probing, understanding, and ultimately treating cancer by studying it in the environment it occurs naturally: the animal.

**Preclinical value of cancer mouse models**

The previous brief historical summary suggests that although insights can be gained by studying the processes that occur in cancer cells *in vitro*, understanding the origins and complexity of the disease required an appreciation for its origin in the context of animal. Understanding cancer at the genomic level opened the door to modeling the spectrum of cancer subtypes with greater precision than with coal tar or oncogenic viruses. A complete picture, however, also necessitates an understanding of the disease in relation to the physiological changes that occur within the animal itself. The goal of cancer mouse models is not only to study the pathways altered by the genetic changes that occur in cancerous cells, but also how the whole organism is affected by and responds to the malignancy. Cancer mouse models offer insights into several categories of questions.
Genotype and disease progression.
How are oncogenes and tumor suppressors involved in transforming cells and influencing disease progression at various stages? Do mutations or genomic rearrangements occur all at once, or do they accumulate slowly over time? Do particular genetic alterations display a tendency towards co-occurrence or mutually exclusivity in a given cancer? Why do particular combinations of mutations occur preferentially in different tissues of origin?

Drug treatment: therapeutic activity and dose response.
What is the maximal tolerable dose of a given anti-cancer compound and what are the side-effects? What is the optimal therapeutic window and dosage strategy? If a range of drugs are known to enzymatically disrupt an important pathway, does the on-target activity of the set of drugs actually correlate with overall survival in the context of the maximum tolerable dose? Despite the importance of these questions, a major issue in animal research is that unlike in human clinical trials, no best-practice standards exist for preclinical mouse models. Mouse cancer research lacks any universal standards for preclinical protocols, proper record-keeping, training, and planning pilot studies.30

Metastasis.
How and why does cancer metastasize, colonizing distant organs? What are the changes in gene expression or the mutational landscape that imbue a tumor with metastatic properties? Can metastases be prevented or detected early? Do metastases have unique properties that can be targeted directly?

The immune system.
Does the body recognize cancer as foreign or self, and why? How do cancers escape immunosurveillance? Does the immune system play a supportive role in tumorigenesis and maintenance? Is there a combination of suppression and support that occurs? Does the role of the immune system depend on the context and stage of the disease? Can certain types of immune cells be exploited or triggered to help target cancer cells?
Microenvironmental factors.

How does the process of tumorigenesis affect the cells surrounding a tumor? Are they reprogrammed due to the environmental and mechanical cues they receive from the tumor? Can they recruit pro- or anti-tumorigenic factors? Can they act as a barrier or facilitator of drug delivery?

The purpose of mouse cancer models is to gain actionable insight into the human disease by making deductions from the observations that appear from mimicking it in mice. Mouse models not only allow scientists to study the disease as it progresses and evolves, but also to make genetic and pharmacological perturbations to the tumor and/or mouse. Studies *in vivo*, however, can be challenging to interpret and can take several years. Indeed, the importance of the duration of the model cannot be understated: in every hour that passes by, about 700 people die of cancer.\(^{31}\) The phrase “time is of the essence” is particularly applicable to cancer mouse models. Temporal considerations aside, the value of a given cancer model corresponds to the amount of information that can be obtained from observing and perturbing the system. The following criteria\(^{32,33,34,35}\) are commonly used to gauge the preclinical value of a given model:

1) Initiation and disease progression
2) Microenvironment factors
3) Host immune status
4) Predictive of clinical outcome
5) Modularity of the system
6) Reproducibility
7) Cost
8) Duration

Many of these criteria can be summarized two simple questions: how well does the mouse model recapitulate the human disease, and how practically feasible is the model? Taking these factors into consideration, I will describe the various model systems used in cancer and discuss the advantages and disadvantages that are associated with each.
1.2 Types of mouse cancer models

Mouse cancer models come in a variety of flavors. Although the particular classes and subclasses of model systems and their respective merits can be divided and defined categorically, they are not mutually exclusive. Many insights into cancer biology have come from the clever integration of multiple types of models within the same mouse. The past two decades have seen an explosion of innovative models, in part due to an increased appreciation of the complexity of cancer biology and the emergence of novel methods to manipulate the genome.

**Figure 1** Categorization of mouse models of cancer

**Transplantation models**
Transplantations models of cancer are straightforward in premise; a group of tumorous cells are moved from an animal or cultured media and placed onto or inside of another cancer-free mouse such that the cancer continues to propagate in its new host. The aforementioned experiments by Rous first described a transplantation model of cancer when he injected the breast of a chicken with a small bit of tumor from another chicken. It is important to note, however, that when he generated a cell-free extract that could give rise to tumors in other chickens, this model was not a
transplantation model. It was a virus-induced model, a type of carcinogenic model that will be discussed further on.

The first consideration of a transplantation model is where to transplant the cancer in the host. The most common transplantation models are subcutaneous, intraperitoneal (IP) and orthotopic. The subcutis is the layer of skin below the dermis and epidermis but above the muscle layer; in mice, transplantation into the subcutis is achieved by injecting into locations where the folds of the skin are loose. Common sites of transplantation are in the flanks of the mouse. IP injections bypass the muscle layer and transplant the cancer into the peritoneal space, which is the body cavity containing the internal organs. Finally, orthotopic transplantation is to place a tumor at the site where it is thought to have originally risen from, such as transplanting pancreatic cancer cells directly into the pancreas. The aforementioned subcutaneous and IP models, in contrast, are ectopic models because they arise from an abnormal location.

The site of transplantation offers various advantages and disadvantages. Subcutaneous models benefit from a quantitative assessment of tumor growth; calipers can be used to record the dimensions of the bulging tumor. While subcutaneous models are useful for quantitating tumor proliferation over time, IP models provide a more accurate recapitulation of disease progression and dissemination in vivo. On the other hand, IP transplantations are more difficult to monitor in a living mouse, limiting analyses of tumor kinetics. Ectopic models are generally more amenable to rapid drug screening. Although orthotopic transplantations can be more time-consuming and difficult than ectopic models, they are generally more predictive of clinical response to a given drug. They also benefit from tissue site-specific pathological information and more accurate models of metastasis; ectopic models tend to preferentially metastasize to the lung and in many cases do not metastasize at all. Orthotopic models often require invasive surgery, which can cause trauma to the mouse, and reproducibility issues can occur due to lack of precision with respect to the location of injection.\textsuperscript{36,37}

\textit{Allografts}

Once the location in the host is decided, the second consideration of a transplantation model is which species of origin of the cancer to use. The type of transplantation involving moving cancer
from one animal to another of the same species is called an allograft. Transfer within the same species alone is insufficient for tumors to form robustly. For allografts to form tumors, the animals must be syngeneic: genetically similar enough that the immune system of the host does not recognize the transplanted tissue as foreign and subsequently destroy it. This phenomenon was the reason why Rous was unable to transplant bits of tumors from his chickens onto store-bought chickens but was successful when he transplanted them onto the same purebred chickens; they were too genetically distinct. The advantage of using allografts is that the tumor can be passaged onto different animals without provoking an immune response; the mice are otherwise healthy. This process is quick and inexpensive given that the minimum requirements are an animal with a tumor and a syngeneic host.

**Xenografts**

The transplantation of a cancer between species of animals is called a xenograft. The concept of xenografts is more attractive than allografts from a therapeutic perspective because it enables the study of actual human cancers in an *in vivo* setting. Unlike with allografts, a xenograft would invariably provoke an immune response in a normal host, leading to its immediate destruction. To circumvent this issue, one type of xenograft model uses mice that are immunodeficient. Examples include “nude” mice and SCID mice, which were identified in the 1980s as being immunocompromised. As time progressed many types of elaborate genetic alterations have been made to further increase or alter the immunodeficiency of certain strains. Without an intact immune system in the host, a xenograft is free to grow without interference. Another version of a xenograft model generates “humanized” mice, a process involving the injection of human hematopoietic stem cells into the host in order to reconstitute a human-like immune system in the mouse. 

Although the time and cost of generating a humanized mouse are greater than breeding immunodeficient mice, humanized mouse models offer unique advantages. By swapping the mouse immune system with a human one, humanized mouse models bypass the immunity issue and better recapitulate the human disease. A barrier to the reproducibility of immunodeficient mice is that the degree of immunocompetence varies between strains of mice and the nature of the immunodeficiency. Additionally, only in humanized mouse models is it possible to study the
interactions between human cancer and human immune cells in an in vivo setting. Moreover, human immunotherapies can be tested for safety and effectiveness in mice, which is particularly relevant given recent high-profile success of immune checkpoint blockade therapies.\textsuperscript{40}

In an early review of transplantation models, it was found that fewer than a third of compounds that demonstrated efficacy in a human tumor xenograft displayed similar results in syngeneic models. This study suggested that xenograft models were more effective at identifying potentially useful drugs, but the correlation between preclinical and clinical efficacy of these drugs was still low. Generally, a large number of patient cancer-derived xenografts (PDX) are required to show an effect, and tissue availability could be a limiting factor for rare cancer types. Moreover, patient cancer-cell derived xenografts tend to lose some of their human-relevant qualities as a result of multiple passages.\textsuperscript{41} Many of the barriers fundamental to transplantation models make their broad applicability across a range of cancers highly limited.

**Autochthonous models**

Autochthonous models are models in which tumors form due to genetic changes in individual cells of the mouse genome. Although transplantation models are useful for rapidly testing different types of drugs in a variety of cancers, a major criticism of these models is that they replicate primarily the cell-intrinsic properties of the disease, without regard for its tissue-specific context or whole-animal physiological changes.\textsuperscript{42} Autochthonous models recapitulate the full spectrum of the disease as well as the physiological adaptations that occur in the host as it progresses. The two broad categories of autochthonous models can be subdivided into carcinogenic models and genetically engineered mouse models (GEMMs).

**Carcinogenic models**

Carcinogenic models involve the use of any external substance or exposure that results in the spontaneous rise of a tumor. A major class of carcinogenic models involve mutagens: agents that induce tumors by causing DNA damage to cells. The list of mutagens in expansive but a few cancer modeling examples involve the delivery of chemical mutagens, exposure to tobacco smoke, and UV irradiation.\textsuperscript{43} Other carcinogenic models include infection with oncogene-carrying viruses,
nutritional changes, immunosuppressive drug, and even disrupting the circadian rhythm by changing mouse sleeping patterns!\textsuperscript{44}

Carcinogens are often biased towards generating one particular type of tumor. For the case of UV irradiation, the bias towards giving rise to skin cancers is obvious because the epidermal cells experience the bulk of the genetic insult.\textsuperscript{45} For many carcinogenic compounds, the preferential bias is often unclear. Despite the lack of clarity, knowledge of the carcinogen’s mechanism of action, or preference for particular organs, is not always supremely important. Rather, the bias grants the model reproducibility and robustness, which are critical for making meaningful interpretations from a given observation. For example, a mouse model of lung cancer induced by N-ethyl-N-nitrosourea and N-nitrosodiethylamine consistently leads to \textit{KRAS}-driven lung cancer. In all cases \textit{KRAS} exclusively contains point mutations in either codon 12, which is the most common substitution found in humans, or codon 61.\textsuperscript{46} Another major advantage of using a chemical carcinogenic model is that the time of delivery and concentration of the carcinogenic compound can be determined precisely, granting some level of control over the time of tumor induction and the number of tumors formed. The availability of a wide variety of chemical carcinogens and the ease of administration allows a degree of reproducibility for certain cancer subtypes.

\textbf{Genetically engineered mouse models}

GEMMs are unique in that mice are altered at the genomic level to be predisposed in some way to cancer before they are even born. These animals are transgenic, meaning that they contain genetic information that was manipulated through the artificial introduction of exogenous DNA. Unlike carcinogenic models, though also autochthonous, the mouse genome itself is hardwired to give rise to tumors. For example, GEMMs overcome the inherent variability in carcinogen-induced models; tumors from GEMMs do not harbor the aberrant mutational landscape generated by DNA-damaging carcinogens at initiation. GEMMs are generally more elegant models because of the precision in which individual pro-tumorigenic mutations and deletions can be engineered to give rise to tumors of a defined genetic context.
The ability to generate cancer GEMMs began in the 1980’s with the concurrent development of techniques to create transgenic mice. The first cancer GEMM using a directed approach was the \( E\mu-Myc \) model, described in 1985, in which mice harbored a transgenic construct expressing the oncogene c-Myc (Myc) from an immunoglobulin enhancer element (\( E\mu \)). Immunoglobulins, better known as antibodies, are only expressed by B-cells. These transgenic animals robustly developed B-cell malignancies, supporting a pro-tumorigenic function for c-Myc overexpression in cancer. The role of tumor suppressor genes can also be studied in GEMMs using “knock-out” mice, which are engineered to delete specific regions of the genome. The innovative adaptation of gene targeting at the single nucleotide level further paved the path for engineering the specific gain of function point mutations that frequently occur in particular oncogenes. GEMMs fall under three general categories that are by no means mutually exclusive: spontaneous, inducible, and endogenous.

**Spontaneous GEMMs**

A spontaneous mouse model of cancer is one in which the mouse genome is engineered to develop tumors without any external influence. In a spontaneous model, every cell in the mouse starts off genetically identical and some cells ultimately develop tumors, often in multiple organs. Heterozygous or homozygous deletions of tumor suppressor genes fall under the category of spontaneous GEMMs. Such GEMMs often mimic human disorders involving familial predisposition to cancer such as Li-Fraumeni syndrome and hereditary retinoblastoma, which involve inactivating mutations in the human genes \( TP53 \) and \( RB \), respectively. A spontaneous mouse model in which murine \( p53 \) is deleted in the germline gave rise to a spectrum of tumors that provided insights into the tissue-specific consequences and latency of tumor formation caused by loss of \( p53 \) protein. Widespread expression of particular oncogenes, generated by targeted mutations, often leads to embryonic lethality. This feature can make them difficult to study in spontaneous GEMMs. To get around this problem, some spontaneous GEMMs have employed clever tricks to stochastically activate latent oncogenic alleles \textit{in vivo}. A notable example is the stochastic Kras-LA model, in which a mouse with one copy of wild-type \( Kras \) with another allele containing substantial homology to the wild-type allele upstream and downstream of the mutation in exon 1 of \( Kras^{G12D} \). The property that makes the oncogenic allele latent is that in its native form the allele is inactive: the exon 1 is duplicated but the sequence order is re-arranged such that the
gene produces no functional protein product. Through spontaneous intrachromosomal recombination, which occurs at a very low rate, the region between the duplicated exons becomes rearranged such that the final result is a single exon with the oncogenic point mutation. Alone, and in combination with the spontaneous models involving loss of p53, a wide spectrum of tumors formed that provided even deeper insight into the type of tumors that form spontaneously due to the combination of these genetic alterations.\textsuperscript{53}

\textit{Inducible GEMMs}

Inducible models are often described as transgenic models, given that their construction involves the transfer of genetic material into the host genome. Semantically, all GEMMs are transgenic models given that the introduction of genetic material occurs at some point as a consequence of genetic engineering. The reason for making a distinction is that inducible models, as opposed to spontaneous and endogenous models, involve the regulation and expression of oncogenes or dominant-negative tumor suppressors from transgenic elements at non-physiological levels. The inducing agent need not be external. The \textit{E\textmu-Myc} model described previously is an example of an inducible GEMM; in real tumors, c-Myc is never under the control of an immunoglobulin enhancer element. Generally, the transgene is placed under the control of a tissue-specific promoter in order to examine its function in a particular cell type or tissue type. Whenever the tissue-specific promoter is expressed, so is the oncogene, thereby controlling the location of oncogene expression. Oncogene expression can also be controlled at the temporal level by the delivery of chemical compounds; tissue-specific promoters can drive transgenes such as reverse tetracycline transactivator (\textit{rtTA}) and tetracycline transactivator (\textit{tTA}) whose transcriptional activity is regulated by binding to the compound doxycycline (dox), which then drive expression of the oncogene from a "dox-inducible" promoter. The ability to spatially and temporally drive oncogene expression allows for the targeted development of particular tumor subtypes and to examine the role of the oncogene in tumor maintenance. Multiple observations that some tumors undergo apoptosis or senescence when expression of the driving oncogene is lost has led to the theory of "oncogene addiction," which posits that tumors rely on the sustained expression of an oncogene in order to maintain their proliferative state.\textsuperscript{54} Common genetic engineering techniques employed in inducible GEMMs are depicted in the following schematic.

20
Constitutive transgenes

Tetracycline-inducible transgenes

Figure 2 Common techniques used in inducible GEMMs

Endogenous GEMMs

Endogenous GEMMs offer a degree of spatio-temporal control and the expression of relevant genes at physiological levels that cannot be achieved through spontaneous or inducible GEMMs alone. Indeed, endogenous GEMMs employ many of the techniques in genetic engineering involved in spontaneous and inducible GEMMs. The distinction that makes endogenous GEMMs unique is that the mouse is primed to develop the naturally occurring tumors in spontaneous models by expressing the relevant genes at physiological levels, but with a conditional trigger that causes the initiating event to occur in a defined location. Endogenous GEMMs take advantage of transgenic DNA recombination technology, most commonly the Cre recombinase, in order to generate conditional alleles. This technology allows for the generation of conditional knockout of endogenous alleles (flox’d or FL alleles) and conditional activation of oncogenic alleles using stop cassettes (LSL alleles). Cre recombinase specifically interacts with short stretches of DNA called loxP sites, which upon Cre-induced recombination allow gene expression to be controlled by endogenous regulatory elements.

Figure 3 Common conditional alleles used in endogenous GEMMs

21
Using exogenous delivery of Cre recombinase, activation and/or deletion of the conditional alleles is decoupled from any information already present in the mouse genome. For example, the observation that the spontaneous Kras-LA model, in conjunction with loss of p53, gave rise to lung adenocarcinoma at 100% penetrance formed the basis for the perhaps the most well-known endogenous GEMM today, the “KP” model of non-small cell lung cancer (NSCLC). In this model, conditional activation of oncogenic Kras and deletion of p53 is achieved by intratracheal delivery of viral particles carrying Cre recombinase.\textsuperscript{56}

Essentially, endogenous GEMMs combine the best qualities of the other transgenic models with an additional layer of spatio-temporal control added by the method of tumor initiation. In organs that are difficult to access by exogenous recombinase delivery, driving Cre expression from a carefully chosen tissue-specific promoter is the next best option. Notably, certain models of pancreatic cancer employ expression of Cre from tissue-specific promoters in the pancreas in order to allow pancreas-specific expression of mutant Kras and p53, which form pancreatic adenocarcinomas at high penetrance.\textsuperscript{57} Endogeneous GEMMs are generally the most amenable to genetic tumor intervention strategies and best recapitulate the disease in humans because other than the transformed cells, the rest of the mouse is essentially wild-type.
**Hybrid models**

Most GEMMs today combine multiple modeling strategies in order to answer questions that a single type of model cannot. A notable example of a clever hybrid model utilized a mouse harboring a conditionally active \( p53 \) allele in combination with a carcinogenic model to examine the role of \( p53 \) in tumor maintenance. The human tumor suppressor \( TP53 \) is of particular interest given that it is the most frequently altered tumor suppressor in human cancer.\(^{58}\) A long-standing controversy continues still today over whether or not there is a difference between complete loss of function of the wild-type and mutant gene in humans; like oncogenes, \( TP53 \) harbors mutations in hotspots, positing speculation over whether these mutations cause a dominant negative protein product or a protein with altered function.\(^{59}\)

In an attempt to engineer a germline mutant \( p53^{LSL} \) allele, an unintended homologous recombination event occurred that resulted in a wild-type \( p53^{LSL} \) allele. Rather than being discouraged by the unfortunate mishap, Ventura et al. employed this allele to ask an interesting question: what happens when you restore \( p53 \) function in an established \( p53 \)-deficient tumor? This allele was crossed into mice harboring the \( \text{Cre-ER}^{T2} \) allele, which induces recombination of loxP sites in the genome upon the delivery of tamoxifen. \( \text{Cre-ER} \) is a fusion protein of Cre recombinase and an estrogen receptor that is tethered to the nuclear membrane in the absence of tamoxifen but shuttles to the nucleus in the presence of tamoxifen, allowing Cre to interact with DNA and perform its recombinase activity. This genetically engineered mouse lacked any \( p53 \) protein in all tissues until tamoxifen was delivered, which recombined the stop cassette and caused expression of the wild-type \( p53 \) gene. This mouse was combined with the carcinogenic model of irradiation-induced tumorigenesis and when tumors were visible by magnetic resonance imaging (MRI), tamoxifen was delivered and the effects of \( p53 \) reactivation were observed in a variety of tumor types.\(^{60}\)

The reactivation of wild-type \( P53 \) in humans is not a very realistic clinical possibility, though pharmacological attempts to restore function of the mutant version have been attempted.\(^{61}\) The reason I provide this example is because it illustrates the potential that GEMMs can have to ask questions about the nature of cancer that would otherwise be impossible. Although GEMMs themselves will always be inherently limited by the technological toolkit available at the scientist’s
disposal for genetic manipulation, the true potential that GEMMs have is only limited by the imagination. I read this paper from Tyler Jacks’ group many years ago in a college cancer biology course; it was an inspiration, of sorts. The power of modeling cancer with GEMMs became clear to me for the first time.

*Endogenous GEMMs have the highest preclinical value*

Endogenous GEMMs fulfill the majority of the criteria for preclinical value previously mentioned. They best recapitulate the disease progression, the microenvironment, and the immune system. Many are highly robust and they offer a great degree of spatio-temporal control in the form of the timing of the initiating event and the number of transformed cells. Most importantly, they are the best predictors of response to pharmacological agents in clinical trials.

An analysis in 2001 of *in vitro* models and xenografts performed by the National Cancer Institute found poor correlation between activity in these models and in phase II clinical trials, and only when a large number of tumor xenografts were used for a specific agent did the correlation improve at all. Similar findings in 2003 comparing allografts and xenografts determined that allografts were not predictive at all and recommended the abolishment of their preclinical use. Notable early failures of particular GEMMs to predict clinical response raised the question of their utility and some concluded that mice were primarily useful simply as a vehicle for human xenografts. In my opinion, some of the lack of preclinical-to-clinical success in earlier GEMMs was not that GEMMs are fundamentally not predictive of clinical outcome; rather, they highlighted the necessity and importance of building better mouse models. Indeed, recent endogenous GEMMs performed in a pre- or co-clinical trial setting have correlated with clinical outcome incredibly well and also provided mechanistic insights into why and how particular therapeutic agents work.

The Pdx1-Cre KP model of pancreatic ductal adenocarcinoma (PDAC) provided insight into the cooperation of gemcitabine and nab-paclitaxel, the first improvement to the standard of care for PDAC patients since 1997. In the same model, the observation that CCR5 antagonists caused tumor-specific accumulation of effector T cells that synergized with immune-checkpoint inhibitors formed the basis for ongoing early phase clinical trials for these combinations. The KPL model of NSCLC validated the improved response of adding Selumetinib, a MAPK pathway-specific
inhibitor, to Docetaxel, the standard of care for KRAS mutant patients and helped elucidate the underlying genetic factors at play in therapeutic response rate.\textsuperscript{67} The KP model of NSCLC has also provided a number of pre and co-clinical insights into why synergy occurs in some combination therapies and identified novel combinatorial strategies.\textsuperscript{68} These examples represent just a small subset of endogenous GEMMs that have validated findings in co-clinical trials and formed the basis for many current clinical trials.\textsuperscript{69}

Although the preclinical insights and predictability of endogenous GEMMs is undeniable, the main limitations come in the form of time and cost. The aforementioned examples highlight the power of GEMMs in identifying genotype-specific responses to certain therapies, but human cancers tend to be far more complex. The entire spectrum of human tumors originating in the lung and pancreas cannot be represented by the KP model of mutant Kras\textsuperscript{G12D} and loss/mutation of p53. Recent whole genome analysis of hundreds of tumor samples has uncovered many novel putative tumor suppressors and oncogenes, each of which may modulate response to a given therapy. The high cost, monetarily and temporally, to engineer new conditional alleles, breed mice to contain the right combination of alleles, generate a sufficient number of mice to do statistically significant preclinical experiments and simply maintain large mouse colonies has proven to be a substantial barrier to the use of GEMMs.\textsuperscript{70}

1.3 Lentiviral vectors in the post-CRISPR era

“Personalized medicine,” the concept of developing treatment regiments tailored to each individual patient, has been espoused as the future of cancer therapy for very many years. In theory, personalized medicine in cancer would be optimal for maximizing patient survival. In practice, the traditional rate-limiting steps in GEMMs have made this concept little more than a pipedream.

This dream recently found its way back into the realm of possibility in the form of a technological renaissance. As it was once famously said, “As if a miracle, the clouds passed, the sun came out, and delivered unto us was CRISPR.”\textsuperscript{71} The CRISPR/Cas9 system, a tool modified from the adaptive immune systems of certain bacteria, allows for unprecedentedly modular and rapid site-directed nuclease activity.\textsuperscript{72} A short guide RNA (sgRNA) is easily designed to be homologous to almost any particular sequence of DNA, and the Cas9 protein binds to the sgRNA and generates a
double-stranded break at the desired site. When targeting a gene, this can lead to an incorrectly repaired gene product such as an insertion or deletion, effectively knocking it out.

The ability to delete any gene of interest in a transformed cell has dramatically improved the efficiency of interrogating the cancer genome. Unravelling the functions of the newly described tumor suppressor genes using the traditional flox’d allele systems would have had enormous costs and taken years to generate. Now, to generate a new mouse model harboring a deletion in a tumor suppressor gene is cheap and takes a matter of months. CRISPR essentially redefined in vivo preclinical cancer research and the field of biology as a whole. The following schematic depicts how CRISPR has been used to modify the KP model of NSCLC:

![Diagram of CRISPR modification in Cas9 mice]

**Figure 5** “KP” model of NSCLC in Cas9 mice
Advantages of lentiviral vectors in GEMMs

Lentiviral vectors have a number of properties that make them superior to other types of vectors, viral or otherwise, in cancer biology. The four types of viral vectors commonly used in GEMMs are gammaretrovirus, lentivirus, adenovirus, and adeno-associated virus (AAV). Although gammaretrovirus and lentivirus are both subtypes of retroviruses, the former is generally referred to as just a “retrovirus” despite the misleading nomenclature. Generally, the following criteria for an optimal viral vector take into consideration ease of production, capability to infect non-dividing and dividing cells, modular tissue-specificity, sustained expression of cargo post-delivery, avoidance of an immune response, packaging size and faithful replication of cargo during cell division.\textsuperscript{73} I will highlight the key properties of lentiviral vectors in the context of the aforementioned criteria and briefly cross-compare these properties against other vectors.

Many of these viral vectors have a broad host range and through techniques of directed evolution the tropism can be modified, tailored to infect only specific tissues.\textsuperscript{74} The ideal vector would be episomal, one that remains in the nucleus and replicates during cell division without interfering the host genome. Some vectors such as the AAV remain in the cell as an episome but do not replicate and the genetic material is quickly lost as cell division proceeds. The most significant advantage to using lentiviral vectors is that these vectors faithfully integrate into the host genome of the infected cell, unlike adenovirus or AAV vectors. The integration of lentiviral vectors allows for the tracking and tagging of all progeny of the infected cell. This property distinguishes the infected cells from non-infected cells; such clarity has been enormously useful in tracking and monitoring tumor progress GEMMs. A few visual markers or quantifiable genetic tags that are incorporated by integration events include fluorescent proteins, DNA or RNA barcodes, and others. Lentiviral vectors are more advantageous than retroviral vectors despite sharing many similar properties such as packaging limit of cargo and capacity to integrate into the host genome. Unlike lentiviral vectors, however, retroviral vectors cannot infect non-dividing cells, they elicit a substantial immune response, and show a preference for integration into open regions of DNA.\textsuperscript{75} This preference can lead to unwanted overexpression of endogenous proto-oncogenes, which had disastrous results in a retroviral-based gene therapy clinical trial for SCID patients, leading to high rates of lymphocytic leukemia.\textsuperscript{76}
Insights from genetic screens using lentiviral vectors

Genetic screens using RNA interference (RNAi) or CRISPR components typically involve delivery using a lentivirus. The reason for using the lentivirus is that it integrates and remains expressed over the relatively long time of the screen in vitro: roughly three to four weeks depending on the double time of the cell line in question. The methodology of genetic screens has been reviewed extensively, but in short; a specific cell line is infected at a multiplicity of infection (MOI) of 1 with a pooled lentiviral library targeting every gene or a subset of genes in the genome in equal proportions, the cell line is passaged for several population doublings, and at the end the genomic DNA is extracted and the integration sites are PCR-amplified and subjected to high-throughput sequencing. The relative abundance of reads for each targeted gene are measured and used to generate a plot that ranks genes according to their capacity to influence cell proliferation. For example, essential genes are expected to be completely depleted from the population, while genes that affect fitness positively or negatively will be enriched or reduced, respectively. Genetic screens in vitro using the CRISPR/Cas9 system to repress or activate genes provide an important framework for identifying cancer cell subtype-specific synthetic lethal partners and vulnerabilities, as well as genetic modifiers of drug resistance and susceptibility to specific inhibitors. An excellent review of these applications in the context of cancer was reported recently and a figure from this review is presented below.

Figure 6 Genetic screens in vitro to identify cancer-specific vulnerabilities
Viral silencing: evidence and mechanisms

A potential barrier to using lentivirus is the question of how long the cargo is actually expressed, both in culture and in the cells of infected animals. Lentivirus silencing, a phenomenon involving loss of expression from the integrated provirus, has been well-documented both in vitro and in vivo. Whether or not silencing occurs is not up for dispute. The factors that remain unclear are the variability in cell- and tissue-specific kinetics of silencing, why certain subtypes of retrovirus display differential silencing activity, and the precise molecular mechanisms involved in silencing. These mechanisms include host and viral transcriptional activity, host and viral proteins, epigenetic modifications, DNA methylation, and specific sequences of the viral genome.

A lentivirus is a subtype of the retrovirus family. Though they share much sequence homology with the closely related gammaretroviral vectors, they have the capacity to infect and integrate into the genome of non-dividing cells, while gammaretroviral vectors are limited to only dividing cells. Much of the early work exploring retroviral silencing was performed on the class of gammaretroviral vectors. The first report of silencing was demonstrated in 1977 after infection of embryonic carcinoma cells with the murine leukemia virus (MLV). Jaenisch’s group demonstrated in the early 1980s that silencing involved methylation at the DNA level, rather than during translation or due to repression of viral proteins. Consequently, other groups demonstrated that DNA methylation is preceded by another silencing event and the kinetics involved tissue-specificity at some level. Cellular reprogramming in the form of differentiation has been noted to decrease lentiviral expression in a time-dependent manner in ES cells, yet some cells retain expression in a stochastic fashion.

At the turn of the century it was determined that the preceding silencing event was a result of epigenetic modifications specific to the viral integration site, and certain human epigenetic modifiers such as histone deacetylases (HDACs) were involved. The sequences that mediate repression were determined to be primarily the long-terminal repeats (LTRs), which act as viral promoters, among other things. Many pathways have been demonstrated to be involved in mediating viral silencing including but not limited to inflammation, heat shock, cell-state changes, Notch signaling, and more. Although many general pathways have been described, recent studies using genetic screens have identified many of the exact proteins and mechanisms involved in
mediating provirus silencing. These proteins include histone modifiers, sumoylation factors, epigenetic cofactors, and scaffolding proteins, which cross-talk in a variety of complex manners in order to orchestrate silencing events.\textsuperscript{91}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Transcription factors and epigenetic events involved in lentivirus silencing\textsuperscript{91}}
\end{figure}

Importantly, it has become clear that the LTRs are primarily responsible for initiating provirus silencing as determined from chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq); silencing factors are predominantly enriched at the LTRs. Therefore, recruitment of such factors to the LTRs is the first step, followed by \textit{cis}-acting silencing of the region between the LTRs.

\textit{Lentiviral vectors in vivo}

The adaptation of endogenous lentivirus for use as vectors for gene therapy was developed in 1996 and by 1998 packaging systems were created to generate self-inactivating (SIN) lentiviral vectors that integrated into the host genome but did not produce more lentivirus.\textsuperscript{92} Essentially, the human immunodeficiency virus (HIV) genome was split into a three-component plasmid system in order to create a replication-incompetent virus. The two “packaging vectors,” which encoded the proteins required create viral proteins, could be transfected into cells along with the “transfer vector,” in order to create HIV viral particles with a genome encoded by the transfer vector, which on its own could not create more HIV particles. The lentiviral particles could infect cells, and following infection integrate the contents of the transfer vector between and including the LTRs into the recipient cell’s genome. The machinery produced by the packaging vectors was required
to infect and integrate the cargo based on cues from the LTRs, but the integrated provirus lacked the components necessary to make more HIV.93

Lentiviral vectors were applied to the production of transgenic animals by direct infection of embryonic stem cells (ESCs) in 2002 by several groups. Generation of lentiviral vectors by replacing the cytomegalovirus viral promoter with a mammalian promoter reduced the rate of silencing; regions of the cytomegalovirus (CMV) promoter in particular are well-documented to influence the kinetics of silencing.94 Efficient transgenesis was observed by several groups including those of Inder Verma and David Baltimore. The title of one paper from Verma’s group explicitly states: “Lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos.” However, it should be noted that in order to achieve close to 100% of retained transgene expression, an MOI of 50 was used. Baltimore’s group reached similar conclusions but performed a deeper copy number analysis for provirus integration at a range from 1-15 and concluded that the lentivirus was expressed consistently in animals carrying at least 2 copies, however a single copy was not expressed consistently.95,96 Furthermore, in 2004 James Ellis’ group isolated mouse ES cells that had been transduced with exactly one copy and found that during differentiation these cell lines became completely silent, variegated, or displayed low expression.92

Figure 8 Schematic of 3-component lentiviral vector system in 199693
The copy number caveat has been obscured to some extent over time, in large part because SIN lentiviral vectors generally work for most purposes and have been adapted for use successfully in many aspects of biology distinct from virology. Verma’s group declared in 2003 that “The transgenes delivered by lentiviral vectors are capable of escaping gene silencing and expressing stably *in vivo.*” This claim is true, but the choice of words here is important; the fact that lentiviral vectors are “capable” of escaping gene silencing is very far from the idea that lentiviral vectors *always* escape gene silencing. This distinction is nontrivial but studies outside of the field of virology have not been exhaustive. By 2005 it was suggested that roughly half of all SIN lentiviral vector provirus sites are either immediately silenced, prone to silencing over time or variable in expression. Multiple integration events were recommended for gene therapy in stem cells. However, SIN lentiviral vectors tended to work for most purposes such as infection of ES cells for transgenesis, transducing cell lines for other purposes, and in the case of GEMMs the delivery of a recombinase to initiate tumor formation.

*Lentiviral silencing in GEMMs*

Lentivirus silencing has not been rigorously studied in cancer GEMMs but a handful of examples have been described that support sustained transgene expression. These cases involve the delivery of a driving oncogene by the vector such that the readout of silencing is relatively simple: do tumors form? These vectors leverage the concept of “oncogene addiction,” the postulation that without expression of a driving oncogene, a tumor will no longer be able to sustain its pro-proliferative program and consequently regress. Based on this assumption it can be inferred that the presence of a tumor requires expression of the integrated vector.

In a mouse model of melanoma, subcutaneous injection of Ink4a/Arf<sup>fl/fl</sup> mice with a virus containing NRAS<sup>Q61R</sup>-IRES-Cre resulted in tumor formation in 63% mice, though this observation can be interpreted in several different ways. The virus may be silenced in the negative mice, but it is just as possible that the transformation efficiency using this method was low. A more convincing example from Inder Verma’s group involved the lentiviral delivery of vectors containing oncogenic HRAS<sup>V12</sup> and/or AKT in conjunction with green fluorescent protein (GFP) to induce glioblastoma in adult mice. In this report, neither vector alone induced tumors but in combination tumors were robustly formed and 98% were positive for GFP and both proteins.
To my knowledge, no rigorous study of silencing kinetics has been studied \textit{in vivo} using endogenous GEMMs initiated by a single lentiviral integration site as is the case with the lentiviral-induced KP model of NSCLC. All of the observations I am aware of regarding silencing in GEMMs are either anecdotal or subject to alternative interpretations. A deeper exploration into the origins of SIN lentivirus vectors will be discussed further on in the context of the coming results.
2. CRISPR screens in the KP model of NSCLC

2.1 Rationale

The aim of this work is to identify and characterize KRAS synthetic lethal partners in vivo using the CRISPR/Cas9 system in the “KP” autochthonous KRAS-driven lung adenocarcinoma GEMM. The rationale for this project is to generate a system that optimally embodies the aforementioned ideals of preclinical cancer modeling: to gain the most amount of clinically relevant information in the least amount of time. The rationale is informed by some of the key points highlighted in the literature review:

1. Endogeneous GEMMs have the highest preclinical relevance.
2. Cas9 mice eliminate the time and cost barrier of breeding conditional knockout (KO) alleles through viral delivery of sgRNAs targeting genes of interest.
3. Lentiviral vectors are optimal for use in Cas9 GEMMs:
   a. Large packaging size and efficient cloning.
   b. Modularity of lentiviral backbone.
   c. Robust correlation between viral titer and number of tumors formed.
   d. Integration into the genome of infected cells.
   e. Retained transgene expression in vivo.
4. CRISPR screens in vitro have revealed promising lists of putative target genes.
5. Cell-line based screens are often poor predictors of clinical efficacy.

There remains an unmet need to rapidly validate and rank the importance of these putative target genes in endogenous GEMMs in order to translate in vitro findings to the clinic. The utility of endogenous Cas9 GEMMs, combined with the efficiency and modularity of lentiviral vectors, have offered the first real opportunity to feasibly address this issue. The gap that remains to be filled is to leverage both the data acquired from in vitro screens and the preclinical value intrinsic to endogenous GEMMs.

Therapeutically relevant targets are genes required for tumor maintenance, rather than those required for initiation. In the clinic, cancer is not typically caught in early stages of disease
progression. Therefore, deleting the gene of interest while simultaneously transforming the cell has no fully interpretable preclinical value. In order to delete genes in established lung tumors using this technique, a lentiviral vector is required. In addition to its role in tumor initiation, integration of the lentiviral vector into the host genome is essential; it carries inducible sgRNA components to delete any gene of interest at a defined time-point. The stable long-term presence of the vector permits unperturbed tumor progression until the gene is targeted in tumor-bearing mice. Using this technology and the KP model, the proposed solution is to develop a technique to perform meaningful genetic screens in vivo.

2.2 Methodology

Screening a library of genes

Targeting putative maintenance genes one at a time, or targeting one gene per mouse, grows increasingly inefficient and less interpretable as the number of genes examined increases. Factors such as mouse-to-mouse variability, actual number of viral particles inhaled, and extremely precise titers across virus are just a few limitations to such a method. To control for these factors, a more desirable method is to generate a pooled virus with equimolar ratios of each unique sgRNA in the library. Conceptually, for a library targeting 10 genes, each unique sgRNA would be represented in 10% of the total virus population. During the infection event in vivo, the probability of a given cell to receive a particular sgRNA would therefore be 10%. As the cell proliferates and eventually becomes a tumor, the integration event is carried on through each cell division. Therefore, a single mouse infected by this pooled library would have a collection of tumors, each composed of cells harboring a single unique inducible sgRNA.

PCR-based readout for sgRNA abundance

After the tumors have developed to a clinically relevant stage, the first step is to simultaneously induce transcription of all unique sgRNAs in vivo. This process could be best compared to the initial infection event with in vitro screens; the cells that received sgRNAs against genes important for proliferation will deplete from the population over time. After several population doublings, the cells harboring these important sgRNAs will be represented at a lower proportion than originally. To assay for the genes whose depletion most affects tumor growth and viability in vivo, tumors must be allowed to progress in a similar manner until an appropriate endpoint is reached.
Building on the previous example of targeting 10 genes, if the number of cells containing an sgRNA against a particular gene is less than 10% of the total number of cells across every tumor, then this gene would be more important for tumor growth than average and more attractive as a therapeutic target. At the defined endpoint, mice are euthanized and whole lung genomic DNA for each mouse is harvested. Afterwards, the region containing the sgRNA sequence is amplified by specific primers, and these products are submitted for high-throughput sequencing to calculate the abundance of reads at least 100x coverage. The relative abundance of sgRNAs therefore acts as a surrogate for the number of tumor cells containing an sgRNA; the total number of cells is unknown but the percentage of cells harboring a given sgRNA can be determined. Based on relative abundance, the rank in physiological importance of each candidate gene in an in vivo setting can be determined.

**Figure 9** Basic conceptual steps of a genetic screen in vivo.

### 2.3 Technical considerations

**Size of library**

The first consideration in any screen is how many genes to target. For statistical reasons, any results from a genome-wide screen in vivo cannot provide any actual meaningful information. An excellent illustration of this point was published recently in 2015. The maximum size of a library

---

102
is influenced by many factors, including the number of genes targeted, the number of cells infected, and an appropriate MOI for statistical purposes.

In the KP model of NSCLC, it has been demonstrated that only a single integration event occurs per cell that leads to tumorigenesis. This fact is an advantage in this model because it means that the MOI for every single cell is exactly 1; there is no need to factor in what effects may happen when more than one gene is deleted in the same tumor. With an appropriate titer, a given mouse can harbor at least 50 macroscopic tumors. The number of tumors per mouse multiplied by the number of mice in the experiment is equal to the total number of tumors across all of the mice. The total number of tumors divided by 100 represents the maximum size of the library, such that each sgRNA is represented on average 100 times. From these rough calculations, I propose to screen a library of 50 genes simultaneously using 100 mice.

**Library selection**
After the maximum size of the library has been determined, the next consideration is the library composition. In this project, the putative target gene list for use in the KP model was curated based on CRISPR screens *in vitro* on over one hundred human lung cancer cell lines performed by collaborators at the Broad Institute. The library cannot be composed exclusively of target genes, however. In order to set a reasonable ranking system for the final abundance of sgRNAs, the library should include sgRNAs against 2 or 3 essential genes, and 2 or 3 non-targeting sgRNAs or sgRNAs that target non-coding regions of DNA to control for the effects of double-stranded breaks due to Cas9 activity. The sgRNAs against essential genes should be present at low or undetectable levels while the sgRNAs expected to have no activity should be the most-detected sgRNAs, representing average tumor proliferation over the time-schedule.

**Data deconvolution**
A consideration that is unique to *in vivo* screens is the inherent stochasticity of tumor development. Cell lines have a defined doubling time and maintaining appropriate coverage of the library is straightforward. *In vivo*, however, a substantial proportion of the library can be lost based on genotype-independent and cell-extrinsic factors. For example; if one were to barcode 100 cancer cells from the same line, transplant them into a mouse, wait a period of time, and then assess the
representation of each barcode in the final cell population, the likelihood that each barcode would be represented perfectly equally would be extremely low. Some cells may die as a product of the transplantation, others may find their way into a nutrient-rich niche, others may be outcompeted based on position effects, etc. Therefore, an in vivo screen must weigh these uncontrollable factors alongside the requirement for an appropriate library representation.

Tumor spectrum of grade and size at time of sgRNA induction and endpoint
Based on previous studies, I propose that the optimal time of sgRNA induction and consequent deletion is 8 weeks post-initiation when the spread of tumors is most heterogeneous, of similar size and generally grade 2 or 3.\textsuperscript{56} The defined endpoint is essentially how long to let the tumors grow before harvesting lung genomic DNA. An endpoint of 12 weeks was decided for several reasons. First, the endpoint must be before the time when the mice succumb to the disease because they must be euthanized simultaneously in order to minimize noise due to temporal variation. The tumors must proliferate long enough for noticeable differences in growth to be seen, factoring in considerations such as the amount of time it takes for Cas9 to delete the gene after sgRNA induction, but they cannot proliferate so long that the inherent stochasticity of tumor growth leads to excessive over-representation of subsets of sgRNA-containing tumors. Since the readout is proliferation-based and lung-specific, it is important not to let the tumors progress to advanced stages such that metastases are frequent. Harvesting lung gDNA in addition to gDNA from metastases would require an inconsistent protocol for each mouse that depends on the presence or absence of metastases and the number of metastases. Moreover, metastases would bias the representation of a particular sgRNA if they were included given the different proliferation rates and space to grow in a given metastatic niche. Although genetic screens for metastasis-related factors would be interesting and possible, although with a much smaller library, this technique is focused on genes that are relevant to the primary tumor.

Non-unique sgRNAs in the same mouse
The raw readout for the screen is measured in total sgRNA abundance based on high-throughput sequencing of PCR-amplified products from lung gDNA. The relative sgRNA abundance alone, however, can be skewed by the fact that non-unique sgRNAs may be present in the same mouse.
To take a basic example, imagine a mouse with 4 tumors but 3 unique sgRNAs, depicted by the following schematic in green, yellow, and blue:

![Figure 10 Illustration of non-unique sgRNAs in the same mouse](image)

The additive effects from unique tumors harboring the same sgRNA presents a problem with calculating total sgRNA abundance. Next-generation sequencing (NGS) platforms would detect 3 unique sgRNAs and therefore one may assume that there were 3 total tumors. By adding up raw sgRNA counts, one might conclude that the yellow gene was the least important for tumor growth. Based on the image above, however, it is clear that the blue and green tumors are larger; the average of the two yellow tumors would be lower than that of the blue and green tumors.

To get around this issue, DNA barcodes can be incorporated into the pooled virus to allow for each tumor and the sgRNA it received to be marked genetically by its unique lentiviral integration event. Each sgRNA is still represented equally in the pooled virus but each individual virus has its own unique tag. Amplification of the barcode and the sgRNA together as a single PCR product would allow for sequencing reads that included both the sgRNA and the tumor it came from. Therefore, additive effects are eliminated by distinguishing between tumors and taking the average or median sgRNA abundance across the population. Continuing the example above, the NGS platform would register 4 total tumors and 3 unique sgRNAs: 2 containing the yellow sgRNA and the other 2 containing the blue and green sgRNA, respectively (Figure 11).
Mouse-to-mouse variability

The advantage of using a single, pooled virus in a cohort of mice is to reduce the mouse-to-mouse noise in terms of tumor-initiating events due to virus delivered and inhaled, the noise from titer and freeze/thaw cycles of using many different unique viruses in a large cohort, the stochasticity of intra-animal tumor development, and many other factors. This technique proposes a screen in an autochthonous model with 5000 tumors spread across 100 mice such that variability and noise is considered over the average of the 5000 tumors. In contrast, a total of 5000 tumors can also be obtained by having 50 cohorts of 2 mice, but given the aforementioned factors the noise and stochasticity would dramatically increase despite having the same number of total tumors.

In order to fully minimize mouse-to-mouse variability from a statistical perspective, ideally a given PCR product of the pooled DNA submitted for NGS will contain the sgRNA, the barcode from the tumor it came from, and information about the exact mouse the tumor came from. After extracting gDNA, amplifying the region of interest, pooling the PCR products in equimolar ratios from each mouse and ensuring that the entire library is represented, the information regarding which mouse the tumor came from would be lost. Attempting to individually barcode 100 mice in the germline would be little more than exercise in futility, but taking advantage of the concept of “dual-index sequencing reads” in NGS can overcome this issue. Sequencing adapters allow a mouse to be identified uniquely by adding an additional PCR overhang containing another DNA barcode in the amplification step. This requires a PCR reaction for each mouse and a total of 20
unique overhang primers; the 5’ and 3’ ends are sequenced so 10 unique 5’ and 10 unique 3’ primers are required for a combination of 100 total PCR products representing 100 mice.105

**Lentiviral vector considerations**

Controlling as many factors as possible for the experiment is essential for obtaining interpretable, statistically significant meaningful results from the genetic screen. Thus far, information regarding the abundance of sgRNA reads in every unique tumor and the mouse it came from is possible. Although some of these factors have been outlined and addressed theoretically, designing the components of the lentivirus itself to make these conditions possible is of utmost importance.

**Packaging limit**

Lentiviral vectors have a relatively large packaging limit (~10kb) but more components in the system lead to lower titers and can also have unpredicted consequences. Given the large size of the Cas9 coding sequence, having Cas9 in the germline is crucial for freeing up space.106 Additionally, nuclease activity would be presumably higher and more uniform given that its expression is driven by a strong promoter in the same open locus in the germline of every mouse.

**Pooled cloning**

To generate a lentiviral vector that with a unique barcode and a unique sgRNA requires several rounds of cloning. The vector is designed to contain a single cloning site such that upon digestion, a pooled library of 15bp unique DNA fragments can be cloned into the vector, making each one unique. The rationale behind choosing 15bp is based on size and uniqueness considerations. The number of unique barcodes generated will be $x^4$, with $x$ being the length of the DNA fragment and 4 being the number of possible bases at each position. Using a 15bp library, calculating $15^5$ yields 50,526 unique viral barcodes. For 5,000 tumors, the probability that the same barcode appears more than once is therefore approximately 10%. However, taking into consideration that the library harbors 50 unique sgRNAs, the total number of unique combinations becomes 50,526 multiplied by 50; therefore, the probability that the same combination appears more than once in the library (5 thousand tumors divided by 2.5 million combinations) is effectively null at 0.2%

The cloning process requires that the barcoding step occurs after the unique sgRNA-harboring vectors are cloned. Essentially, the 50 unique vectors are cloned by conventional methods,
sequenced to confirm accuracy, and lastly pooled in equimolar ratios. The pooled vector is then digested by a restriction enzyme unique to the barcode entry site, and the barcodes are cloned into the open site rapidly by Gibson Assembly; the vectors and the barcodes are designed to have Gibson-compatible 5’ and 3’ ends. Finally, the barcoded, pooled vector is subjected to deep-sequencing to confirm the equimolar presence of all sgRNAs and the successful insertion of the barcodes. The general outline of this process is depicted visually below.

1. Digest pooled plasmid at pre-defined restriction enzyme site
2. Clone barcode library built with Gibson Assembly-compatible ends into the digested plasmids

![Diagram of the process]

Figure 12 Generation of a barcoded pooled vector

**Inducible promoter**

An important factor to consider is what the basal expression of the sgRNA is in the off-state of the promoter, commonly referred to as the promoter “leakiness.” Since Cas9 gene deletion is a binary event, minimizing sgRNA expression is crucial prior to tumor initiation; it is difficult to perform an appropriate analysis if the time a given gene is deleted is not uniform across all tumors. Expression of sgRNAs is typically driven by a Pol III promoter, which robustly transcribes non-coding RNAs. The dox-inducible version of the Pol III promoter is a basally active promoter that is repressed by the tet-repressor (TetR) protein in the absence of doxycycline. Administration of doxycycline relieves repression and causes activation. The problem with a basically active promoter is that it is leakier; it requires expression and sufficient levels of the TetR protein to prevent any sgRNA expression. A more desirable promoter would be the Pol II promoter TRE, which is a basally inactive promoter in the absence of doxycycline and requires doxycycline to induce a conformational change in the rtTA protein to activate transcription. The catch is that Pol
II promoters typically transcribe mRNA while Pol III promoters typically transcribe noncoding RNA such as sgRNAs. To overcome this problem, I adapted a method developed by the Lu lab that leverages the RNA endonuclease capability of the protein Csy4 with a triple helix architecture to assist in noncoding RNA stability expressed from a Pol II promoter.\footnote{108}

\begin{center}
\includegraphics[width=\textwidth]{figure13.png}
\end{center}

\textbf{Figure 13} Schematic of inducible portion of vector

In this system, doxycycline is required to activate expression of the tet-responsive-element (TRE), which then leads to transcription of the Csy4 protein and an inactive sgRNA. The triple helical structure at the 3′ end of the Csy4 mRNA maintains stability of the transcript. Following translation of Csy4, it cleaves the 28bp Csy4 recognition sites flanking the sgRNA and produces an active sgRNA that can be bound by Cas9 to cleave the gene of interest. This architecture controls the tightness of Cas9 activity with a built-in AND gate that requires doxycycline to express the protein from the transcript, followed by sufficient amount of Csy4 to cleave the sgRNA, and finally Cas9 binding to the sgRNA and cleaving DNA (Figure 14).

\textit{Efficiency of homozygous deletion}

The efficiency of homozygous deletion of a gene for a given sgRNA is difficult to predict despite recent improvements in algorithms made for sgRNA design. Efficiencies per sgRNA are variable and for this reason CRISPR screens \textit{in vitro} recommend at least 6 unique sgRNAs per gene in order to control for this variability.\footnote{109} Studies involving deletion of a large portion of DNA by using two sgRNAs have demonstrated an improved efficiency of nuclease activity at the site of interest.\footnote{110,111} In order to improve the efficiency in this system, the Csy4 architecture is further applied in order to include multiple sgRNAs in the same vector targeting different regions of the same gene (Figure 15) to increase the probability of double KO.
Figure 14: A Csy4 architecture creates an AND-gate for Cas9 cleavage

Figure 15: Increasing efficiency of deleting a single gene by tiling multiple sgRNAs

The number of sgRNAs required to achieve >95% double KO can be roughly modeled mathematically:
\[ a + ar + ar^2 + ar^3 + \cdots + ar^{n-1} = \sum_{k=0}^{n-1} ar^k = a \frac{1 - r^n}{1 - r}. \]

**Figure 15** Equation for a geometric series

In this equation, \( a \) is the average probability of double KO success for a given sgRNA, \( n \) is the number of sgRNAs tiled in the vector, and \( r \) is equal to probability of double KO failure for an sgRNA, or \((1 - a)\). Setting an average sgRNA efficiency of 60%, employing 2, 3 or 4 sgRNAs against the same gene leads to an overall probability of double KO at 84%, 94%, and 97%, respectively. Obviously this model is highly simplistic but it gives a reasonable framework to think about how many sgRNAs to use in order to achieve a high efficiency of double KO.

**Lentiviral silencing**

Last, but not least, an important variable is the phenomenon of lentivirus silencing. In the KP model, whether or not this phenomenon occurs has only been speculated upon anecdotally, and a quantitative assessment of the kinetics has not been performed to my knowledge. More generally, studies of lentivirus transgene expression *in vivo* in cancer GEMMs are sparse and the few that exist are qualitative and open to many interpretations. Therefore, one of the goals of this project is to assess whether this issue occurs in the model and if so, find ways to resolve it.

In the following two pages, a more complete schematic of the steps involved in genetic screens involving one cohort or two cohorts in the context of a pharmacological agent for drug sensitivity are presented.
Proposed screen in the KP model

Mice are infected with pooled library of top Kras* SL candidates

Tumors progress normally and sgRNAs are not expressed

Induce sgRNA expression in established tumors

Cas9-mediated deletion and further tumor progression

Sacrifice, assess sgRNA abundance by NGS

Figure 16 Detailed schematic of proposed in vivo screen
Screen for drug sensitivity in the KP model of NSCLC

**Figure 17** Schematic of split-cohort pharmacological *in vivo* screen
2.4 Results

Due to the technique-based nature of this project, the experiments are largely not hypothesis-driven. Therefore, the major experiments are controls to address the technical considerations of the project before proceeding with the screen itself. In this section, I will discuss the experiments performed or planned in order to address these considerations.

In vitro experiments

Much of the work performed in vitro involved designing, cloning, testing, and optimizing viral vectors. The components of the vector were assembled from PCR-amplified parts of previous vectors used by the Jacks lab or gene-blocks ordered from IDT. These gene-blocks were codon-optimized for expression in mouse with minor changes in base-pairs to remove unwanted restriction enzyme sites. The parts were generated through a combination of standard, assembly, and extension polymerase-chain-reaction (PCR) techniques followed by Gibson Assembly to ligate the components of the insert into the lab backbone to produce TERC (TRE-Csy4-iRFP EFS-rtTA-Cre). The vector schematic without any sgRNAs or barcodes is visualized below:

Figure 18 Schematic of dox-inducible backbone
Vector components

The vector has all of the components required to inducibly express and cleave sgRNAs, measure expression via fluorescence, incorporate a library of barcodes, and lastly perform recombinase activity. The vector utilizes a two-promoter system in order to express two separate gene products. The first promoter (TRE) is inducible while the second promoter (EFS) is constitutive. The rationale and value of using the following parts are listed in order from 5' to 3':

a) TRE
The TRE promoter was chosen as the inducible promoter for its robust induction in response to doxycycline and its low degree of leakiness, as described in the previous section. The TRE is the most upstream component in the vector.

b) Csy4
The Csy4 protein was chosen as the optimal choice for multiplexing sgRNAs for a variety of reasons. The Csy4 protein itself is very small and the system allows for the rapid cloning of a string of sgRNAs simultaneously. In contrast, other methods for multiplexing such as dual promoter systems, sgRNA release via endogenous ribozymes, tRNAs, etc., were avoided due to space considerations and cloning inefficiency. Moreover, Csy4 is the nuclease that is responsible for cleaving tandem sgRNAs in the native CRISPR system and consequently adds a degree of orthogonality to the system by avoiding using endogenous mammalian machinery.

c) P2A-iRFP670
Porcine teschovirus-1 2A (P2A) is a self-cleaving polypeptide that acts as a linker between Csy4 and iRFP670. iRFP670 is a fluorescent protein in the far-red spectrum and allows for both in vivo imaging and as a surrogate for measuring Csy4 expression. The purpose of using the P2A, as opposed to using an internal ribosome entry site (IRES), is that generally cleavable linkers express both proteins from the same promoter more efficiently. Since termination of translation occurs at the stop codon in iRFP670, the ratio of Csy4 to iRFP670 expressed is 1:1 and therefore the kinetics of induction and degree of Csy4 expression can be measured with higher precision than with an IRES.
d) Restriction enzyme site Pmel
The Pmel restriction enzyme site is unique and upon digestion by Pmel acts as a Gibson-compatible entry site for the barcoded library.

e) “Filler” region flanked by BsmBI sites
The filler region contains junk DNA that is excised by restriction digest during the process of cloning sgRNAs into the vector. The filler region is essentially an entry site for sgRNA cloning.

f) Efl-a core promoter (EFS)
The EFS promoter was chosen as the constitutive promoter due to its small size, mammalian origin, and its robust and homogeneous expression across a panel of cell lines.\(^{113}\)

g) rtTA
The rtTA protein is a third generation dox-inducible trans-activating protein that is constitutively expressed but only becomes active and binds to the TRE to induce expression upon the addition of the chemical compound doxycycline.

h) Cre
The recombinase Cre is the component required for tumor initiation. In the KPC9 model, it activates an oncogenic \textit{Kras} allele, deletes \textit{p53}, and induces Cas9 expression such that its activity is limited to tumor cells.

\textit{Component testing}
In order to proceed forward at all, the activity of each component had to be confirmed. Cre activity was confirmed by transient transfection of the vector into a murine cell line harboring a LSL-GFP transgene; in the presence of Cre, the stop cassette is excised and GFP-fluorescence can be detected visually using fluorescence microscopy. Cre expression was also observed by immunoblotting. Robust induction from the TRE promoter was observed by transient transfection of the vector into 293FS* cells. Mean fluorescence intensity (MFI) was measured by flow cytometry, validating the functionality of rtTA and iRFP670 expression. In the presence of doxycycline, shown in red
below, expression from the TRE promoter was equivalent to that of the CMV promoter (data not shown), which is one of the highest-expressing promoters.10

The increase from baseline to maximum induction of the MFI in dox(-) vs dox(+) was roughly 6.6-fold. The relatively high basal expression is likely due to the nature of transient expression; massive amounts of the vector are transfected and each cell will tend to harbor many copies of the plasmid. In contrast, a single viral infection event will have only one copy and although the induction levels may be lower, the basal expression will most likely also be lower. Transient transfection after 48-72 hours while the plasmid is still retained may be comparable to a stably transduced cell line with a very high MOI.

**sgRNA multiplexing**

Due to the lack of commercial antibodies against Csy4, its presence was measured indirectly by whether or not the vector was capable of cleaving target genes when Cas9 was also expressed. To assess the relative efficiency of double KO of 1x, 2x, 3x, 4x, or 5x sgRNAs against the same gene, three readouts were chosen. The first is at the protein level by immunoblotting for the Yap1 protein, the second was a flow cytometry based screen using a secondary antibody against a non-essential cell surface protein, B2m, and the third was a viability-based assay using sgRNAs against a previously described essential gene, Polr1b.77 In order to rapidly clone multiple sgRNAs into the same backbone simultaneously, a Golden Gate assembly strategy was performed. Essentially, each sgRNA was flanked by Golden Gate-compatible BsmbI sites. This TypeIIIS restriction enzyme leaves a 4n base pair overhang past its recognition sequence, and the 4bp sequences were designed to have homology to either the backbone or its position in the string of sgRNAs. This cloning
system is superior because it is a one-pot reaction: the backbone and any number of sgRNAs are placed together with the Type IIS restriction enzyme and a DNA ligase. After multiple rounds of digestion and ligation in a thermocycler, the mix is ready to immediately transform bacteria and positive colonies can be screened following day.

Initial attempts at cloning multiple sgRNAs simultaneously showed a dramatically low efficiency, despite many optimizations of the thermocycler settings. When running the mix or individually digested parts by gel electrophoresis it was clear that the poor efficiency was due to slow or weak activity of the enzyme itself, as the majority of products were undigested. Additionally, another speculation as to the inefficiency of the reaction was that the BsmbI enzyme is active at 55°C while the ligase was active at 16°C. Perhaps the difference in temperatures lead to lower activity or minor ligase inactivation during the cycling process; moreover, the DNA ligase used is normally heat inactivated at 65°C. Because BsmbI appeared to be inefficient, BsmbI was replaced with the Esp3I isoschizomer, a nuclease that recognizes the same restriction site. Esp3I is active at 37°C and is reported to be dramatically more efficient than BsmbI. Indeed, switching to this isoschizomer and re-optimizing the PCR conditions resulted in an extremely high efficiency of ligation with very low background.

After the plasmids were cloned and verified by sequencing, generating virus yielded less than 1% lentivirus-positive cells in the total population. Packaging constraints due to the size of the construct were ruled out by making virus in parallel with a completely unrelated plasmid of roughly the same size. Even when sorting the 1% positive cells, no nuclease activity was observed by immunoblotting even after over a week in doxycycline-containing media (data not shown). To rule out possible issues with the plasmid itself, a murine cell line containing a Cas9 transgene and a Cre-inducible GFP was transfected with plasmids containing 0, 1x, or 2x sgRNAs against an essential gene. 72 hours post-transfection, the relative viability of 1x or 2x sgRNAs vs. the control was measured by GFP-positivity. This experiment (Figure 20) demonstrated roughly that efficiency of double KO appeared to increase with 2x compared 1x sgRNAs but other questions regarding leakiness, inducibility, time for nuclease activity to occur, etc., could not be answered without generating virus.
Transient transfection (72 hours)

Figure 20 Efficiency of multiple sgRNAs in double KO

Although it perhaps seems obvious in retrospect, one possibility that virus could not be produced is that expression of Csy4 from the transcript during the viral production process leads to cleavage of the viral RNA genome and therefore makes it unable to be packaged.  

Figure 21 Likely error during packaging process
To overcome this issue, two strategies were in development. The first was to invert the inducible portion over the vector so that very little Csy4 protein is translated during the viral production process. The 5’ CMV enhancer element before the 5’ LTR will no longer transcribe Csy4 RNA and since the 3’ LTR has very little promoter activity in and of itself, the goal would be that insufficient Csy4 protein would be produced to cleave the viral genome.

**Figure 22** Inverted Csy4 strategy to overcome packaging issues

The second strategy would require more breeding steps, employing a germline Csy4 allele in the mouse to completely bypass the packaging issue due to Csy4.

**Figure 23** Csy4-germline strategy to overcome packaging issues
These experiments are in progress and it remains to be determined whether the Csy4 platform for rapid Golden Gate assembly of multiple sgRNAs will be a feasible solution.

**In vivo experiments to probe lentivirus silencing**

In order to address the question of whether or not lentivirus silencing occurs in the KP model of NSCLC, I designed a straightforward dual-fluorescence reporter for lentivirus activity *in vivo*. This first fluorescent marker was red and came endogenously from the KPT mouse: this mouse harbors a Rosa26<sup>Lsl-Tom</sup> allele which allows for the expression of a very bright red fluorescent protein called tdTomato (Tom) in cells that receive Cre recombinase. All cells that become transformed will be red due to recombination of the stop cassette in the *Tom* transgene. As is the case with a germline allele expressed from the Rosa26, cells should retain expression of the *Tom* allele and remain red permanently; germline alleles in the Rosa26 locus are not subject to lentiviral silencing effects. The mice were then to be infected with a vector “ECG” containing Cre and a bright green fluorescent protein, mNeonGreen (EFS-Cre-p2a-mNeonGreen); the fraction of green to red cells over time was to be a surrogate to measure lentiviral silencing quantitatively and qualitatively.

In parallel, I also attempted a strategy derived from the aforementioned concept of oncogene addiction. Indeed, it has been shown that withdrawal of doxycycline from a mutant *Kras* transgene leads to tumor regression in a mouse model of NSCLC. Although the strategy was less elegant than the endogenous model, I added a codon-optimized mutant *Kras<sup>G12D</sup>* to the end of the previous vector to make “ECGK” (EFS-Cre-p2a-mNeonGreen-p2a-<sup>G12D</sup>*Kras*). Given the relatively small size of Kras protein, I was concerned that some altered function may result from the extra peptides that would much more be difficult to detect; Cre and mNeonGreen have simple readouts but a slight modulation of Kras may produce subtle and unpredictable changes in its binding affinity or activity. The rationale for putting Kras last in the series was that due to the nature of P2A linkers, the cleavage event actually leaves a few peptides at the C terminus of the protein that precedes it; the N terminus of the protein that follows it is fully native, generating an unaltered Kras<sup>G12D</sup> protein product. I confirmed the expression of all components by immunoblotting and fluorescence microscopy. I used an antibody specific for mutant *Kras* in a wild-type *Kras* cell line in the absence
or presence of the vector to confirm the expression of mutant Kras and distinguish between the wild-type protein.

![Figure 24 Schematic of lentivirus silencing experiments and cross-comparison](image)

A total of 8 mice were included in the preliminary study *in vivo*: 4 with genotype KPT and 4 with PT, with some carrying the Rosa26<sup>LSL-Luciferase</sup> transgene as well. KPT(L) and PT(L) mice were infected with 200K viral particles of either EFS-Cre-p2a-mNeonGreen or EFS-Cre-p2a-mNeonGreen-p2a-Kras<sup>G12D</sup>, respectively. The purpose of the luciferase allele was to monitor and compare growth kinetics *in vivo* between the two cohorts and to assess whether or not an oncogenic Kras transgene was capable of overcoming silencing and mimicking the KP model. Mice were imaged at 4, 6, 8, and 10 weeks. Of the 4 in each cohort, 2 were sacrificed at 8 and 12 weeks each for other assays.

*In vivo imaging via bioluminescence*

KP(L) or K+PT(L) infected with ECG and ECGK, respectively, were monitored over time for *in vivo* tumor growth. At 4, 6, and 8 weeks the signal appeared relatively similar. The images below appear to have different intensities but this apparent difference is actually based on the automated exposure time; normalizing the signal based on raw photon count, the tumors appeared to grow at similar rates however at 8 weeks the signal was slightly lower in the K+PT(L) group (Figure 25).
At 10 weeks, a marked difference was observed between the two groups. It appeared as if the cohort infected with the Kras transgene had suddenly stopped growing or the kinetics of growth had rapidly diminished. The remaining 4 mice from both cohorts that were not sacrificed at 8 weeks were placed in the same chamber together to compare bioluminescence and epifluorescence based on tdTomato simultaneously (Figure 26). This observation is subject to multiple interpretations; it is possible that the Kras transgene had been silenced but it is also easily imaginable that non-physiological levels of the Kras protein product expressed by the EFS promoter were simply insufficient to drive further tumor growth.
Flow cytometry

Data based on flow cytometry was inconclusive largely in part due to a lack of proper controls in the experimental design. Optimally, the cohort would have included mice without tdTomato infected with mNeonGreen alone, mice with tdTomato infected without a fluorescent protein, and mice without a fluorescent protein in them at all. By flow cytometry at 12 weeks, it appeared that very few cells contained mNeonGreen; however, the inability to perform fluorescence compensation for spectral overlap in the flow cytometer made the results uninterpretable. KPT mice infected with ECG displayed roughly 3% double-positive cells, but this is likely to be an artefact due to the lack of necessary controls. In one of the mice infected the Kras transgene, no detectable evidence of any positive cells were found. Representative raw data from the flow cytometer plots are presented in the following page (Figures 27 and 28).
Figure 27 Representative FACS plot of KPT mouse infected with ECG
(Q1: Red only Q2: Double positive Green and Red)

Figure 28 Representative FACS plot of PT mouse infected with ECGK
Fluorescence microscopy

Images obtained from frozen sections of lung tissue using fluorescent microscopy were difficult to interpret for similar reasons as with flow cytometry. Using confocal microscopy, the signal from mNeonGreen (green) was substantially weaker than that of the DAPI mounting media (blue) and tdTomato (red). Crosstalk between channels made any meaningful interpretations very difficult. Using ImageJ analysis for spectral deconvolution I was able to partially separate the contribution but due to the inherent subjectivity of post-image processing the results remained inconclusive. Below, a representative image obtained from a 12 week KP tumor is shown; the unprocessed image is shown on the left and the right is the same image after applying software for spectral deconvolution. In the right image, patches of green become more visible.

![Figure 29](Image)

**Figure 29** *Left:* Original image. *Right:* Process image using spectral unmixing algorithm.

By zooming in more closely on certain regions, the limitations of fluorescence microscopy in this experiment become evident. Blue staining of nuclei and red cells are clearly visible. A couple of cells display evidence of a green signal but paradoxically, there are locations in which there is green and no red. After all, no cells should be single-positive for green given that tdTomato should be robustly expressed in all cells that have received Cre, and therefore mNeonGreen. Green autofluorescence in the lung tissue is likely to play a role in obscuring the potential information to be obtained in the image. An alternative interpretation is that macrophages engulfed an mNeonGreen(+) cell but even still one would expect to see tdTomato as well.
**Immunohistochemistry (IHC) for Cre recombinase**

The most convincing data regarding the lack of sustained expression from the lentiviral vector came from IHC analysis of tumor sections. By performing IHC for Cre recombinase and comparing matching hematoxylin and eosin (H&E) slides, a qualitative sense of inter- and intratumoral expression could be determined. For clear reasons, if Cre is not expressed then the vector is silenced. In general, tumors fell into three classes of expression based on Cre staining: retained, variegated, or extinct. Qualitatively, it was observed that while some tumors maintained almost complete expression of Cre, some tumors displayed variable expression and in others Cre expression was almost completely lost or encountered only in the peripheral portions of the tumor. Representative images for 8 week and 12 week KP tumors at various magnifications are presented in the following pages.
Figure 31 Wide-field image of spectrum of 8 week tumors

Figure 32 zoomed image of 8 week tumors

Figure 33 Wide field image of 12 week tumors
Figure 34 Zoomed images of tumors with retained, variegated expression at 12 weeks

Figure 35 Zoomed example of tumor with largely extinct expression at 12 weeks

Figure 36 Cre expression of K+PT tumor at 12 weeks
From the previously described results, the following four key points can be inferred:

1) Tumors display extensive intra- and intertumoral heterogeneity in Cre expression at 8 and 12 weeks and appears to be mouse-independent.

2) Three subcategories of tumors appear with respect to Cre expression: mostly retained, variable, or mostly lost.

3) Intratumoral lentiviral Cre expression appears qualitatively to decline with time but more data points are required to substantiate this claim.

4) Tumors initiated by vectors harboring the Kras transgene fail to recapitulate the histological features of the KP model.

**Future directions**

Necessary future experiments unrelated to the silencing data presented include:

1) Infection of KP mice with barcoded lentiviral vector and harvesting DNA at various time points to determine the precise number of unique barcodes detected; this number is equal to the total number of tumors per mouse that are formed and will provide some more insight into determining the optimal size of the library.

2) Infection of KPC9 mice with the TERC vector containing sgRNAs tiled against several essential genes. In response to doxycycline at a given time point, the expected results are that tumors will disappear completely; this experiment serves as a positive control and also determines the degree of responsiveness to doxycycline.

3) Infection of KPC9 mice with TERC vector targeting more than one non-essential gene, including a cell-surface marker. The presence of these genes could be detected by IHC, immunofluorescence, or flow cytometry. This experiment will validate that Csy4 works and can allow the cutting of multiple targets in vivo.

4) Pilot experiment with KPC9 mice using a small library of 5 genes (including negative and positive controls and genes whose disruption is known to affect tumor development) to optimize the technique and determine the minimum amount of gDNA required in order to acquire 100x coverage of the library by NGS.

Given that the key obstacle at this stage of the project appears to be inconsistent expression of the integrated virus at the necessary time-points, further work must be performed to attempt to address this issue. One possibility is to change the system such that sgRNAs are expressed constitutively.
at initiation, though this option is undesirable because the screen no longer asks the fundamental question of what genes are responsible for tumor maintenance. Given that the transgenic Kras approach appeared to fail, the previous assays will be repeated in KPT mice and remain the same save for one component; iRFP670 will replace mNeonGreen to make ECR (EFS-Cre-p2a-iRFP670). Substituting the fluorescent protein carried by the vector with one in the far red spectrum will substantially reduce data convolution due to overlapping excitation and emission spectra that result from a dramatic difference in the expression of tdTomato. Previously, exciting mNeonGreen also lead to a minor excitation of tdTomato, but since there was an overwhelmingly disproportionate amount of tdTomato, some signal from tdTomato was read in the channel for mNeonGreen. With a strong DAPI to stain nuclei in the blue channel, some interference was also observed between the blue and green channels. iRFP670 has an excitation frequency which is beyond that of the excitation frequency of tdTomato and therefore complications due to spectral unmixing will no longer present a problem for fluorescence-based readouts. Increasing the size of each cohort will be beneficial as well. Sorting single and double positive cells and subjecting them to epigenetic analysis to determine methylation at the proviral integration site could prove helpful, as well as DNA and RNA fluorescence in situ hybridization for the lentiviral sequence to see if genetic deletions occurred that caused the loss of the provirus itself. Additionally, including additional time points such as 2, 4, 6, and 10 weeks to sacrifice mice should yield data points to give information regarding the actual rate of silencing with respect to time.

Many modifications to the vector itself are possible, including swapping the promoter EFS with other commonly used promoters like PGK, UBC, etc., or using various chromatin insulator elements reported.\textsuperscript{118} The time and number of mice required to try each different combination prohibits testing every possible combination so the precise modifications to the vector must be chosen very carefully. My leading hypothesis is that modifications to the LTR of the SIN lentiviral vector used are required to ultimately address the fundamental issue of lentiviral silencing. In the following section, I will elaborate on the previous discussion of lentiviral silencing and speculate as to possible problems and resolutions that arise from the investigation of the nature of lentivirus; the factors that are independent of the transgenic components added to generate the vector.
3. Discussion

3.1 Lentivirus silencing in vivo and implications

Epigenetic silencing of the long-terminal repeats of the lentiviral vector followed by methylation between the LTRs is almost certainly the cause for the results observed in my system. A multitude of evidence in the literature points to the fact that the LTRs are generally responsible for mediating lentivirus silencing. Indeed, transcription factor binding sites for known mediators of lentiviral silencing are present in the vector used in these studies. Given all of this information, a deeper look into the phenomenon of lentiviral silencing is warranted in order to better understand its role in cancer GEMMs and to explore possible methods to overcome it.

The Verma paradox

A persistent and puzzling question arose from my research: why were Verma’s results using an oncogene delivered by a lentiviral vector to generate glioblastoma so different from mine? After all, contradictions cannot exist. In this section, I will postulate as to why we obtained different results using similar strategies and discuss possible interpretations.

Leveraging the principle of oncogene addiction by adding oncogenic Kras to the lentiviral vector did not appear to share the same characteristics as the standard KP model. The kinetics of tumor progression appeared similar by luciferase assay at 8 weeks but by 12 weeks proliferation slowed dramatically despite being infected with the same number of viral particles. Positive cells displayed a distinct morphology and tissue architecture, including increased immune cell infiltration (Figure 33). A deeper analysis of the histological features of the tumor cells and surrounding tissue may be warranted to pinpoint the precise differences between the models. Another possibility is that the transgene was not expressed at sufficiently high levels or lacked the complex regulatory features that tightly control endogenous Kras.

Another possibility is that in Verma’s model the cells in the tissue of origin are less prone to viral silencing. The neuronal cell type could have played a role in long-term sustained expression; it is a well-documented fact that neuronal cells generally retain transgene expression for longer periods of time. Lentiviral vectors were originally applied to neurons and the cargo is expressed at high
levels. It is worth noting, however, that in early experiments using neural stem cells (NSCs) the MOI was on average 13. Furthermore, during in vitro differentiation of NSCs, SIN lentiviral vectors were found to be silenced rapidly. 

Taking a deeper look into Verma’s paper itself, one possibility is that some of the interpretations regarding lentiviral silencing were inaccurate. A figure from the Verma paper displays an image of the FITC (GFP channel) alongside the corresponding H&E-stained section. The darker region reflects the increased density of the tumor.

![Figure 37](image)

**Figure 37 left:** FITC confocal image **right:** H&E section

Note that the GFP observed is heterogeneously expressed. The lack of a positive or negative control of the normal or tumorous cerebrum in the FITC channel was not shown and the raw files generated by the confocal microscope itself were not included. In my hands, mNeonGreen, which is substantially brighter than GFP, is difficult to distinguish from background auto-fluorescence in the lung. With no negative or positive control, the background intensity specific to the precise conditions (intensity, field of view, exposure time, pinhole size, etc.,) of a given shot can be extremely difficult to gauge accurately. Without the raw files, how the image was processed cannot be determined either. The normal variability of Hras expression within tumors cannot account for this heterogeneity because in this case, mutant Hras is driven by a promoter in the lentiviral vector. Regardless, the mice did indeed form tumors that recapitulated the biology of the normal tumor well. The degree of expression and prolonged kinetics were far greater than what I observed in the KP model.

One very likely possibility in my opinion comes from the observation that oncogenic Hras and Akt
were insufficient to induce tumors alone. Two integration events were required to form tumors. Recall that early studies on SIN lentivirus pointed to a key feature involving lentivirus MOI in transgenesis; single copy integration sites resulted in variable expression or complete extinction while high numbers of integration sites lead to robust expression. Based on much of these data, it was postulated over a decade ago that the probability of a single integration site being silenced was roughly 50%. Therefore, the fact that two lentiviral vectors were required in order to induce tumors in Verma's paper suggest that the number of integration sites correlates with the capacity for long-term tumor development. A caveat here is that in the glioblastoma model both Akt and Hras (tagged with HA and FLAG, respectively) was observed in 98% of cells; if there were only 2 integration events then one would imagine that on average one would become silenced and therefore a heterogeneous population of AKT+ and/or HRAS+ positive cells would appear. Below is a representative image taken from the supplementary data:

![Image](image.png)

**Figure 38** Left to right: Immunofluorescence images for Hras, Akt, and merged with nuclei staining

Although clear co-localization is observed in some cells, it appears to be that many cells (red) are only Akt positive. Furthermore, besides the two patches of heavy co-localization, expression is much weaker and without a negative control to subtract background auto-fluorescence, the claim is arguable. A possible interpretation is that the cells that happened to receive at least one copy of both Akt or Hras that did not become silenced grew out and eventually formed glioblastomas.

**The doctrine of original SIN**

There is one more reason that Verma's paper showed different results than mine, and it has to do with the nature of the vector, not the cargo. Delving more deeply into the history and evolution of SIN lentiviral vectors, a critical but substantially overlooked factor regarding the nature of SIN lentiviral vectors and their propensity for silencing will be discussed.
The foundation for the use of lentiviral vectors for stable gene transduction *in vitro* and *in vivo* was laid in 1996 by Inder Verma and Didier Trono. Although vectors for gene therapy had been developed previously, they were of limited utility because they could only transduce dividing cells. Verma and Trono chose a system based on HIV because of its capacity to infect non-dividing cells. Two years later, Verma and Trono published two separate papers describing several distinct SIN lentiviral vectors. In the following decade the concept of a ubiquitous singular SIN lentiviral vector emerged that culminated in a sense of interchangeability between these vectors without consideration of their differences. The devil is in the details; in this case, the details are in the U3 region of the 3’ SIN lentiviral LTR. Below is how SIN vectors and their relationship to wild-type HIV are generally annotated today:

In 1998 the groups of Verma and Trono both made modifications of the U3 region of the transfer vector in order to reduce *cis*-acting transcriptional activity from the LTR promoters. Because the provirus integrates into random regions of DNA, site-specific interference of the surrounding endogeneous elements was undesirable. It was known that the U3 element contained transcription factor binding sites such a TATA box, NF-kB, and Sp1. In order to reduce basal activity, Verma’s group deleted 133bp of the “3’ LTR” U3 region that was found inessential for packaging and integration. In the normal HIV vector, there is no need for a 5’ and 3’ annotation for the LTRs;
the provirus that integrates contains the exact same flanking LTRs. SIN vectors, however, leverage the fact that if a U3 region is removed from one of the LTRs, the viral genome RNA will be reverse transcribed and integrated with the U3 from the other end in the same orientation (Figure 39b). For this reason, the terminology of 5' and 3' LTRs was introduced in order to provide orientation in SIN vectors. For reasons that will become clear shortly, I will refer to any SIN lentiviral vectors harboring a 133bp deletion in the U3 region as “Verma vectors.” The schematic of an example transfer vector from this paper is presented below, with the triangle representing the 133bp deletion.

![Figure 40 Verma vector: 133bp deletion](image)

Trono's group took a similar strategy for similar reasons. However, Trono's group was less conservative with the U3 deletion. Noting that there were many more transcription factor binding sites in the HIV U3, they deleted an entire 400bp from the vector. Due to the difference in the amount of information deleted from the U3 region, I will refer to any lentiviral vectors harboring the 400bp deletion as “Trono vectors.”

![Figure 41 Trono vector: 400bp deletion](image)
An extremely important point that must be emphasized is why they chose to generate these deletions. The deletions had nothing to do with lentiviral silencing. Silencing was not mentioned in these papers. The primary reason for making these changes was to reduce the risk of positional effects of the LTRs leading to activation of proto-oncogenes. Other factors considered were safety reasons such as preventing mobilization of replication competent viruses or recombination to create a wild-type U3 region. Silencing was not studied in these papers because at the time the role of the U3 region in mediating silencing or activation of HIV was unknown or unclear. To reiterate the difference between the vectors; Verma’s vector deletion is shorter than Trono’s vector, therefore Verma’s vector has more transcription factor binding sites in the U3 region than Trono’s vector. Over time, an appreciation for the distinction was lost and they became essentially synonymous. Addgene and common plasmid maps annotate Verma and Trono vectors in the same way. They both seem to work fine, however, so when presented with this information a fair enough question to ask is “Who cares?”

Let us take a moment to consider why we should care. The reasons go back to the nature of HIV itself and the concept of viral latency. Latency is the capacity of a virus to become dormant in a cell for a period of time and reactivate under certain conditions; in terms of SIN vectors, the concept of dormancy is similar to that of silencing. Epigenetic alterations occur under particular conditions that cause the virus to stop producing viral particles and halt the viral life cycle. HIV is infamous for its extremely high mutation rate due to replication errors resulting in a rapid selection for fitness-related traits; this feature makes HIV extremely difficult to target pharmacologically or vaccinate against. Despite this rapid selection for fitness, it at first seems counterintuitive that HIV would have the property of latency given that latency acts as a limitation on the viral lifecycle and results in fewer viral particles. Yet why would HIV have human transcription binding site factors in its LTRs? The prevailing hypothesis among HIV virologists has long been that this latency is merely a quirk in evolution that gives no fitness advantage; some epigenetic changes occur during CD4+ T cell (HIV’s target) state transitioning that happens to reactivate the virus.

An alternative theory was proposed in 2015, supported by a mathematical model of HIV latency based on human data. An exhaustive discussion of the details is beyond the scope of this work but
the general idea is that latency evolved in HIV as a “bet-hedging” strategy to optimize survival and transmission; during mucosal infections there is an incentive to minimize viral extinction in order to optimally ensure long-term survival.\textsuperscript{126} This means that the transcription factor binding sites in the U3 region of HIV are there on purpose; they were evolved to respond to cell state changes and establish a state of dormancy and reactivation when necessary. Putting these pieces together, one could imagine that the fewer transcription factor binding sites there are in the U3 region of a SIN lentiviral vector, the less prone to silencing or variegation the vector would be.

Today, most SIN lentiviral vectors commonly used are derived from the Verma vector, containing the 133bp deletion that leaves several transcription factor binding sites in the U3 region. These commonly used backbones include pSico, pLKO.1, lentiCRISPRv2, etc., many of which are used for short hairpin RNA (shRNA) knockdown to create stable cell lines, RNAi screens, CRISPR screens, and in many GEMMs. The vector used in this work is also derived from the Verma vector. This is not to say that vectors derived from Trono’s 400bp deletion are not in use today, but the difference is not acknowledged. Presumably, Trono’s vector would be less prone to silencing given that it has fewer transcription factor binding sites.

Returning to the Verma paradox, the 2009 paper in which 98% expression of the lentiviral vector was observed in a glioblastoma GEMM, I examined the map of the vector generated for this work called pTomo by this group. In this paper, Verma’s group used a modified version of Trono’s vector containing the 400bp deletion! Perhaps the resolution to the paradox is that this Trono vector used by Verma’s group lacked the transcription factor binding sites contained in the Verma vector I used to perform silencing studies. To my knowledge, up until 2009 Verma’s group had used lentiviral vectors derived from Verma’s original vector. The reason for the switch was not stated in the paper or in any review in the literature. It is unclear whether it was intentional or accidental, but all things considered, the nature of the vectors used appears to be the most pronounced difference between our methodology. This concept remains to be tested, but if true it could have far-reaching consequences for the use of lentiviral vectors in all contexts.
Final thoughts on viral LTRs

Today, lentiviral LTRs appear to be the biggest contributor to lentiviral silencing. Despite this fact, no innovations have been made since 1998 to reduce LTR-induced lentiviral silencing. Evidence from studies of endogenous retroviral silencing, HIV virology, and SIN lentiviral vectors in gene therapy, when taken together, suggest that novel modification of the LTRs is required in order form single copy lentiviral vectors capable of prolonged transgene expression in vitro and in vivo.

Shortening the U3 region of the LTR even further may prove to be a plausible strategy to prevent the binding of transcription factors that silence the site of integration. During the process of writing this thesis and contemplating the nature of viral LTRs, one possible solution occurred to me to prevent silencing specifically in the KP model: simply replace the 400bp deleted region of the Trono U3 with the partial murine Kras promoter, which is about 380bp. It is difficult to imagine that Kras-driven lung cancer cells could successfully silence a region flanked by 2 endogenous Kras promoters. One could also imagine putting the mutant Kras transgene back in the vector with the same flanking configuration. A positive aspect is that the different oncogenic variants of Kras could be tested rapidly but the model is of course no longer an endogenous GEMM since the allele comes from an outside source. To save space, one could just include the 30bp GA-element of the KRAS promoter, which is required for 94% of the transcriptional activity of the aforementioned Kras reporter. Generally, including the binding motifs for transcriptional activators like c-Myc, or other epigenetic activators would seem to be a plausible strategy to tip the epigenetic balance towards maintenance of expression.

With respect to genetic screens, recent evidence strongly suggests that CRISPR screens are far superior to RNAi based screens in vitro. A number of valid reasons have been posited for this; variability is hairpin knockdown, interference with endogenous machinery, full knockout is superior to partial knockout, Cas9 is more effective, etc. On the other hand, sgRNAs vary in their ability to generate homozygous deletions and a minimum of 6 sgRNAs per gene is recommended in order to obtain statistically significant information. However, recall that genetic screens require a very low MOI close to 1 in order ensure that the same cell does not receive multiple vectors, convoluting the data. In both RNAi screens and CRISPR screens, a short round of selection for integrated vectors by FACS or antibiotic resistance (so as not to enrich or deplete
genes involved in antibiotic resistance) is performed. Most of the vectors used in such screens are
derived from the Verma vector. In my hands, I have observed loss of expression of fluorescent
markers at low MOI over the time it takes to complete a CRISPR screen. I would go so far as to
posit that one reason CRISPR screens are superior is because CRISPR does not require sustained
expression of the vector for long periods of time. The event is binary: Cas9 cleaves the target gene
while the sgRNA is expressed, and it either deletes the gene or does not. With RNAi screens,
sustained expression of the shRNA over the course of the experiment is required in order to mimic
ture repression of the gene of interest. If expression of a given shRNA diminishes or becomes
extinct over time, the noise in the system increases dramatically.

If my theory regarding the U3 region of the LTR is correct, modifications to SIN lentiviral vectors
could also substantially benefit clinical trials in gene therapy. Lentiviral vectors are the safest
option but are prone to silencing in humans as well.

I strongly recommended that further work be pursued to modify the U3 region of SIN lentiviral
vectors in order to remove and replace the components that are responsible for mediating viral
latency.

3.2 Significance

Considerations for silencing in vivo

For experiments performed with lentiviral vectors that require long-term expression or retention
in vivo, I strongly suggest using multiple methods to confirm that the transgene is still expressed
at any relevant time points. For example:

1) IHC for transgenic protein product and immune markers.

2) Include a fluorescent protein in the vector for IF. Far-red proteins are recommended due to
visibility in vivo, compatibility the nuclear staining (DAPI) in the blue channel and
minimization of auto-fluorescence issues.

3) Include several negative and positive controls in the appropriate assays.
Preclinical significance of successful CRISPR screens in GEMMs

The “gap” between identifying putative cancer drug targets and translating discoveries to the clinic is more aptly described as an abyss. Less than 10% of animal models with positive findings translate to clinical cancer trials. Despite successful pre-clinical testing, 85% of early clinical trials for novel drugs fail; of those that survive through to phase III, only half become approved for clinical use. A systematic review of 30% of the most highly cited papers involving animal models demonstrated that under 50% displayed good methodological quality. While a third were replicated in clinical trials, a fifth contradicted results from clinical trials. A successful FDA approval process takes on average at least 11 years and costs range from 500$ million to 2$ billion per drug.

The overarching aim of this project, lofty though it may be, is to address this problem. Rather than solving a single issue, the goal is to create a technique that could be used by many cancer biologists to assess the context-specific role of putative drug targets across a range of lung cancer GEMMs and ultimately to GEMMs of cancers arising from other tissue types. The goal is to build a technique that is meant to be spread and innovated upon.

A single academic lab would invariably lack the resources to scale up a project of this magnitude to include a larger library of targets and a greater variety of genetic backgrounds and cancer types. However, large scale-ups could be more economically feasible for the biotechnology industry and on a smaller scale, academic labs could answer more precise questions. From a basic science perspective, independent labs could use the tool to ask specific questions about a pathway of interest to gain insight into tumor progression, maintenance, and the role of the cancer microenvironment.

The efficacy of certain pharmacological agents, which often target multiple members of the same protein family, could be gauged against the effect of genetic deletion of all family members. This form of analysis could serve to explore two fundamental questions. First, how effective is the agent in inhibiting the enzymatic function of its targets in vivo? Second, do these proteins play other roles that are independent of function that the drug targets? An example experiment would be to assess the effects of MEK1/2 kinase inhibition via Trametinib against complete genetic ablation.
of both genes simultaneously. It is known that deletion of Mek\textsubscript{1/2} or Erk\textsubscript{1/2} at initiation prevent tumor initiation in a murine model of lung adenocarcinoma, but whether or not both Mek\textsubscript{1/2} or Erk\textsubscript{1/2} are both required for tumor maintenance is an open question. By assessing a kinase inhibitor against genetic deletion of its targets, a metric for in vivo drug efficacy can be established. If Mek\textsubscript{1/2} are essential in Kras mutant tumors in GEMMs but still retain sufficient kinase activity to survive in the presence of current kinase inhibitors, then a more focused effort into developing superior kinase inhibitors would be warranted. A similar co-experiment in which sgRNAs are designed to delete particular “hotspots” of the kinase domain specifically could prove helpful as well. If increasing the on-target effects of new kinase inhibitors yield no survival benefits, then the exploration of a non-enzymatic role for the proteins could yield new insights into MAPK biology and novel targets. Such findings would not be too surprising given the major roles of Mek\textsubscript{1/2} as scaffolding proteins in multiple pathways.

The unprecedented speed, modularity and flexibility of this system combined with the substantial preclinical relevance of endogenous GEMMs would, in theory, open up a world of questions for the imaginative scientist. The ability to ask these questions and obtain meaningful answers could offer enormous insight into the unknown vulnerabilities of different types of cancer and into the nature of biology itself. In this way, the hope of the project is to play a small role in shrinking the abyss: to help prevent the tragic and unnecessary loss of human life.
**Materials and methods**

**Mice**
Mice were housed in the Unit for Laboratory Animal Medicine at the Koch Institute for Integrative Cancer Research and all the experiments were performed in accordance to the Massachusetts Institute of Technology Institutional Animal Care and Use Committee (IACUC) guidelines. All animals were maintained on a mixed C57BL/6J × 129SvJ genetic background. \(K-ras^{LSL-G12D/+/p53^{fl/fl}}\) or \(p53^{+/+}\) mice were bred into mice harboring the \(Rosa26^{LSL-Tom}\) and \(Rosa26^{LSL-Luc}\) mice and were infected intratracheally with 200K transforming units of lentivirus as described\(^5\) and sacrificed at 8 and 12 weeks.

**Viral production and infections**
For transfection experiments, HEK-293FS* cells and murine KP lung adenocarcinoma cancer cell line 1233-Cas9 were transfected using TransIT-LT1 (Mirus Bio) in Opti-MEM (Life Technologies). Lentivirus was produced by transfection of HEK-293FS cells with A8.2 (gag/pol), CMV-VSV-G, and the corresponding lentiviral construct as previously described.\(^5\) To concentrate virus, supernatant was collected 48 and 72 hours post-transfection and centrifuged at 25,000 RPM for 2 hours and resuspended in an appropriate volume of OptiMEM (Gibco).

**Molecular cloning**
Lentiviral vectors were constructed by combining compatible vector parts designed as gene blocks (gBlocks) and ordered from IDT. Amplification of PCR products, ligation by Gibson Assembly and bacterial transformation were performed using standard techniques as described.\(^1\) Golden Gate Assembly of multiple sgRNAs was performed as described\(^8\) by designing complementary overhang sites specific for the Esp3I restriction enzyme built into the vector.

**IVIS imaging**
*In vivo* bioluminescence images were acquired with the IVIS Spectrum (Xenogen) after intraperitoneal injection of 1.5 mg beetle luciferin (Promega) as described.\(^3\)

**Cell culture**
Human embryonic kidney cells (HEK-293FS) cells and 1233-Cas9 cells were cultured in DMEM (Corning) supplemented with gentamicin 0.5mg/mL, and 10% fetal calf serum (FCS; Gibco) at 37 °C in a 5% CO\(_2\) humidified atmosphere. To induce iRFP670 expression, cells were treated with varying doxycycline concentrations for 48-72 hours.
**Immunohistochemistry**

Mice were euthanized by carbon dioxide asphyxiation. Lungs were perfused through the trachea with 4% paraformaldehyde (PFA), fixed overnight, transferred to 70% ethanol and subsequently embedded in paraffin. Sections were cut at a thickness of ten micrometers and stained with H&E for pathological examination as described. Antigen retrieval was performed with Borg Solution (Biocare Medical) in a pressurized Decloaking Chamber (Biocare Medical) for 3min. Samples were blocked in two steps: first, samples were incubated for 20min with 5% donkey serum solution in antibody diluent (Vector Laboratories) supplemented with Avidin D solution (Vector Laboratories) (4 drops per ml), rinsed with Tris-Tween and then incubated other 20min with 5% donkey serum solution in antibody diluent supplemented with Biotin solution (4 drops per ml). Samples were subsequently rinsed with Tris-Tween and incubated overnight at 4°C with primary antibody in antibody diluent solution. Anti-Cre antibody was a gift from the Yilmaz lab. Samples were then washed 3x for 5min with Tris-Tween, incubated with secondary antibody (1:500) in antibody diluent for 35 min, rinsed 3x 5min with Tris-Tween and incubated for 1h with VectaStain Elite ABC immunoperoxidase detection kit (Vector Labs PK-6101) followed by Dako Liquid DAB+ Substrate (Dako) for visualization.

**Cryosections and immunofluorescence**

Mice were euthanized by carbon dioxide asphyxiation. Lungs were perfused through the trachea with 4% paraformaldehyde (PFA), isolated, and were incubated in 15mL conical tubes in the dark overnight at 4°C in 4% PFA in PBS. After being fixed, samples were rinsed 3x 10min with PBS, incubated in 30% sucrose for 5h at 4°C and embedded and frozen in OCT medium (Tissue-Tek). To perform IF staining, 10μm samples were cut in a cryostat machine, attached to microscope slides and fixed for 10min in -20°C acetone. Subsequently, samples were washed 3x 5min with TNT (0.1M Tris pH 7.5, 0.15M NaCl and 0.05% Tween-20), fixed with 1% PFA in PBS 2min at RT, rinsed 3x5min with PBS, and mounted in Vectashield media.

**Flow cytometry**

Mouse lungs were isolated for flow cytometry analysis as described. For culture, cells were centrifuged 5min at 1000rpm at RT, re-suspended with roughly 1mL with 1:500 7-aminoactinomycin D (7-AAD) in 2mM EDTA SMEM and filtered through a 40μm mesh before flow cytometer analysis. Data was analyzed using Flowjo software (Tree Star).

**Microscopy and image analysis**

Immunofluorescence images were acquired on an Olympus FV1200 Laser Scanning Confocal Microscope and the DeltaVision microscope. Images were processed using ImageJ (NIH, Bethesda, MD) and spectral unmixing was performed using the “Spectral Unmixing” algorithm.
Abbreviations

AAV - Adeno-associated virus
B2M – Beta-2-microglobulin
cDNA – Complementary DNA
ChIP-Seq – Chromatin immunoprecipitation sequencing
CMV promoter – Cytomegalovirus promoter
Dox – Doxycycline
FACS – Fluorescence-activated cell sorting
gDNA – Genomic DNA
GEMMs – Genetically engineered mouse models
GFP – Green fluorescence protein
HDACs – Histone deacetylases
HIV – Human immunodeficiency virus
HSC – Hematopoietic stem cells
IF - Immunofluorescence
IHC - Immunohistochemistry
IP - Intraperitoneal
IRES – Internal ribosome entry site
iRFP – Infra-red fluorescence protein
KO – Knockout
LTRs – Long-terminal repeats
MFI – Mean fluorescence intensity
MLV – Murine leukemia virus
MOI – Multiplicity of infection
MRI – Magnetic resonance imaging
NGS – Next-generation sequencing
NSCLC – Non-small cell lung cancer
P2A – Porcine teschovirus-1 2A
PCR – Polymerase chain reaction
PDAC – Pancreatic adenocarcinoma
PDX – Patient cancer-derived xenografts
Pdx1 – Pancreatic and duodenal homeobox -1
Polr1b – RNA polymerase I subunit B
RNAi – RNA interference
ROI – Region of interest
RSV – Rous sarcoma virus
rtTA protein – Reverse tetracycline transactivator (tTA) protein
SCID – Severe combined immunodeficient
sgRNA – Short guide RNA
shRNA – Short hairpin RNA
SIN – Self-inactivating
Tet - Tetracycline
tetR protein – Tet repressor protein
Tom – tdTomato
TRE – Tet-responsive-element
tRNA – Transfer RNA
tTA protein – Tetracycline transactivator protein
YAP1 – Yes-Associated-Protein 1
References

10 Woglon, William H. Experimental Tar Cancer--. 1926.
13 "The Discovery of the Molecular Structure of DNA - The Double Helix". Official Website of the Nobel Prizes.
17 Stehelin, Dominique, et al. "DNA related to the transforming gene (s) of avian sarcoma viruses is present in normal avian DNA." (1976): 170-173.
31 http://www.medindia.net/patients/calculators/world_cancer_clock.asp


http://webcast.aacr.org/console/player/26968?mediaType=podiumVideo&


