FUNCTIONAL INTERROGATION OF GENETICALLY SIMPLE AND COMPLEX CANCERS

by

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A Dissertation

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Scott W. Lowe Date

Dissertation Mentor

Dedication

To my parents, for all their love and support.

ABSTRACT

The rapid expansion of cancer genomics over recent years has provided a valuable catalog of the events that drive malignancy, but functional dissection of oncogenes and tumor suppressor genes are needed to fully appreciate the roles of cancer-associated genes. Cancer types vary dramatically in genomic complexity, with pediatric cancers tending towards low mutation rates and certain other diseases tending towards hypermutation and highly rearranged genomes. Targeting driving oncogenes for therapy has precedent, particularly in low complexity cancers, and pre-clinical models provide an opportunity to refine such strategies. Treating high complexity tumors remains particularly challenging in part due to intratumoral heterogeneity, presumed to be linked to enhanced adaptability of cancer cell populations. Patterns of evolution supporting intratumoral heterogeneity have been extensively observed, but functional dissection of heterogeneity has been limited.

Fibrolamellar hepatocellular carcinoma (FL-HCC) is a rare but lethal liver cancer that primarily affects adolescents and young adults. It has been described to harbor only one recurrent genetic event: somatic deletion of a segment of chromosome 19, resulting in *DNAJB1-PRKACA* gene fusion. Efforts to understand and treat FL-HCC have been confounded by a lack of models that accurately reflect the genetics and biology of the disease. Herein, CRISPR/Cas9 genome editing and transposon-mediated somatic gene transfer is implemented to demonstrate that expression of both the endogenous fusion protein or chimeric cDNA leads to the formation of indolent liver tumors in mice that closely resemble human FL-HCC. Notably, overexpression of the wild type PRKACA

was unable to fully recapitulate the oncogenic activity of *DNAJB1-PRKACA*, implying that FL-HCC does not result from enhanced PRKACA expression alone. Tumorigenesis was significantly enhanced by genetic activation of β-catenin, an observation supported by the discovery of recurrent Wnt pathway mutations in human FL-HCC, as well as treatment with hepatotoxin DDC, which causes tissue injury, inflammation and fibrosis. Our study validates the *DNAJB1-PRKACA* fusion kinase as an oncogenic driver and candidate drug target for FL-HCC and establishes a practical model for preclinical studies to identify strategies to treat this disease.

In contrast, many cancers do not contain a single identifiable driver. Instead, evolutionary processes govern cancer initiation, metastasis, and therapeutic resistance. A better understanding how initiating driver events affect intratumoral heterogeneity, and how diversity affects the robustness of a population, is central to understand the evolution. Our hypothesis is that p53 deficiency tends to permit the accumulation of greater subclonal diversity than in p53 wild type cancer cell populations. *TP53,* the most frequently mutated gene in human cancer, is activated by various stress stimuli and acts to cull damaged cells. By using derivatives of the Eμ-myc model of B-cell lymphoma, we examined how p53 inactivation affects the heterogeneity of resulting disease. We aim to resolve whether p53 loss allows the outgrowth of a single aneuploid "jackpot" clone, or does it allow the long-term survival of a wider field of sub-optimally fit clones, which could serve a diverse reservoir of potential resistance to changing conditions, as in dissemination and treatment?

BIOGRAPHICAL SKETCH

Edward Kastenhuber grew up in Newtown, Pennsylvania and attended the University of Pittsburgh, where he graduated with a degree in bioengineering. He enjoyed a number of research experiences in engineering and medicine. With Gregory Armstrong (Children's Hospital of Philadelphia), he explored the epidemiology of optic nerve gliomas arising in children with neurofibromatosis. With Yong He and Sanjeev Shroff, he studied how mechanical stress can influence gene expression and endothelial cell behavior in the context of high blood pressure. Under the direction of Mark Gartner, Edward joined a senior design project team that designed, prototyped and installed an ultra-low cost neonatal incubator for Hôpital Albert Schweitzer Haiti.

After reading about cancer immunotherapy for glioblastoma in the university newspaper, Edward was inspired by the work of Hideho Okada and knew he wanted to become involved in this work. Dr. Okada accepted Edward into his lab as a technician where postdocs Ryo Ueda, Xinmei Xhu, and especially Mitsugu Fujita contributed greatly appreciated mentoring. It was here that Edward really got to know the joy of scientific investigation and the culture of the laboratory.

Having taken a minor concentration in Italian studies during his undergraduate studies, Edward found an exciting and rewarding opportunity working in *S. Aureus* vaccine development for Novartis in Siena, Italy for which he is indebted to Fabio Bagnoli for his mentorship.

Upon returning to the United States, Edward was hired as a research technician by Jason Huse as he was starting up his lab focused on molecular profiling and pathogenesis

of glioma. Dr. Huse provided Edward a great deal of independence and responsibility while bringing the lab from empty benches to a productive scientific endeavor. Along with the generous support by Cameron Brennan to learn the R programming language and incorporate more data analysis into his work, Edward published a first author paper with the Huse lab.

In 2011, Edward was accepted by the Gerstner Sloan Kettering Graduate School and joined the laboratory of Scott Lowe the following year. He has been supported by the Geoffrey Beene Foundation and the NIH Ruth L. Kirschstein National Research Service Award.

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I appreciate the consistent insight that I have been fortunate to receive from my thesis committee members Timothy Chan and Charles Sawyers. I have always been impressed by how broad their knowledge base extends and have always been inspired by their enthusiasm and encouragement. I would especially like to thank collaborators Gadi Lalazar and Sanford Simon, Linas Mazutis, Roshan Sharma and Dana Pe'er, J. Erby Wilkinson, Nitin Shirole and Rafaella Sordella, Carol Prives, Neal Rosen, Luke Dow, and Ghassan Abou Alfa.

I would also like to thank some of the other people that have taken the time to teach me something, provide me with useful tools and methods, to edit my work, to help brainstorm, and to generally help me enjoy the last few years. Thanks to Charles Sherr (for his advice, stories, and tough questions), Yadira Soto-Feliciano, Viraj Sangvi, Robert Bowman, Yilong Zou, Joana DeCampos Vidigal, Andrea Ventura, Johanna Joyce, Leila Akkari, Oakley Olson, Alberto Schumacher, Dan Marks, Robert Benezra, Zhirong Bao, Steve Albanese, Manu Vanaerschot, Maria Skamagki, Javier Carmona.

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LIST OF ABBREVIATIONS

PKA	Protein kinase A
HSP	Heat shock protein
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
HCC	Hepatocellular carcinoma
ICC	Intrahepatic cholangiocarcinoma
FL-HCC	Fibrolamellar hepatocellar carcinoma
FISH	fluorescent in situ hybridization
PDX	Patient-derived xenograft
CRISPR	clustered regularly interspaced short palindromic repeats
SBase	Sleeping beauty transposase
EV	Empty vector
ssGSEA	Single sample gene set enrichment analysis
EM	Electron microscopy
ARF	Alternate reading frame
BCL2	B-cell lymphoma 2 gene
PUMA	p53 upregulated modulator of apoptosis
TSG	Tumor suppressor gene
CIN	Chromosomal instability
HSC	Hematopoietic stem cell
SNV	Single nucleotide variant
LOH	Loss of heterozygosity
CNV	Copy number variant
DDR	DNA damage response
MDM2	Mouse double minute 2 homolog
LINE	Long interspersed nuclear *elements*
TCGA	The Cancer Genome Atlas
t-SNE	t-Distributed Stochastic Neighbor Embedding
scRNAseq	Single cell RNA sequencing
PCA	Principal component analysis

INTRODUCTION

Decades of intensive research in cancer genetics have yielded a monumental advance in our understanding of cancer. The proliferation and advances in high throughput sequencing has fundamentally changed the field of cancer genetics. At the time that cancer genome sequencing became commonplace, many of the most common, and most potent, cancer drivers had been discovered and characterized for many years. Tools were developed for the purpose of validating candidate genes as functionally important and explore the molecular mechanism of their function(s). The gold standard of a bona fide cancer driver gene is the capacity for the activation of oncogenes or the knockout of tumor suppressor genes to generate tumors in genetically engineered mouse models (Sharpless and Depinho, 2006). Based on knowledge of biochemical mechanisms and the generation of faithful *in vitro* and *in vivo* platforms, advances in cancer genetics have led to numerous rationally designed clinical applications to inform treatment of cancer progression and recurrence (Druker et al., 2001a).

A panoply of sequencing studies, exemplified by large consortia including the Cancer Genome Atlas and the International Cancer Genome Consortium, have produced a fairly comprehensive catalog of somatic mutations in human cancer(Golub, 2010). This explosion in the molecular characterization of cancer has far outpaced the capacity to functionally characterize newly identified candidate cancer drivers. While many genes involved in mitogen signaling, proliferation, and survival were well-known, new classes of genes have been highlighted for their importance in cancer. For example, the expansion of the epigenetics field has coincided with the understanding that epigenetic

modified are commonly dysregulated in cancer. Cancer metabolism has again come to the fore with the discovery of IDH mutations and small molecule inhibitors that may show clinical benefit. Additionally, a remarkably 'long tail" containing a wide variety of potential cancer genes has been uncovered. Therefore, platforms to generate experimental systems need to be far more time- and cost-efficient to take advantage of the wealth of these cancer genomic catalogs. One major class of drivers that has been illuminated by cancer sequencing efforts are fusion oncogenes (Gao et al., 2018). In CHAPTER I, we tackle one such candidate fusion oncogene, *DNAJB1-PRKACA*, which is ubiquitously and specifically altered in a rare type of liver cancer, fibrolamellar hepatocellular carcinoma (Kastenhuber et al., 2017). These cancers harbor remarkably few other mutations, virtually none of them recurrent, implying that the key to understanding this disease is through its potentially druggable fusion kinase.

On the other end of the spectrum, p53 is the most extensively scrutinized and also the most commonly altered gene in cancer (Kastenhuber and Lowe, 2017). Even though cancer genetics has been extensively catalogued, the biological consequence of most cancer-associated genes has yet to be fully appreciated. Surprisingly, new discoveries in p53 biology are continuously being reported. Perhaps if other more obscure genes were to receive the same amount of attention, they would also reveal this startling level of complexity. It remains relevant to pursue novel mechanisms and vulnerabilities linked to established cancer driver genes, particularly concerning subjects like p53, where there are no clinical tools to grapple with many p53 mutant cancers. In CHAPTER II, we will explore a novel consequence of p53 deficiency: the enhanced tolerance of elevated intratumoral heterogeneity. Beyond what *TP53* mutations can do to an individual cell, the

emergent properties of how TP53 mutant cells behave at the population level may hold significance to cancer progression, metastasis, and drug resistance, which can be viewed as evolutionary processes.

CHAPTER I: DNAJB1-PRKACA fusion kinase interacts with β-catenin and the liver regenerative response to drive fibrolamellar hepatocellular carcinoma in mice.

Summary

A segmental deletion resulting in *DNAJB1-PRKACA* gene fusion is now recognized as the signature genetic event of fibrolamellar hepatocellular carcinoma (FL-HCC), a rare but lethal liver cancer that primarily affects adolescents and young adults. Here, we implement CRISPR/Cas9 genome editing and transposon-mediated somatic gene transfer to demonstrate that expression of both the endogenous fusion protein or a chimeric cDNA leads to the formation of indolent liver tumors in mice that closely resemble human FL-HCC. Overexpression of the wild type PRKACA was unable to fully recapitulate the oncogenic activity of *DNAJB1-PRKACA*, implying that FL-HCC does not simply result from enhanced PRKACA expression. Tumorigenesis was significantly enhanced by genetic activation n of β-catenin, an observation supported by evidence of recurrent Wnt pathway mutations in human FL-HCC, as well as treatment with hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), which causes tissue injury, inflammation and fibrosis. The intact kinase domain of PRKACA is required for tumor initiation. Our study validates the *DNAJB1-PRKACA* fusion kinase as an oncogenic driver and candidate drug target for FL-HCC and establishes a practical model for preclinical studies to identify strategies to treat this disease{Kastenhuber, 2017 #6}.

Introduction

Liver cancer and basic liver function

Although it not as extensively studies as many other malignancies, primary liver cancer is the third leading cause of cancer mortality worldwide, at more than 750,000 deaths (Bray et al., 2018). Liver tumorigenesis is a product of the convergence of environmental and genetic drivers with the cell-of-origin.

The normal liver is organized into functional units called lobules (**Figure 1.1A)**. Blood flows into the acini from the portal venule (from the intestines, stomach, and spleen) and hepatic arteriole (from the abdominal aorta via the celiac artery) and passes through sinusoids, fenestrated blood vessels lined by endothelial cells (Lautt, 2009). Blood flow exits the lobule though the central veins, which drain into the hepatic vein and then into the inferior vena cava. A substantial oxygen and nutrient gradient exists from the periportal region to the pericentral region and hepatocyte metabolism is segregated along this axis in 3 zones (Kietzmann, 2017). Bile flows counter to blood from zone 3 to zone 1 and carried to the gallbladder by bile ducts, where it is concentrated and later injected into the duodenum.

The liver is composed of heterogeneous cell types, several of which undergo risk of malignant transformation (**Figure 1.1B)**. The most numerous type of cells are hepatocytes, which not only account for the majority of liver cells, but also serve as the cell-of-origin for the most common type of primary liver cancer, hepatocellular carcinoma. Hepatocytes are specialized carry out many of processes for which the liver is responsible, including the breakdown of metabolites, production of bile, detoxification,

and liver regeneration. Cholangiocytes make up the bile ducts and can undergo malignant transformation to cholangiocarcinoma.

Liver cancer is strongly linked to epidemiological and lifestyle factors, namely chronic viral hepatitis infection and alcoholism. Aflatoxin and parasitic liver flukes are epidemiological factors that specifically drive hepatocellular carcinoma and cholangiocarcinoma, respectively.

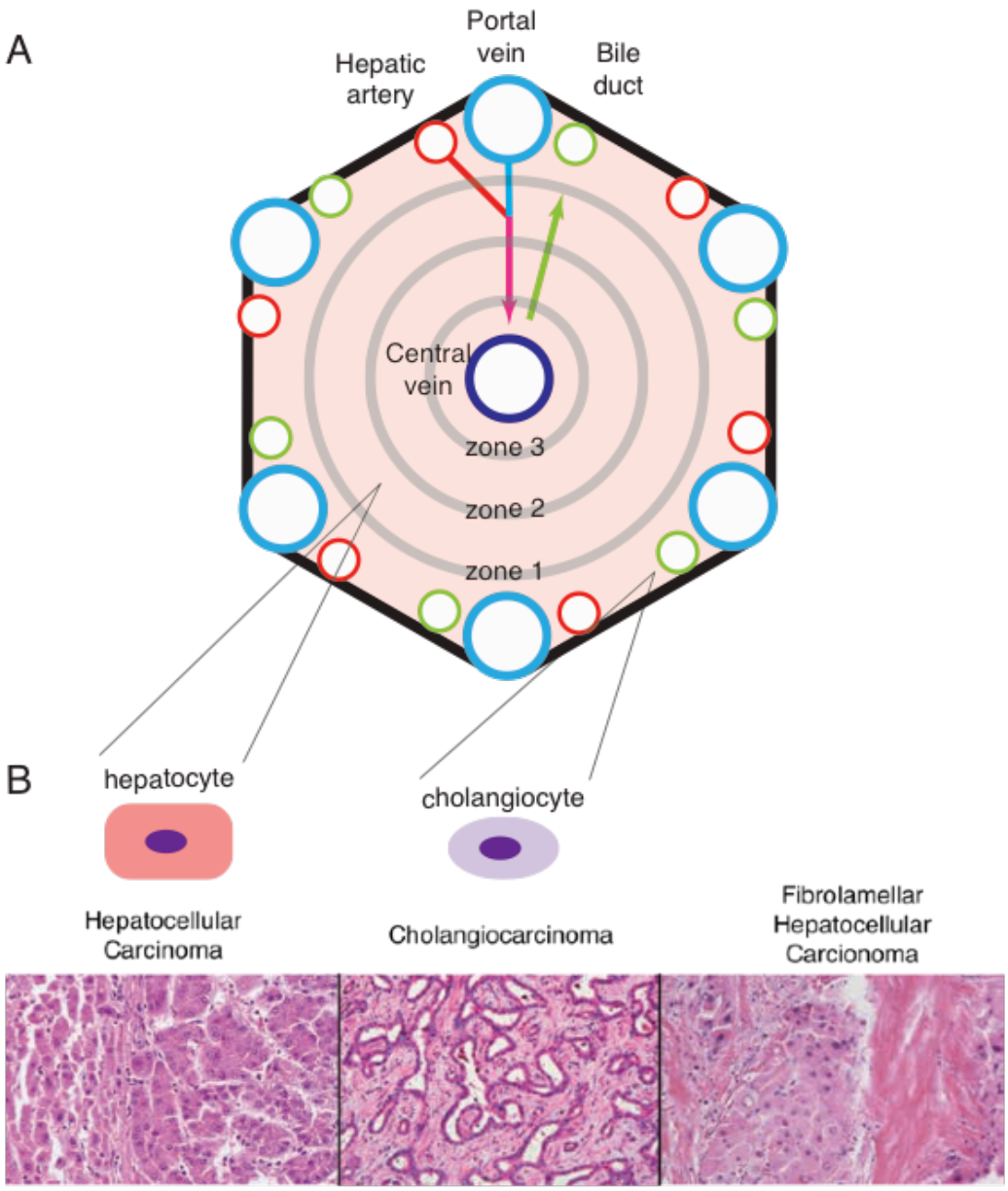

Figure 1.1. Liver structure and function

(A) The liver lobule is the functional unit of the liver. Blood mixes from portal veins and hepatic arteries and pass through sinusoids until it is drained through central veins.

(B) Hepatocellular carcinoma is derived from hepatocytes. Cholangiocarcinoma is derived from cholangiocytes. The cell-of-origin of the fibrolamellar variant of HCC is unknown.

Fibrolamellar hepatocellular carcinoma

Fibrolamellar hepatocellular carcinoma was initially described by Edmonson in 1956 (Edmondson, 1956). The first extensive case series to further define the characteristics of FL-HCC did not come until 1980 (Craig et al., 1980b). A major turning point in the study of this disease came with the discovery of *DNAJB1-PRKACA* gene fusions as a nearly universal characteristic of fibrolamellar hepatocellular carcinoma (Honeyman et al., 2014).

FL-HCC is extremely rare and accounts for less than 1% of primary liver cancer diagnoses. Aside from its low rate of incidence, a number of characteristics of FL-HCC make it biologically and demographically unusual. Also unlike the vast majority of liver cancers, which are typically diagnosed in middle-aged and elderly patients, FL-HCC specifically afflicts adolescent and young adult patients (**Figure 1.2**).

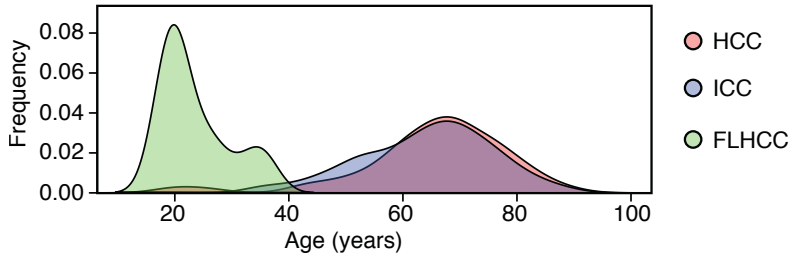

Figure 1.2. Age at incidence of liver cancer by histological subtype.

Hepatocellular carcinoma and cholangiocarcinoma are typically diagnosed in older patients. Fibrolamellar hepatocellular carcinoma is found in younger patients. Data from MSK-IMPACT (Zehir et al., 2017). HCC n=129, ICC n=165, FL-HCC n=16.

Unlike liver cancer in older adults, FL-HCC is not associated with any known etiological risk factors such as alcoholism, chronic viral hepatitis infection, liver flukes, or underlying chronic liver disease (Craig et al., 1980b; Torbenson, 2012). Cases of FL-HCC that occurred prior to 1939 were not identified in a number of retrospective studies by researchers that discovered and initially characterized the disease (Craig et al., 1980a; Edmondson and Steiner, 1954). This fueled speculation that the incidence of FL-HCC is increasing or is attributable to an environmental factor specific to the recent years of the post-industrial era (Oikawa et al., 2015). This possibility was ruled out on the basis of the identification of multiple archived cases prior to the time period in question (Graham et al., 2017). Furthermore, no significant trend in changes of the rate of incidence over time were observed in the Surveillance, Epidemiology and End Results (SEER) database of clinical outcomes (Eggert et al., 2013). Cases of FL-HCC are distributed globally, with cases studies published in Latin America (Arista-Nasr et al., 2002), Europe (Malondra et al., 1989), Africa (Moore et al., 1997), and Asia (Tangkijvanich et al., 2000).

Likewise, risk for FL-HCC is not usually inherited. The common genetic event, *DNAJB1-PRKACA* fusion, is somatic given that adjacent normal liver does not harbor the gene fusion (Honeyman et al., 2014). The rarity of the disease precludes the ability to identify predisposing germline alleles that may predispose individuals to develop FL-HCC with incomplete penetrance, but FL-HCC does not appear to be restricted to any enthnic or geographical demographic, nor has any shared germline mutation been described in the majority of FL-HCC patients. On the other hand, in multiple cases that lack the prototypical fusion gene, instead were found to be carriers of Carney complex syndrome, an autosomal dominant hereditary disorder, caused by an inactivating

germline mutation in *PRKAR1A* (Graham et al., 2018). *PRKAR1A* is a regulatory subunit of the PKA complex, whose deficiency results in dyregulated PKA activity.

Nevertheless, most FL-HCC patients do not carry Carney complex syndrome (Graham et al., 2018) and individuals that have germline *PRKAR1A* mutations very rarely develop liver cancer (Boikos and Stratakis, 2007). Thus, no known environmental or common inherited genetic driver of FL-HCC has not been identified.

Diagnosis

Currently, FL-HCC is diagnosed on the basis of histological features such as large cells with granular eosinophilic cytoplasm, vesiculated nuclei, and large nucleoli. Ultrastructural studies observe a hyperaccumulation of mitochondria and abundant endoplasmic reticulum (Graham et al., 2017). While onset at a young age and lack of chronic liver disease are suggestive of FL-HCC, classic HCC can also occur in young patients and misdiagnosis is common. In fact, a *post hoc* review of HCC cases included in the Cancer Genome Atlas sample, revealed a number of unannotated FL-HCC cases that were subsequently histologically and molecularly validated (Dinh et al., 2017). Given the specificity of *DNAJB1-PRKACA* fusion for FL-HCC, its detection will likely be decisive for correct diagnosis. Differential diagnosis between FL-HCC and classic HCC is currently based on histology and will likely be aided by the recent development of a fluorescent in situ hybridization (FISH) assay for sensitive and specific detection of *DNAJB1-PRKACA* fusion (Graham et al., 2015).

FL-HCCs often are not detected until they are relatively large, which mean diameter of ~11.8 cm (Do et al., 2014), compared to a mean diameter of 4.5 cm for conventional HCC (Lee et al., 2012). By CT imaging, FL-HCC typically appears with a well-defined outline and heterogeneous contrast uptake, commonly with a large central scar and calcifications (Ganeshan et al., 2014), and sometimes exhibiting biliary dilation, hemorrhage, and/or gross vascular invasion (Do et al., 2014). FL-HCC is hypointense of T1-weighted MRI and hyperintense on T2 weighted MRI (Do et al., 2014). FL-HCCs reportedly exhibit uptake of ^{18}F-flourodeoxyglucose (FDG) in PET imaging studies (von Falck et al., 2008). Beyond radiographic imaging, real-time monitoring of non-invasive biomarkers could serve as early warning signs of disease progression or response to therapy, although not have been clearly established. Anecdotal evidence has been reported for the utility of serum biomarkers including high serum vitamin B12-binding capacity (Kanai et al., 2004; Paradinas et al., 1982; Wheeler et al., 1986), des-γ-carboxyprothrombin (Nakao et al., 1991) and neurotensin (Collier et al., 1984).

Metastasis is a common in FL-HCC pathogenesis, especially after serial recurrence following one or more surgical resections. Nodal metastasis occurs in the majority of patients and is a negative prognostic factor (Stipa et al., 2006). FL-HCC also metastasizes intrahepatically to distal liver lobes, and to the peritoneum, lungs or brain (Do et al., 2014; Hammond et al., 2018).

Other reported signs and symptoms associated with FL-HCC include Budd-Chiari Syndrome, where hepatic veins are occluded (Asrani and LaRusso, 2012; Lamberts et al.,

1992), gynecomastia, (McCloskey et al., 1988), and hyperammonemic encephalopathy (Hashash et al., 2012; Sethi et al., 2009).

Treatment and Prognosis

While prognosis of FL-HCC patients has been reported to be better than classic hepatocellular carcinoma, this no longer holds true when comparing to HCC without background liver cirrhosis (Eggert et al., 2014). Surgical resection is currently the primary treatment for FL-HCC patients. Although often described as a relatively indolent disease, a high rate of recurrence represents a major clinical challenge (Kaseb et al., 2013) and the 5-year survival rate is 34% (Eggert et al., 2013). There is no evidence of survival benefit from adjuvant chemotherapy or any systemic treatment applicable to classic HCC (Riggle et al., 2016). While the unique demographics and genetics of FL-HCC suggest that these patients should be treated differently than those with HCC, there have been few clinical trials that have been tailored to this patient population (www.clinicaltrials.gov). The development of FL-HCC-specific therapies has been further hindered by the lack of genetically and biologically accurate model systems.

Molecular Characterization of the human disease

Fibrolamellar hepatocellular carcinoma (FL-HCC) ubiquitously harbors a ~400kb deletion on chromosome 19 that produces an in-frame fusion of the DnaJ heat shock

protein family member B1 (*DNAJB1)* and protein kinase cAMP-activated catalytic subunit alpha (*PRKACA)* (**Figure 1.6A**) (Graham et al., 2015; Honeyman et al., 2014).

Beyond the presence of *DNAJB1-PRKACA* fusions, FL-HCC tumorigenesis is poorly understood. Few, if any, other significantly recurrent mutated genes have been described (Cornella et al., 2015; Darcy et al., 2015), and while broad copy number alterations have been observed, they do not specifically implicate known oncogenes or tumor suppressors (Cornella et al., 2015).

Like many pediatric tumors, these tumors harbor an exceptionally low number of somatic mutations, very few of which are seen in multiple patients. Gene expression profiling and proteomics have confirmed that this tumor type is indeed biologically distinct from classic HCC, and implicates a variety of biological processes that may underlie disease mechanisms (Dinh et al., 2017; Simon et al., 2015).

The age distribution of exclusively adolescent and young adult patients could be consistent with a yet to be defined reliance on a specific, possibly puberty-associated, endocrine milieu for DNAJB1-PRKACA-mediated transformation. The remarkable specificity of DNAJB1-PRKACA to liver cancer supports the notion that aspect of the hepatic tissue environment may be a critical aspect of pathogenesis. Analogous to cataloguing the cell-autonomous products of FL-HCC tumors, an improved understanding of the types and activities of stromal cells and extracellular signaling molecules would be of great value.

Heat Shock Protein 40

DNAJB1 encodes a heat shock factor 40 (HSP40) protein (**Figure 1.3**), which serves as a molecular co-chaperone and is expressed in a wide variety of cell types (Liberek et al., 1991). HSP40 is part of the highly coordinated heat shock response can be induced by an array of environmental stresses. The HSP40 family is conserved from *E.* coli and has expanded from six *E. coli* homologs to constitute 50 homologs in humans (Kampinga et al., 2009). The primary role of DnaJ/HSP40 is to bind un/misfolded polypeptides and recruit DnaK/HSP70 to refold them (Hinault et al., 2010). DnaJ/HSP40 also has the capacity to independently bind non-native polypeptides and prevent aggregation (Priya et al., 2013). DNAJB1 negatively regulates heat shock factor 1 (HSF1) transcriptional activity by interacting with its transactivation domain during the attenuation and recovery phase of the heat shock response (Shi et al., 1998). DNAJB1, in combination with HSP70 and nucleotide exchange factors, can also stimulate ATP-dependent chaperone activity of HSPA1A/B (Rauch and Gestwicki, 2014).

HSP40/DnaJ family genes are classified into 3 groups based on their domain structure: subfamily A, B, and C. Subfamily A (*DNAJA1-DNAJA4*) contain a J-domain, a Gly/Phe-rich region (G/F), a zinc finger (ZF) motif, a peptide-binding fragment, and a C-terminal dimerization domain (Qiu et al., 2006). Subfamily B (*DNAJB1-DNAJB14*) lacks the ZF motif. Subfamily C (*DNAJC1-DNAJC22*) have only a J domain and J-like proteins (*DNAJC23-DNAJC30*) contain only partially conserved J- domains.

The J-domain is highly conserved and is the contact point with the ATPase domain of DnaK/HSP70, which DnaJ/HSP40 positively regulates. In the DnaJ/HSP40

proteins that contain the G/F region, this domain modulates the substrate binding activity of DnaK/HSP70 (Wall et al., 1995). ZF domains can support the interaction of the DnaJs that contain them with denatured, non-native proteins (Szabo et al., 1996).

DNAJB1 is primarily cytosolic, but can also be found in secreted exosomes (Gonzales et al., 2009) and under certain conditions of stress can also translocate to the nucleus and contribute to folding nuclear proteins (Wang and Bag, 2008).

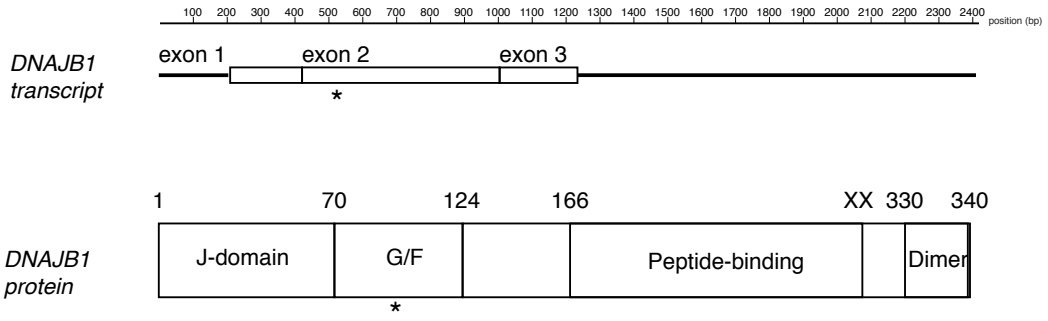

Figure 1.3. Structure and function of DNAJB1 locus.

The DNAJB1 transcript consists of 3 exons. The functional domains of the DNAJB1 protein include the J-domain, the G/F-rich domain, the peptide-binding fragment, and the dimerization domain (Hu et al., 2008; Qiu et al., 2006). An alternate start site in exon 2 allows for the expression of an isoform, which truncates the N-terminal 100 amino acids (denoted by *).

Protein Kinase A

PRKACA encodes PKA C-α, a catalytic subunit of protein kinase A (PKA) (**Figure 1.4**). PKA resides in the cytoplasm in an inactive multimeric complex variably containing catalytic subunits PKA C-α, PKA C-β, and PKA C-γ as well as regulatory subunits PKA RIα, PKA RIβ, PKA RIIα, and PKA RIIβ (Turnham and Scott, 2016). Ligands, such as hormones and neurotransmitters, activate G-protein coupled receptors (GPCRs) to transduce extracellular signals via G proteins and adenyl cyclase by elevating levels of the second messenger cAMP, which in turn activates PKA (Turnham and Scott, 2016). Phosphodiesterases (PDEs) oppose the accumulation of cAMP, favoring its conversion to AMP.

While it was previously believed that cAMP bound the regulatory subunits of PKA, thereby releasing and activating the catalytic subunits, it has now been uncovered that PKA C-α is found largely in the context of PKA holoenzyme and that, surprisingly, dissociation is not required for cAMP-mediated PKA activation (Smith et al., 2017). Rather, cell-type specific expression of the AKAPs can regulate PKA, for instance by binding to PKA RII, and recruiting the kinase to specific subcellular locations without dissociating the C-α:RII complex, thereby regulating the target spectrum of the kinase (Smith et al., 2017). Type I (RI-containing) or type II (RII-containing) PKA complexes can have substantially different biological outputs, perhaps by regulating subcellular localization independent of activity level of the kinase (Constantinescu et al., 2002). PKA has been reported to be anchored to subcellular loci including the nucleus, plasma membrane, ER, mitochondria peroxisome, and cytoskeleton (Pidoux and Tasken, 2010).

Numerous context-specific substrates, with complex biological functions in various tissues, are phosphorylated by PKA resulting in modification of their activity, localization, or stability (Kirschner et al., 2009). An uncharacterized subset of these potential PKA targets could contribute to tumorigenesis. Canonically, cAMP stimulation can lead to PKA nuclear entry, where it phosphorylates transcription factor CREB at serine 133, positively regulating transcriptional activation of genes with cAMP response elements (CRE) in upstream promoter or enhancer regions (Montminy and Bilezikjian, 1987). NF-κB p65 is stimulated by PKA upon phosphorylation at serine 276 (Zhong et al., 1998), although opposing effects have been reported (Takahashi et al., 2002). PKA phosphorylates GSK3β at serine 9, which suppresses its activity and modifies glycogen and glucose homeostasis and Wnt pathway activity (Fang et al., 2000; Whiting et al., 2015). PKA, when anchored to the mitochondria, can inactivate BAD by phosphorylating it at serine 112, enhancing cellular survival (Harada et al., 1999). In a mouse model of activated GNAS in pancreatic tumorigenesis, PKA phosphorylates and suppresses salt-inducible kinases (SIK1-3), which promotes fatty acid oxidation (Patra et al., 2018).

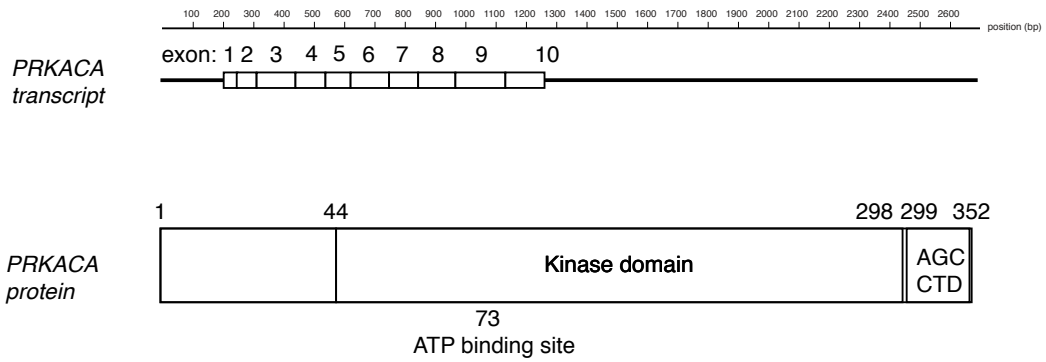

Figure 1.4. Structure and function of PRKACA locus.

The PRKACA transcript consists of 10 exons. This encodes the serine/threonine protein kinase PKA C-α. The kinase domain, ATP binding site, and the ACG-family C terminal domain are indicated.

DNAJB1-PRKACA fusion protein

The crystal structure of the DNAJB1-PRKACA fusion protein shows that the catalytic site, regulatory subunit binding and AKAP protein binding remain similar to the wild type PRKACA (Cheung et al., 2015). The fusion results in the hyperactivation of kinase activity, at least in part by driving elevated expression of the PKAcα component (Honeyman et al., 2014). Thus, expanding the fundamental understanding of native PKA signaling greatly reinforces the chances of productively interfering pathological PKA signaling.

Several possibilities for the molecular mechanism underlying DNAJB1-PRKACA exist. It is possible that the fusion serves only to elevate expression levels of the kinase through high DNAJB1 promoter activity. Otherwise, it is plausible that the DNAJB1-PRKACA fusion acts as a scaffold, bringing together novel binding partners in a kinase-independent mechanism. The specificity of the DNAJB1-PRKACA fusion to FL-HCC and the absence of activating hotspot mutations in PRKACA support the hypothesis that the first exon of DNAJB1 contributes to a putative gain-of-function character of the fusion protein in addition to high levels of expression accomplished by control by the DNAJB1 promoter. The DnaJ segment may lead to enhanced or novel phosphorylation targets of the fusion kinase, through its properties as a molecular chaperone or by altering subcellular localization or stability of the fusion protein.

There may also be some interesting convergence in the biology of FL-HCC and alternative paths to pathogenic PKA signaling, including GNAS mutant cancers of the pancreas (Cancer Genome Atlas Network, 2017) and hotspot PRKACA mutant tumors

seen in Cushing's syndrome (Beuschlein et al., 2014). Germline mutations in GNAS cause McCune–Albright syndrome, which results in endocrine dysfunction and neoplasia via aberrant PKA signaling (Kirschner et al., 2009).

Existing Disease Models:

Studies based on observation and correlation are very powerful to form relevant hypotheses, yet controlled perturbation experiments are required to provide convincing evidence that a given tumor property is important for tumor development or a valuable therapeutic target. To do so, experimental model systems are employed to push the boundaries farther than what could be done by even the most careful examination of human tumor samples. Ideally, a panel of model systems with non-overlapping strengths and caveats can be assembled to best predict what which strategies stand the best chance to be beneficial to patients. Currently, no FL-HCC cell lines are available.

The first FL-HCC model system described was a patient-derived xenograft (PDX) propagated in immuno-compromised mice (Oikawa et al., 2015). In agreement with the relatively indolent growth rate and resistance to cytotoxic drugs of primary tumors, this xenograft exhibits stem-like characteristics. PDX models provide a unique and powerful opportunity for perturbation experiments *in vivo*. Due to the variability from patient-to-patient, multiple PDX lines from independent donors are needed to ensure representation of the FL-HCC population. A small number of additional PDX lines exist, but are not widely distributed or well characterized. For this reason, a central PDX repository for FL-HCC has been proposed to accelerate the search for disease vulnerabilities. Harnessing such resources remains a challenge, given the substantial drift and mouse-specific

evolution that has been observed over serial passage of PDXs (Ben-David et al., 2017). PDXs are typically subcutaneous for convenience of transplantation and tumor tracking. It has not yet been established whether orthotopic seeding of the mouse liver, where tumor cells could interact with hepatic stroma and vasculature, could yield more physiologically relevant versions of PDX models.

Mouse models have been a powerful tool to evaluate the oncogenic potential of candidate drivers, to study the biology of tumorigenesis, and as preclinical systems to test novel therapeutics (Kersten et al., 2017). In this study, we employed hydrodynamic transfection combined with either CRISPR/Cas9-mediated editing of the endogenous deletion or transposon-mediated transgenesis of fusion cDNA and variants, allowing us to introduce genetic lesions in a subset of hepatocytes without the time and expense of producing germline genetic strains (Kawakami et al., 2017; Tschaharganeh et al., 2014). Using this approach, we demonstrate that the *DNAJB1-PRKACA* fusion is a *bona-fide* oncogene and identify genetic and environmental factors that cooperate with the fusion event to drive aggressive disease. Our results further show that the PRKACA kinase domain is required for these effects, providing rationale for targeting kinase activity pharmacologically. We anticipate that the models presented herein will serve as a powerful platform for future biological and pre-clinical studies.

Efforts to understand and treat FL-HCC have been confounded by a lack of models that accurately reflect the genetics and biology of the disease. Here, we demonstrate that the *Dnajb1-Prkaca* gene fusion drives tumorigenesis in mice, and that fusion to DNAJB1 drives FL-HCC initiation more effectively than wild type PRKACA

overexpression. The requirement of the PRKACA kinase domain in tumor initiation establishes the potential utility of kinase inhibitors targeting the fusion. By identifying genetic and environmental factors that can enhance the consistency and aggressiveness of disease progression, we reveal biological characteristics of the disease and advance a robust platform for future pre-clinical studies.

Materials and Methods

Vectors and cloning

One sgRNA was cloned into the px330 vector, which was a gift from Feng Zhang (Addgene plasmid #42230), and a second U6-sgRNA cassette was inserted in the XbaI site with XbaI-NheI overhangs. As indicated, for other experiments, the lenti-CRISPR vector was a gift from David Sabatini (Addgene plasmid #70662). The pT3 transposon and SBase vectors were a kind gift of Dr. Xin Chen, University of California at San Francisco. Sequences for sgRNAs, cDNAs and primers are listed in **Supplementary Table 4** (https://www.pnas.org/highwire/filestream/624718/field_highwire_adjunct_files/2/pnas.201716483SI.pdf).

Animals and Treatments

Female, 6- to 10-week-old C57BL6/N mice were purchased from Envigo (East Millstone, NJ). All animal experiments were approved by the MSKCC Institutional Animal Care and Use Committee (protocol 11-06-011). For hydrodynamic tail-vein

injection, a sterile 0.9% NaCl solution was prepared containing plasmid DNA of either 40μg CRISPR vector or 20μg transposon vector together with CMV-SB13 Transposase (1:5 molar ratio). Mice were injected into the lateral tail vein with a total volume corresponding to 10% of body weight (typically 2 ml for a 20g mouse) in 5–7 seconds (Bell et al., 2007; Tschaharganeh et al., 2014). DDC treatment was administered through a diet containing 0.1% DDC (Sigma-Aldrich, St. Louis, Missouri; Envigo, Madison, WI) until sacrifice (Beer et al., 2008). Transplants were performed by finely mincing freshly isolated tumors, suspending in 1:1 PBS:matrigel, and injecting subcutaneously in a 100 μl volume.

Electron Microscopy

Tissue was fixed in 4% glutaraldehyde and transferred to cold PBS until further processed. The tissues were post fixed in 1% osmium tetroxide in PBS. After washing in water, the tissue was stained with 2% aqueous uranyl acetate for ~2 h at 4°. Tissues were dehydrated through a series of acetones and propylene oxide and embedded in Epon. Ultrathin sections were deposited on grids and stained with uranyl acetate for 15 minutes and lead citrate for 5 minutes.

Immunohistochemistry and Immunofluorescence

Tissue was prepared for histology by fixing in 10% buffered formalin overnight then transferred to 70% ethanol until paraffin embedding and sectioning (IDEXX RADIL, Columbia, MO). Antigen retrieval was performed in a pressure cooker with Sodium Citrate buffer. The following primary antibodies were used: Ki67 (Abcam ab16667 ,1:200), p-S6rp (Cell Signaling 2211, 1:200), E-cadherin (BD 610181, 1:500),

HNF1a (Santa Cruz sc-6547, 1:100), HNF4a (Abcam ab41898, 1:200), CK7 (Abcam ab181598, 1:500), CK19 (Abcam ab133496, 1:1000), CD68 (MSKCC Pathology Core Facility), IBA1 (Wako, 1:500), GFP (Abcam ab13970, 1:200), β-catenin (BD610154, 1:500), p-β-catenin (Cell Signaling 9567,1:100), and AXIN2 (Abcam ab32197, 1:800). Primary antibodies were incubated at 4°C overnight in blocking buffer. Sections were incubated with anti-rabbit ImmPRESS HRP-conjugated secondary antibodies (Vector Laboratories, #MP7401) and chromagen development performed using ImmPact DAB (Vector Laboratories, #SK4105). Stained slides were counterstained with Harris' hematoxylin. Images of stained sections were acquired on a Zeiss Axioscope Imager Z.1. Raw .tif files were processed using Photoshop CS5 software (Adobe Systems Inc., San Jose, CA) to adjust white balance.

RNAseq

Total RNA was isolated from frozen tissue using the RNeasy Mini Kit (Qiagen), quality control was performed on an Agilent BioAnalyzer, 500 ng of total RNA (RNA integrity number > 8) underwent polyA selection and Truseq library preparation according to instructions provided by Illumina (TruSeq RNA Sample Prep Kit v.2) with 6 cycles of PCR. Single-end, 75-bp sequencing was performed at the CSHL core facility. Approximately 8 million reads were acquired per sample. Resulting RNA-Seq data was analyzed as described previously (Liu et al., 2016). Adaptor sequences were removed using Trimmomatic. RNA-seq reads were then aligned to the mouse genome (mm10) using STAR (Dobin et al., 2013) with default parameters and genome-wide transcript counting was performed using subread to generate a count matrix (Liao et al., 2013,

2014). Differential expression analysis was performed by DESeq2 (Love et al., 2014). Genes were considered to be significantly differentially expressed if tumor/normal comparison was greater than 2-fold and FDR-adjusted p value was less than 0.05.

Human-Murine mapping of orthologs was performed based on the Ensembl database accessed through the Biomart R/Bioconductor package (Durinck et al., 2009). Human fibrolamellar HCC signatures were defined as genes with at least 2-fold and significant expression changes in fibrolamellar tumors with respect to normal. Human transcriptional profiling data was obtained from published studies (Cornella et al., 2015; Simon et al., 2015), and the TCGA/Broad GDAC firehose using annotation from Dinh et al (Dinh et al., 2017). For comparison to human datasets and for gene set enrichment analysis, the ssGSEA method was implemented using the GSVA package within R (Hanzelmann et al., 2013). The GSVA outputs were subsequently compared across groups using the limma package (Ritchie et al., 2015). The C2, C3, C5, and Hallmark collections of gene sets from MSigDBv6.0 were queried (Liberzon et al., 2015).

Human Tumor Sequencing Data

The MSK-IMPACT sequencing data (Zehir et al., 2017) were obtained from the MSKCC cBioPortal (Cerami et al., 2012) (http://www.cbioportal.org). Of the 18 *DNAJB1-PRKACA* fusion cases, 14 were annotated as fibrolamellar HCC and 4 were annotated as HCC. We considered all *DNAJB1-PRKACA* positive liver cancers as FL-HCC, given the common of misdiagnosis of this rare cancer type (Dinh et al., 2017), for which the presence of the *DNAJB1-PRKACA* should be considered diagnostic (Graham et al., 2015).

Results

CRISPR-mediated deletion results in fusion oncogene and drives tumorigenesis in vivo

The oncogenic potential of the endogenous *DNAJB1-PRKACA* fusion *in vivo* was assessed. Co-expression of Cas9 with multiple single guide RNAs (sgRNAs) can be used to model chromosome translocations, inversions, and deletions by generating DNA double strand breaks (DSBs) at the breakpoints of chromosome rearrangements, which are subsequently joined by non-homologous end-joining (NHEJ) (Blasco et al., 2014; Choi and Meyerson, 2014; Cook et al., 2017; Han et al., 2017; Maddalo et al., 2014). While such events are rare, an oncogenic rearrangement is expected to be positively selected *in vivo*. We determined whether the FL-HCC-associated rearrangement could be generated in hepatocytes of young adult mice via hydrodynamic tail vein injection using tandem sgRNAs corresponding to the breakpoints of the disease-associated deletion in the first introns of *Dnajb1* and *Prkaca* (**Figure 1.5A**). Importantly, the deleted region on human chromosome 19 in FL-HCC is syntenic to a corresponding region on mouse chromosome 8. In fact, all protein-coding genes present in the human region have orthologs present in the mouse region, and are arranged in the same order (**Figure 1.5A**).

To test the feasibility of this approach, different sgRNAs capable of targeting the first intron of *Dnajb1* and *Prkaca* were co-expressed with Cas9 in NIH3T3 cells or adult livers using lentiviral transduction or hydrodynamic injection, respectively (**Figure 1.6A**), and confirmed to produce a fusion event using PCR (**Figure 1.6B, C**). Next, two sgRNA pairs, targeting different sequences within the same introns (herein CRISPR.1 and CRISPR.2), were introduced into the livers of adult mice and the animals were

monitored over time. A subset of animals transduced with both sgRNA combinations became moribund with liver tumors 16-24 months post injection (**Figure 1.5B**). Tumor-bearing mice typically harbored disease involving multiple lobes, presumably from independent initiating events and ranged from diffuse to macroscopically visible (**Figure 1.5C**). In samples evaluated histologically, 2/9 mice injected with CRISPR.1, and 3/7 mice injected with CRISPR.2, died as a result of tumor burden. Additionally, non-moribund animals that were sacrificed harbored histological evidence of disease (annotated as "asymptomatic") in 2/9 CRISPR.1 mice and 2/7 of CRISPR.2 mice (**Figure 1.5D**). For both guide pairs, RT-PCR and Sanger sequencing with fusion-specific primers confirmed expression of the intended *Dnajb1-Prkaca* fusion oncogene in these lesions (**Figure 1.5E**).

Histologically, the CRISPR-induced mouse tumor cells (**Figure 1.5F**) were strikingly similar to human FL-HCC (**Figure 1.5G**). Like human FL-HCC, the mouse liver tumors were composed of large, pleomorphic polygonal cells with abundant eosinophilic cytoplasm, large vesicular nuclei and prominent nucleoli. Furthermore, the lobular structure of the tumors was clearly disrupted (**Figure 1.5H**) and distinctive cytoplasmic inclusions were observed. However, unlike the human disease, the mouse tumors were not surrounded by detectable fibrosis. Supporting the robustness of these results, tumors with similar latency and histology were recapitulated with an independent Cas9-expressing vector (**Figure 1.6D**). Thus, induction of an endogenous *Dnajb1-Prkaca* fusion through intrachromosomal deletion drives tumors with features of FL-HCC in mice. Independent work from others recently demonstrated an FL-HCC phenotype of lesions in asymptomatic mice using similar methods (Engelholm et al., 2017).

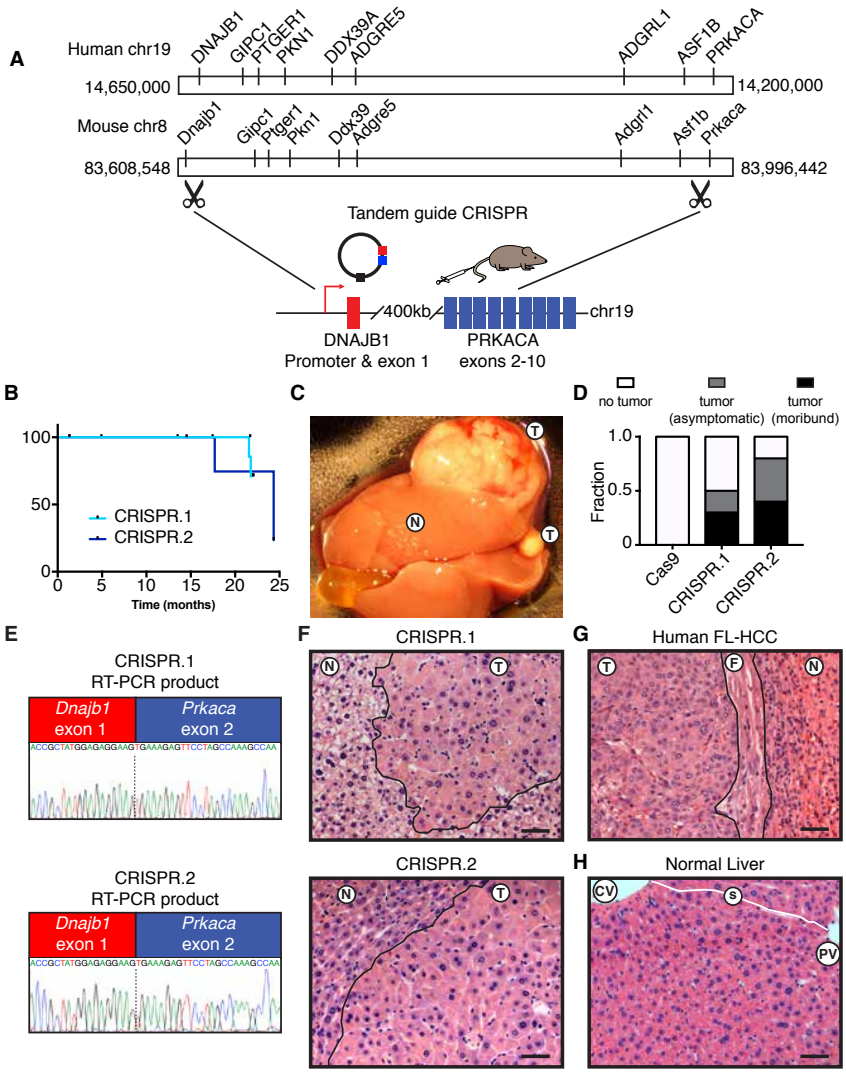

Figure 1.5. CRISPR-mediated deletion results in fusion oncogene and drives tumorigenesis in vivo.

(A) Configuration of human chromosome 19, including *DNAJB1* and *PRKACA*, configuration of mouse chromosome 8, including *Dnajb1* and *Prkaca*, and schematic of endogenous 400kb deletion targeted by hydrodynamic injection of vector containing tandem guide sgRNAs to introns of *Dnajb1* and *Prkaca* and Cas9. (B) Overall survival of mice injected with CRISPR.1 (n=10) or CRISPR.2 (n=9). (C) Macroscopic view of a tumor-bearing liver (D) Fraction of mice harvested with no detectable tumor, asymptomatic mice with histologically detectable disease (asymptomatic), or moribund mice with tumors (moribund) for each indicated genotype. (E) Sanger sequencing of chimeric transcript amplified from tumors generated by CRISPR.1 (top) and CRISPR.2 (bottom). (F) H&E staining of tumor generated by CRISPR.1 (top) and CRISPR.2 (bottom). (G) Human case of FL-HCC (T, tumor; F, fibrosis). (H) Normal mouse liver, where sinusoids trace from central veins to portal triads (CV, central vein; PV, portal vein) with intact sinusoids (white line, s). All scale bars are 50 μm.

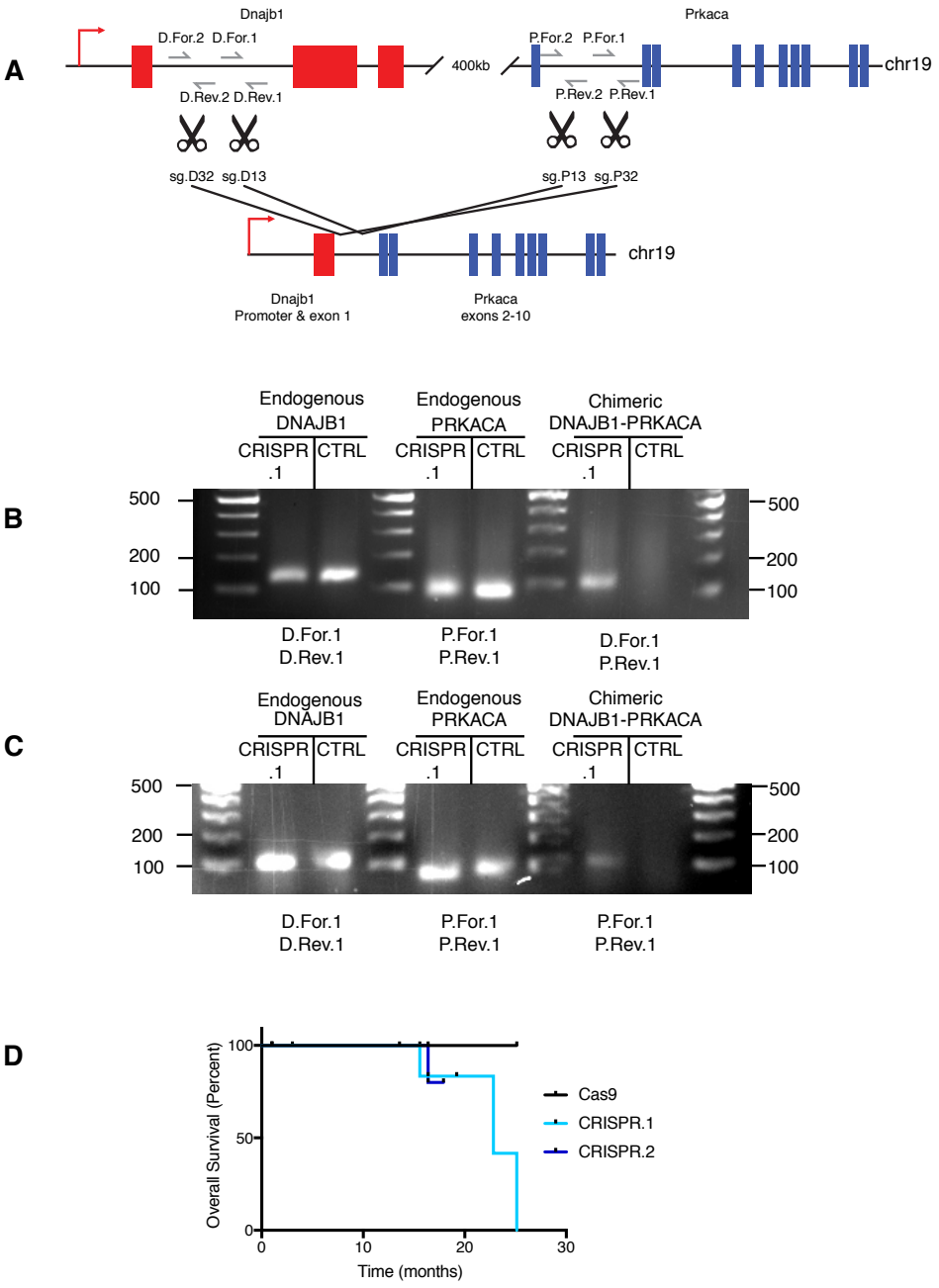

Figure 1.6. Validation of CRISPR-mediated deletion.

(A) Schematic of sgRNAs and primers. **(B)** Detection of genomic deletion in NIH-3T3 cells infected with tandem guide lentiCRISPR construct. (**C**) Detection of deletion in genomic DNA extracts from whole livers 4 days following hydrodynamic tail vein injection of the same construct. (**D**) Overall survival of mice following hydrodynamic tail vein injection of the lentiCRISPR plasmid DNA.

DNAJB1-PRKACA fusion drives liver tumorigenesis

The segmental deletion that results in the *DNAJB1-PRKACA* gene fusion entails heterozygous loss of 7 other coding genes, with unknown functional contribution. In other contexts, such deletions can contribute to tumorigenesis directly through attenuating the function of haploinsufficient tumor suppressors (Liu et al., 2016). To determine whether the *DNAJB1-PRKACA* fusion is sufficient to drive tumorigenesis (uncoupled from the typical genomic deletion), and whether simply the overexpression of the wild-type PRKACA gene could recapitulate this effect, we used hydrodynamic injection to deliver a transposon expressing the human *DNAJB1-PRKACA* fusion cDNA or a full length wild-type *PRKACA* cDNA. Co-transfection of transiently expressed sleeping beauty transposase ("SBase") with a transposon construct allows for stable integration and constitutive overexpression of the cDNA that mimics the high levels in human tumors (Honeyman et al., 2014) (**Figure 1.7A**).

Expression and protein stability are similar between the DNAJB1-PRKACA fusion protein and full-length WT PRKACA, and overexpression of wild type PRKACA produced some changes to hepatocyte histology, but it did not trigger the formation of lethal tumors (**Figure 1.7B-E**). However, expression of the *DNAJB1-PRKACA* cDNA produced tumors with similar kinetics, penetrance, and morphology as CRISPR-driven murine FL-HCC (**Figure 1.7B,C,F**). Again, a spectrum of histological findings supported the similarity between these complementary methods and human FL-HCC (**Figure 1.5,1.7**), including the presence of large tumor cells with granular, eosinophilic cytoplasm and prominent nucleoli (**Figure 1.7F**). Mitotic figures, steatosis, and pale bodies

(Torbenson, 2012) were also observed (**Figure 1.7G**). These results imply that the DNAJB1 portion of the fusion protein contributes to disease beyond facilitating overexpression of PRKACA and that the chromosome 19 deletion event is not required for oncogenesis.

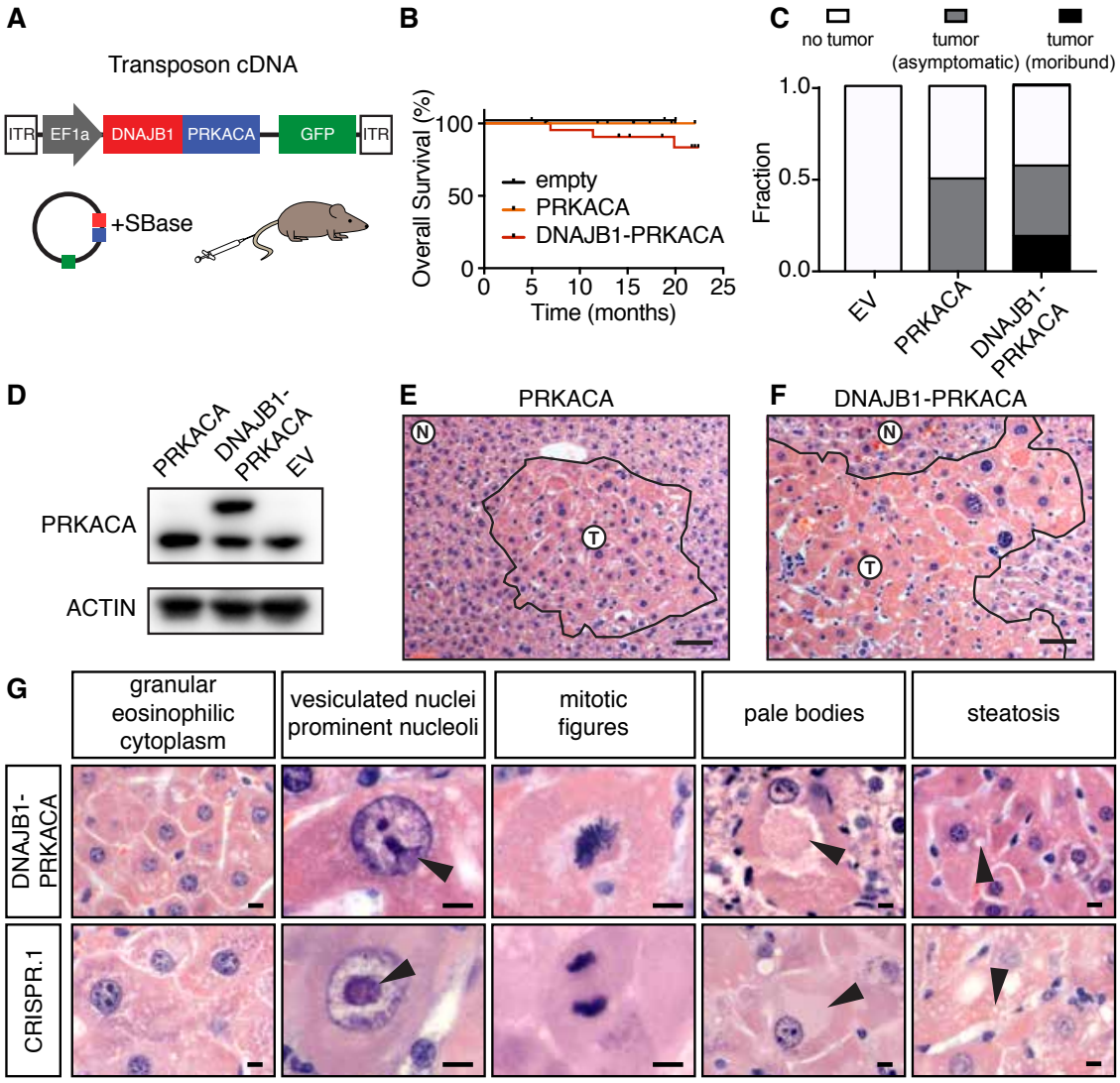

Figure 1.7. *DNAJB1-PRKACA* fusion drives liver tumorigenesis.

(**A**) Schematic of Sleeping Beauty transposon strategy to deliver *DNAJB1-PRKACA* fusion cDNA to young adult livers (ITR: inverted terminal repeats). (**B**) Overall survival of mice injected with cDNA encoding *DNAJB1-PRKACA* (n=23), wild-type *PRKACA* (n=12), or empty vector (n=4). (**C**) Fraction of mice harvested with no detectable tumor, asymptomatic mice with histologically detectable disease, or moribund mice with tumors expressing empty vector (EV), wild type *PRKACA*, or *DNAJB1-PRKACA* fusion. (**D**) Western blot of liver progenitor cells 4 days after transduction with indicated constructs *in vitro* (**E**) Cluster of atypical hepatocytes in liver injected with wild type pT3-PRKACA (**F**) Tumor generated by pT3-DNAJB1-PRKACA (T, tumor; N, adjacent normal; scale bar=50um). (**G**) Higher magnification image highlighting common murine FL-HCC features: large granular, eosinophilic cytoplasm; vesiculated nuclei with prominent nucleoli; mitotic figures; pale bodies; steatosis. Scale bars=10 μm.

Murine tumors display structural and molecular features of human FL-HCC

To validate that the murine model recapitulates other aspects of the human disease, we further characterized the phenotype of murine tumors. Human FL-HCC has consistently recognizable ultrastructural features that were also observed in murine FL-HCC tumors by electron microscopy (EM). Like human FL-HCC, murine tumor cells were typically larger than adjacent normal hepatocytes and contained clumped heterochromatin (**Figure 1.8A-B, Figure 1.9A**), occasional pale bodies (**Figure 1.8B**), and prominent nucleoli (**Figure 1.9B,C**). Most notably, tumor cells exhibited a marked increase in mitochondria with atypical appearance (yellow arrows, **Figure 1.8B,C,D, Figure 1.9E-H**) (Graham et al., 2017; Payne et al., 1986; Torbenson, 2012). Numerous megamitochrondria were observed. The mitochondria were round to oval and homogeneous without obvious cristae and were surrounded by abundant rough endoplasmic reticulum (red arrows, **Figure 1.8C,D, Figure 1.9**). Throughout the tumor, cells had moderate to severe perinuclear and cytoplasmic aggregates of lipofuscin pigment (**Figure 1.8B, Figure 1.9I**). This finding, along with the mitochondrial phenotype, could be consistent with a state of oxidative stress (Hohn et al., 2012; Sohal and Brunk, 1989).

Human FL-HCC is known to often express markers of multiple lineages, including hepatic, billiary, and neuroendocrine (Ross et al., 2011; Ward et al., 2010). The murine tumors were positive for hepatocyte markers HNF4A and HNF1A, with some cells showing reduced expression, consistent with reduced hepatocyte lineage commitment (**Figure 1.10A,B**). However, the murine tumors were negative for other

proteins that are often expressed in FL-HCC, including biliary markers CK7 and CK19 as well as CD68 (**Figure 1.10C-F**), perhaps reflecting the fact that mature hepatocytes are targeted by the hydrodynamic transfection technique and thus are necessarily the cell of origin of the murine FL-HCC model, whereas the cell of origin in human FL-HCC is unknown.

To further validate the mouse model, gene expression analysis by RNA-Seq was performed on murine tumors and control liver tissue (**Table S1**) and compared to human FL-HCC. Sequencing reads that cross the junction were observed, confirming expression of the fusion (**Figure 1.8G**). Principal component analysis demonstrated that the vast majority of the variance between samples described the differences between tumor and normal samples (**Figure 1.8H**). A focused analysis to investigate the similarity between mouse and human tumors was evaluated in two ways. First, single sample gene set enrichment analysis (ssGSEA) (Hanzelmann et al., 2013), using FL-HCC expression signatures from three independent published studies (Cornella et al., 2015; Dinh et al., 2017; Simon et al., 2015), was used to confirm that differentially expressed genes in human tumors were, in aggregate, significantly enriched in our murine tumors (**Figure 1.8I**). Second, a supervised analysis of curated functional gene sets previously reported as enriched in FL-HCC (Simon et al., 2015) was consistent with murine tumor expression data (**Figure 1.11A**). These results provide a global analysis that classifies the murine model as FL-HCC. Overall, the murine tumors arising in the presence of the *DNAJB1-PRKACA* fusion show most, but not all, features of the human disease.

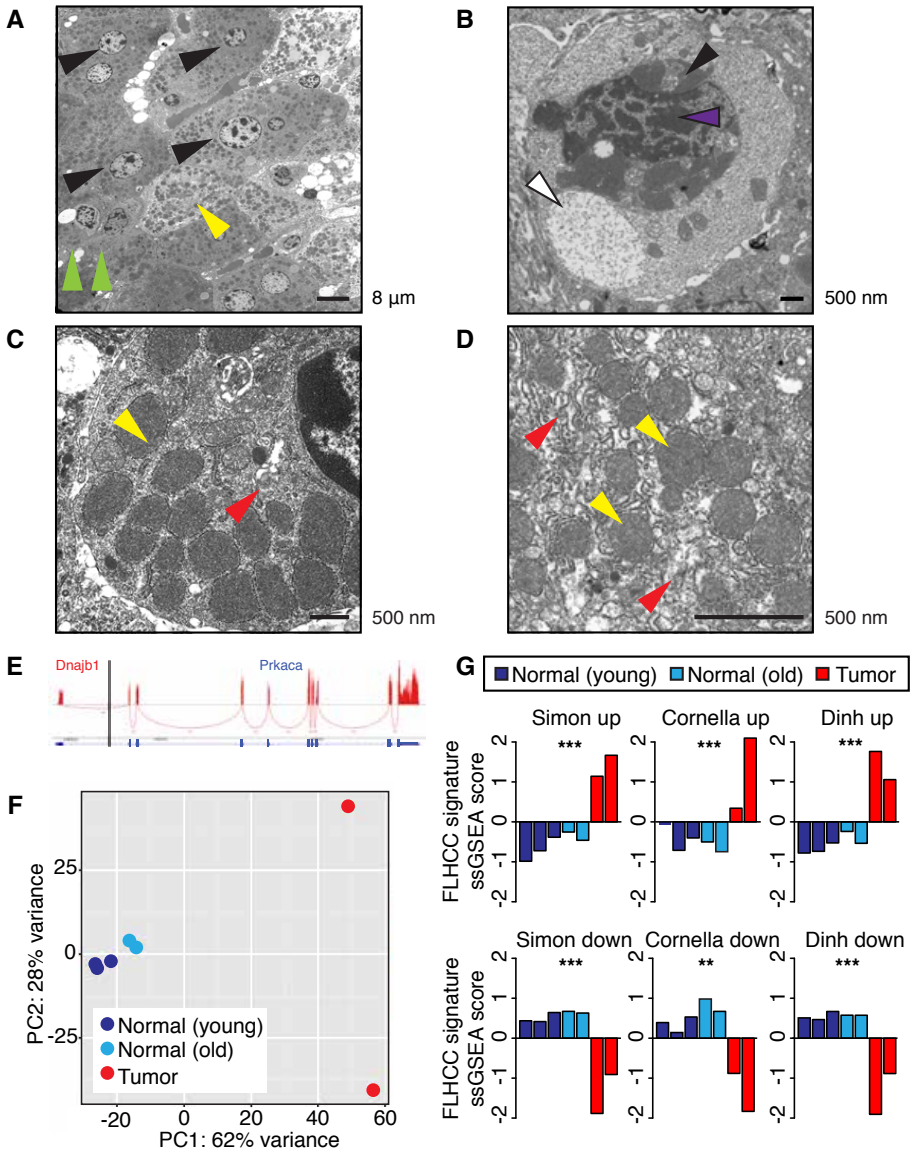

Figure 1.8. Murine tumors display structural and molecular features of human FL-HCC.

Ultrastructural analysis of a transposon-induced tumor by electron microscopy (**A**) Tumor cells (black arrows) of varying larger size than compressed adjacent hepatocytes at tumor margin (green arrows). (**B**) Pale body (white), perinuclear accumulation of lipofuscin (black) and heterochromatin (purple). (**C**) Tumor cell with abundant mitochondria (yellow), Rough endoplasmic reticulum (RER, red). (**D**) Abnormal mitochondria (yellow) with indistinct cristae surrounded in RER (red). (**E**) Sashimi plot indicating RNA-seq reads that cross the *DNAJB1-PRKACA* junction. (**F**) Principal component analysis of young control livers (dark blue), aged control livers (light blue), and tumors derived from CRISPR.1 (red). (**G**) Z scored single sample gene set enrichment analysis (ssGSEA) for normal (black) and tumor (red) samples. **P<0.01 ***P<0.001.

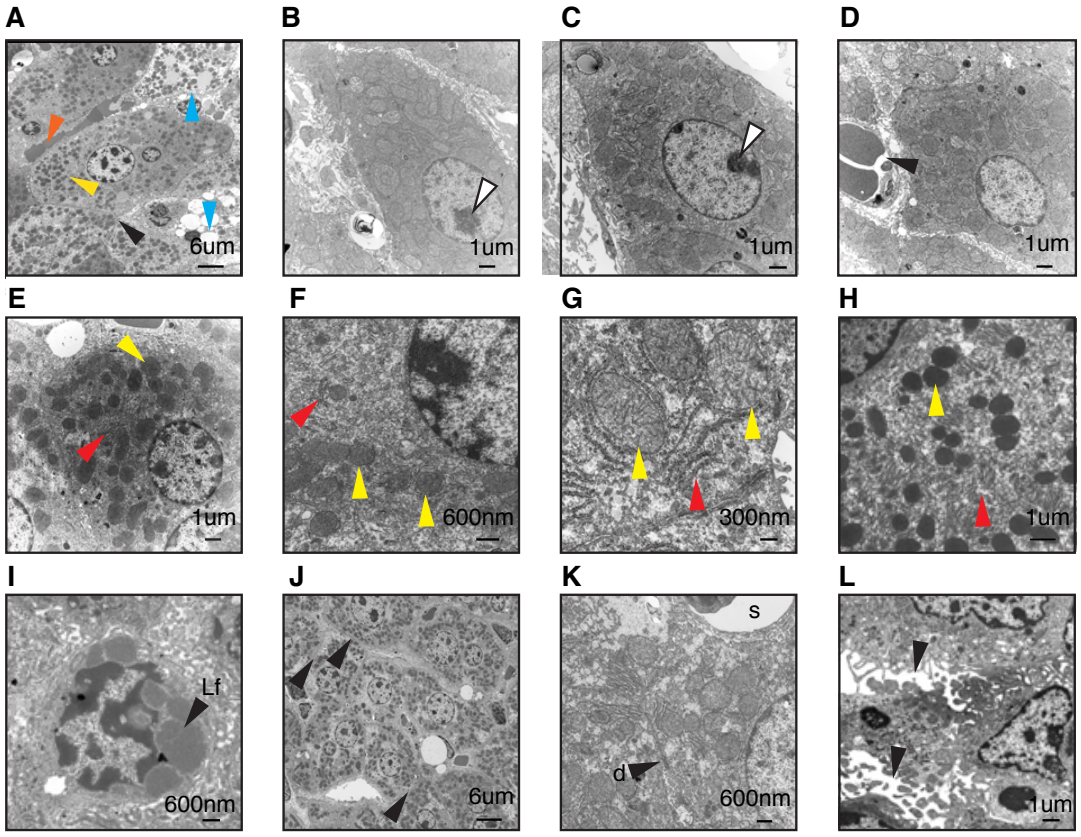

Figure 1.9. Additional ultrastructural analysis of murine FL-HCC.

(A) FL-HCC tumor cells are enlarged with abnormally abundant mitochondria (yellow arrowhead). Necrotic cells (blue) and nonfenestrated vessels (orange), and indistinct cell-cell junctions (black). **(B,C)** Tumor nuclei are large and round to indented with prominent nucleoli (white arrows) **(D)** tumor-associated vasculature (black arrow) **(E-H)** Abundant mitochondria (yellow arrows) surrounded by rough endoplasmic reticulum (red arrows) with scant smooth endoplasmic reticulum **(I)** Lipofuscin "Lf" **(J)** Simple, indistinct cell-cell junctions (black arrows) **(K)** rare desmosome "d" with nearby sinusoid "s" **(L)** Bile canniculi are sometimes widened (black arrows).

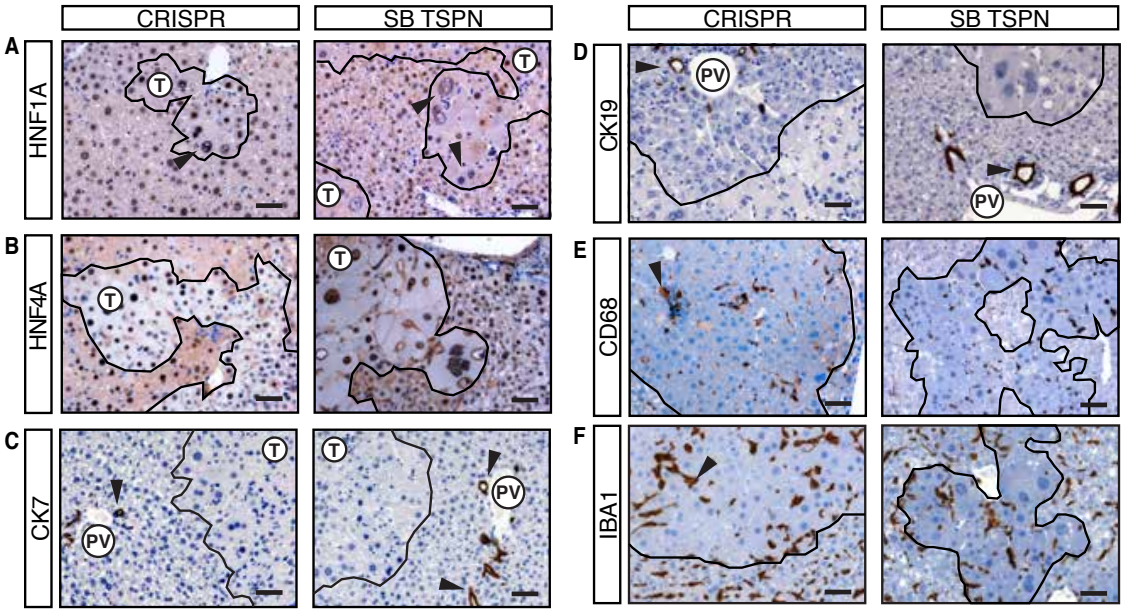

Figure 1.10. Additional molecular characterization of murine FL-HCC.

Indicated genotypes stained for hepatocyte markers (**A**) HNF1A and (**B**) HNF4A, cholangiocyte markers (**C**) CK7 and (**D**) CK19 (arrows: bile ducts, PV:portal vein), (**E**) CD68 (arrows: CD68+ infiltrating macrophages) and (**F**) IBA1 confirming **S3E**. All scale bars are 50 μm.

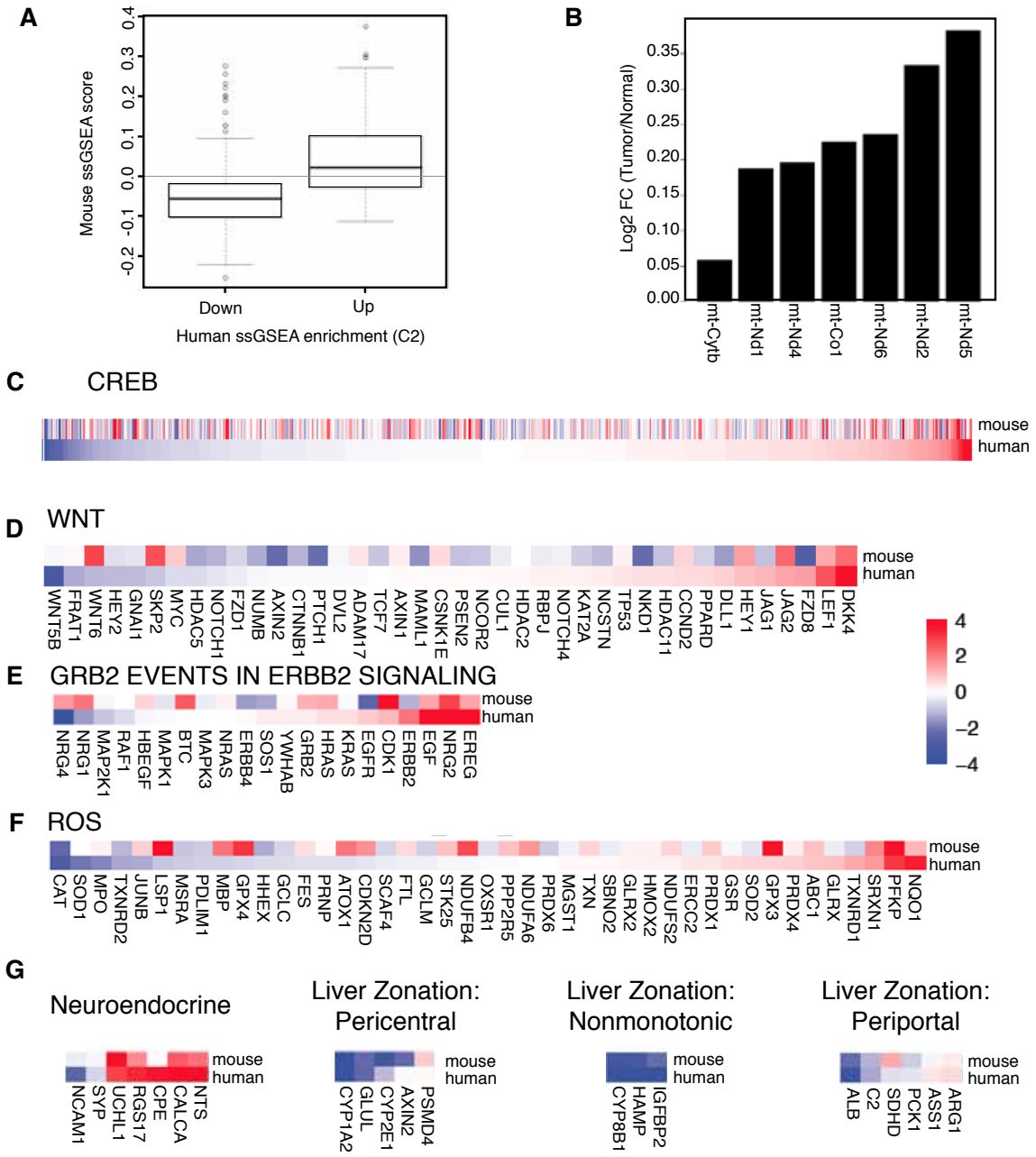

Figure 1.11. Gene expression of FL-HCC-associated gene sets of interest.

(A) ssGSEA enrichment scores from mouse tumor differential expression for gene sets significantly enriched in either upregulated or downregulated human gene expression data (Simon et al., 2015) (p=4.22-17). **(B)** Gene expression for genes encoded by mitochondrial DNA. Gene expression of corresponding mouse and human (Simon et al., 2015) genes in the specified gene sets: **(C)** CREB targets **(D)** Wnt signaling pathway **(E)** GRB2 events in ERBB2 signaling **(F)** genes upregulated by ROS **(G)** Lineage specific genes including neuroendocrine markers (Simon et al., 2015) and (**H**) liver zone-specific genes (Halpern et al., 2017).

Molecular profiling of murine FL-HCC reveals processes linked to tumorigenesis

Unbiased analysis of expression data also suggested yet other biological processes that may be relevant to FL-HCC pathogenesis. A total of 5710 genes were significantly differentially expressed between tumor and normal tissue (**Figure 1.12A, Table S2**) and further global analysis by GSEA was performed (**Figure 1.12B, Table S3**). The gene expression data, in agreement with human signatures, showed evidence of increased proliferation and mitogenic signaling (**Figure 1.12A-C**). For example, cell cycle and DNA biosynthesis gene sets, and specifically *Cdk1*, *Gins2*, and *Cenpa,* were highly upregulated in the experimental tumors (**Figure 1.12D,E**). Accordingly, tumors displayed an elevated Ki67 index (~9%) compared to adjacent normal tissue (~1%) (**Figure 1.12C**). Activation of the PI3K pathway was indicated by downregulation of *Deptor* (a negative regulator of mTORC1 also decreased in human tumors) and upregulation of the RTK ligands *Egf*, *Nrg2*, and *Ereg* in both mouse and human tumors (Simon et al., 2015) (**Figure 1.12A,S4E**). This observation was validated by immunofluorescence showing high levels of phospho-S6rp, a marker of mTOR activity that is highly expressed in most FL-HCCs but rarely in classic HCC (Cornella et al., 2015; Riehle et al., 2015) (**Figure 1.12C**).

Supporting previous reports that dedifferentiation is associated with *DNAJB1-PRKACA*-driven transformation (Oikawa et al., 2015), we observed downregulation of hepatocyte lineage markers and upregulation of some neuroendocrine markers (**Figure 1.12D, S4G**), as has been observed in human FL-HCC (Simon et al., 2015). GSEA further showed downregulation of epithelial cell fate commitment, bile acid biosynthesis,

liver specific genes, and xenobiotic metabolism (Kannangai et al., 2007) and upregulation of a teratoma-associated gene set (**Figure 1.12E**). Similarly, gene expression signatures defining specialized zones of hepatocytes (Halpern et al., 2017) were universally lost, consistent with human data (Simon et al., 2015) (**Figure S4H**).

Murine FL-HCC cells may also have defects in cell adhesion. Tumor cells displayed a decrease in *Cdh1*/E-cadherin expression and cell adhesion- and desmosome-associated gene sets were downregulated (**Figure 1.12A,B**). E-cadherin downregulation was confirmed by IHC and EM revealed indistinct or simple tumor cell-cell junctions (**Figure 1.9J,K**). Loss of cell polarity was indicated by a loss of synchronized glycogen storage evident in neighboring hepatocytes, shown by largely negative Periodic Acid Schiff (PAS) staining (**Figure 1.12D**). An exception to this pattern was the observation of PAS+ Mallory/hyaline bodies, which are commonly found in FL-HCC (Torbenson, 2012) and are reminiscent of a stress-induced phenotype (Celli and Zhang, 2014) (**Figure 1.12D, inset**).

Murine and human tumors also showed evidence of a response to oxidative stress, as indicated by the upregulation of enzymes involved in detoxifying reactive oxygen species (e.g. *Nqo1*, *Gpx3*, *Gpx4, and Acox1*) (**Figure 1.12B,S4F**). The accumulation of mitochondria can be driven by oxidative stress (Luo et al., 2013); upregulation of mitochondrial-encoded transcripts (**Figure S4B**) and an increase in the number of mitochondria observed by EM (**Figure 1.8,FIGURE 1.9E-H)** were also evident in both the mouse model and clinical samples. Whether or how each of these features contributes to the pathogenesis of FL-HCC remains to be determined.

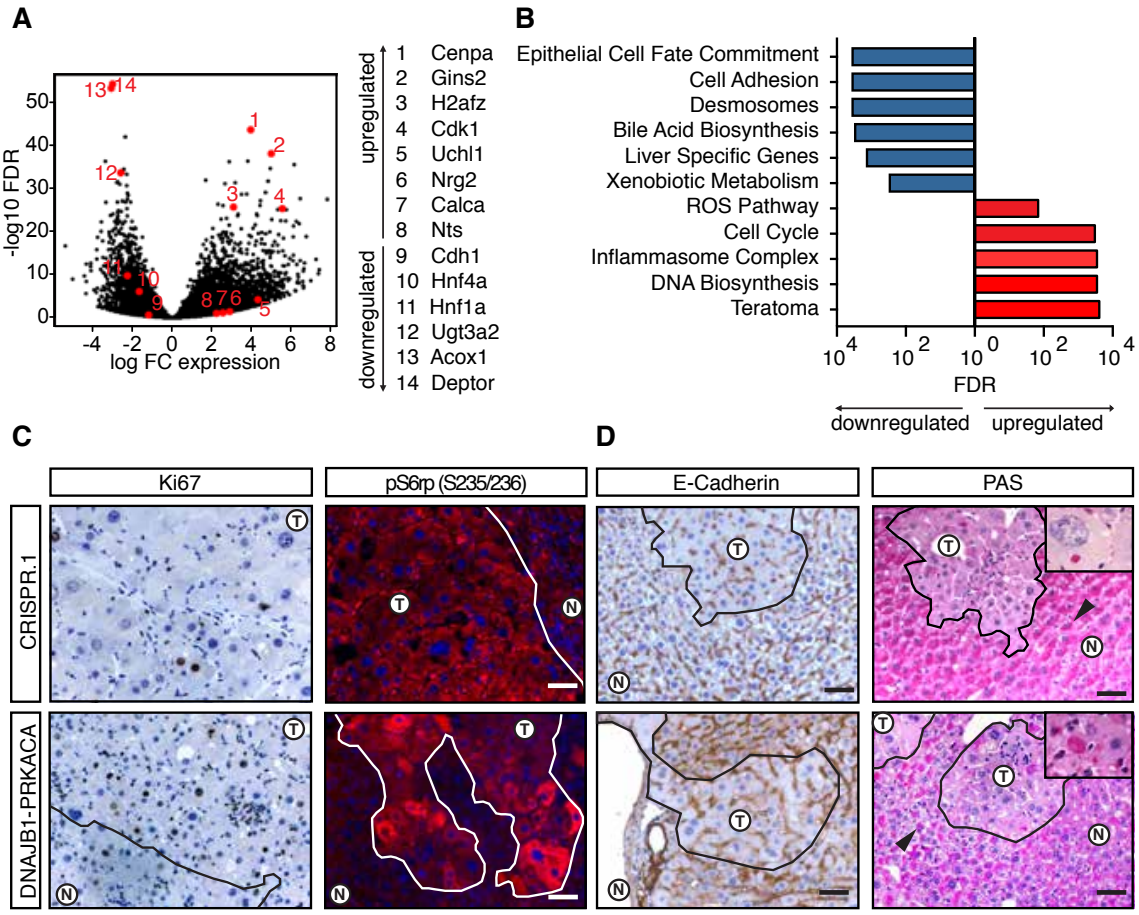

Figure 1.12. Molecular profiling of murine FL-HCC reveals processes linked to tumorigenesis.

(**A**) Volcano plot depicting differentially expressed genes in CRISPR-induced tumors with respect to normal liver. (**B**) ssGSEA analysis for select functionally annotated gene sets. (**C**) Ki67 IHC and p-S6rp S235/236 IF of CRISPR-induced tumor (top), or transposon-induced tumor (bottom). (T: tumor, N: normal) (**D**) E-cadherin (CDH1) staining and Periodic Acid Schiff (PAS) staining. Note adjacent normal hepatocytes positive with asymmetrical subcellular distribution (black arrow). Inset: PAS+ Hyaline bodies. All scale bars are 50 μm.

WNT pathway cooperates with DNAJB1-PRKACA to accelerate FL-HCC

To further understand the genetic basis of FL-HCC and to address the long latency of the single hit models, we investigated additional factors that could accelerate disease. We queried the MSK-IMPACT collection of targeted sequencing data of over 18,000 cancer patients (Zehir et al., 2017). Eighteen liver cancer patients (age 18-36) whose tumors harbored the *DNAJB1-PRKACA* fusion were identified (**Figure 1.13A**). As expected, the fusion was not detected in any liver cancer patient over the age of 36 (0/414 patients) or in any non-liver cancer patient (0/18,367 patients), confirming the remarkable specificity of the *DNAJB1-PRKACA* fusion to liver oncogenesis (**Figure 1.14A**). While HCC and intrahepatic cholangiocarcinoma (ICC) share several common mutations, none of these have been linked to FL-HCC. Surprisingly, we noted previously unreported recurrent mutations in the Wnt pathway in human FL-HCC (**Figure 1.13A,B**). The MSK-IMPACT cohort contains 3/18 (17%) samples of FL-HCC with *CTNNB1* or *APC* mutation (age 18-21 years old). These cases each showed classic histological features of FL-HCC (**Figure 1.14C**).

In parallel, candidate drivers of liver cancer were evaluated for their ability to synergize with *DNAJB1-PRKACA* to transform hepatocytes. Neither transposon based delivery of *MYC*, *AKT*$^{\text{myristoylated}}$, *NOTCH*$^{\text{ICD}}$, *YAP*$^{\text{S127A}}$, *Fgf15*, *Il10*, *Il18*, nor CRISPR-mediated inactivation or knockout of *p19*^{ARF}, *Pten*, *Prkar1a*, *Rb1*, *Cdkn1b*, and *Tsc2*, cooperated with *DNAJB1-PRKACA* (**Figure 1.15**). However, an activated form of β-catenin uniquely cooperated with *DNAJB1-PRKACA* (using a transposon encoding *CTNNB1*$^{\text{T41A}}$ cDNA). Of note, the *CTNNB1*^{T41A} is the same allele that co-occurred with

the *DNAJB1-PRKACA* fusion in a human primary FL-HCC and its corresponding brain metastasis (**Figure 1.13B, Figure 1.14B)**. Both pairs of tandem guide CRISPRs, as well as transposon delivery of fusion cDNA, synergized with transposon delivery of stabilized β-catenin, increasing penetrance and reducing latency of the model (**Figure 1.13C,D**). In all cases, the histology of the resulting tumors matched the single hit models, though some features (e.g. cell size) were more pronounced (**Figure 1.13E**). The acceleration of the model by Wnt signaling was further validated by the combination of *DNAJB1-PRKACA* cDNA and disruption of *Apc* using CRISPR, which yielded tumors with a similar phenotype (**Figure 1.16**). While expression of the *DNAJB1-PRKACA* fusion alone led to increased membranous β-catenin, and phosphorylation of β-catenin at PKA phosphorylation site S675, expression of the canonical Wnt target AXIN2 was negative or weak in samples without genetic manipulation of the Wnt pathway (**Figure 1.16**), which is corroborated by low expression of AXIN2 mRNA (**Table S2**) and the "HALLMARK_WNT_BETA_CATENIN_SIGNALING" gene set (**Table S3**) in samples driven by the fusion only. Of note, tumor cells arising in a mouse injected with CRISPR.1 sgRNA pair and the *CTNNB1*^{T41A} transposon formed tumors upon multiple rounds of subcutaneous transplantation into syngeneic recipients (**Figure S8**). Hence, genetic lesions that activate Wnt signaling occur in the human disease and can cooperate with *DNAJB1-PRKACA* to accelerate FL-HCC.

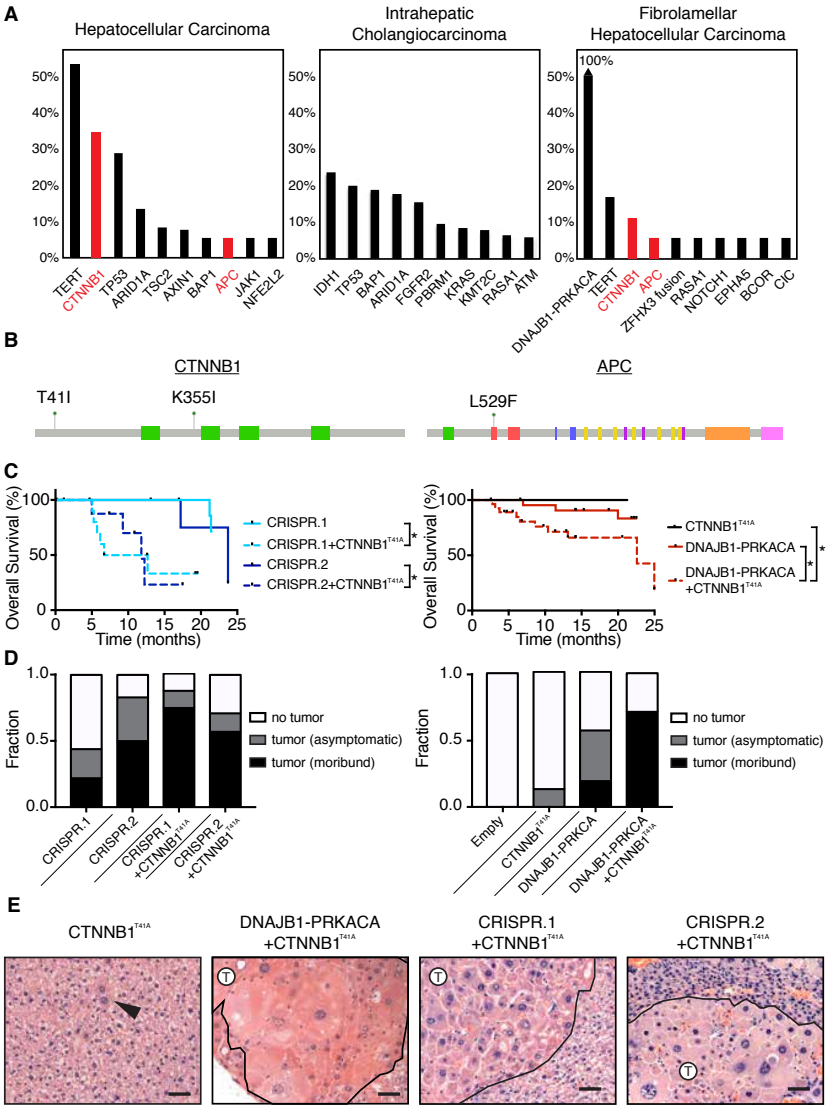

Figure 1.13. WNT pathway cooperates with *DNAJB1-PRKACA* to accelerate FL-HCC.

(A) Frequency of top 10 most commonly mutated genes in hepatocellular carcinoma (HCC, n=142), intrahepatic cholangiocarcinoma (ICC, n=175), and fibrolamellar hepatocellular carcinoma (FL-HCC, n=18) samples from MSKCC IMPACT sequencing. (**B**) Domain schematic of loci of Wnt pathway mutations in FL-HCC. (**C**) Overall survival of mice injected with *CTNNB1*^{T41A} and: CRISPR.1 (n=10, log rank p=0.0042 +/- *CTNNB1*^{T41A}), CRISPR.2 (n=9, p=0.022 +/- *CTNNB1*^{T41A}), and *DNAJB1-PRKACA* (n=30, p=0.069 +/- *CTNNB1*^{T41A}). CRISPR.1, CRISPR.2, and *DNAJB1-PRKACA* alone are repeated from (**Figure 1.5, 1.7**) for reference. (**D**) Fraction of mice harvested with no detectable tumor, asymptomatic mice with histologically detectable disease (asymptomatic), or moribund mice with tumors (moribund) for each indicated genotype. (**E**) H&E images depicting histology of mice injected with *CTNNB1*^{T41A} in combination with CRISPR.1, CRISPR.2, or *DNAJB1-PRKACA*.

A

Age	Gender	Mutations	CNA	Survival Status	Survival (months)
18	Male	DNAJB1-PRKACA, APC, CHEK2	$TERT^{AMP}$	Alive	29
18	Male	DNAJB1-PRKACA, CTNNB1	none	Alive	15
21	Female	DNAJB1-PRKACA, CTNNB1 LOC101928035-ZFHX3, ATR (met only)	$SOX17^{AMP}$ (Met only)	Alive, Brain Metastasis	31

B

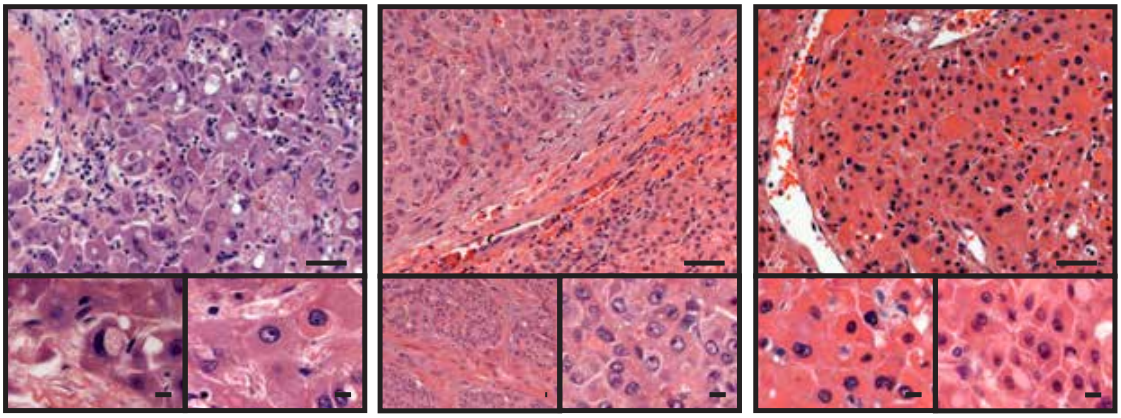

Figure 1.14. Wnt pathway alteration in human FL-HCC.

(**A**) Clinical and genomic characteristics of 3 cases of FL-HCC with Wnt pathway mutation. (**B**) H&E staining reveals classic FL-HCC morphology in cases with Wnt pathway mutations. Top: scale bars are 50 μm**,** bottom (insets): scale bars are 10 μm.

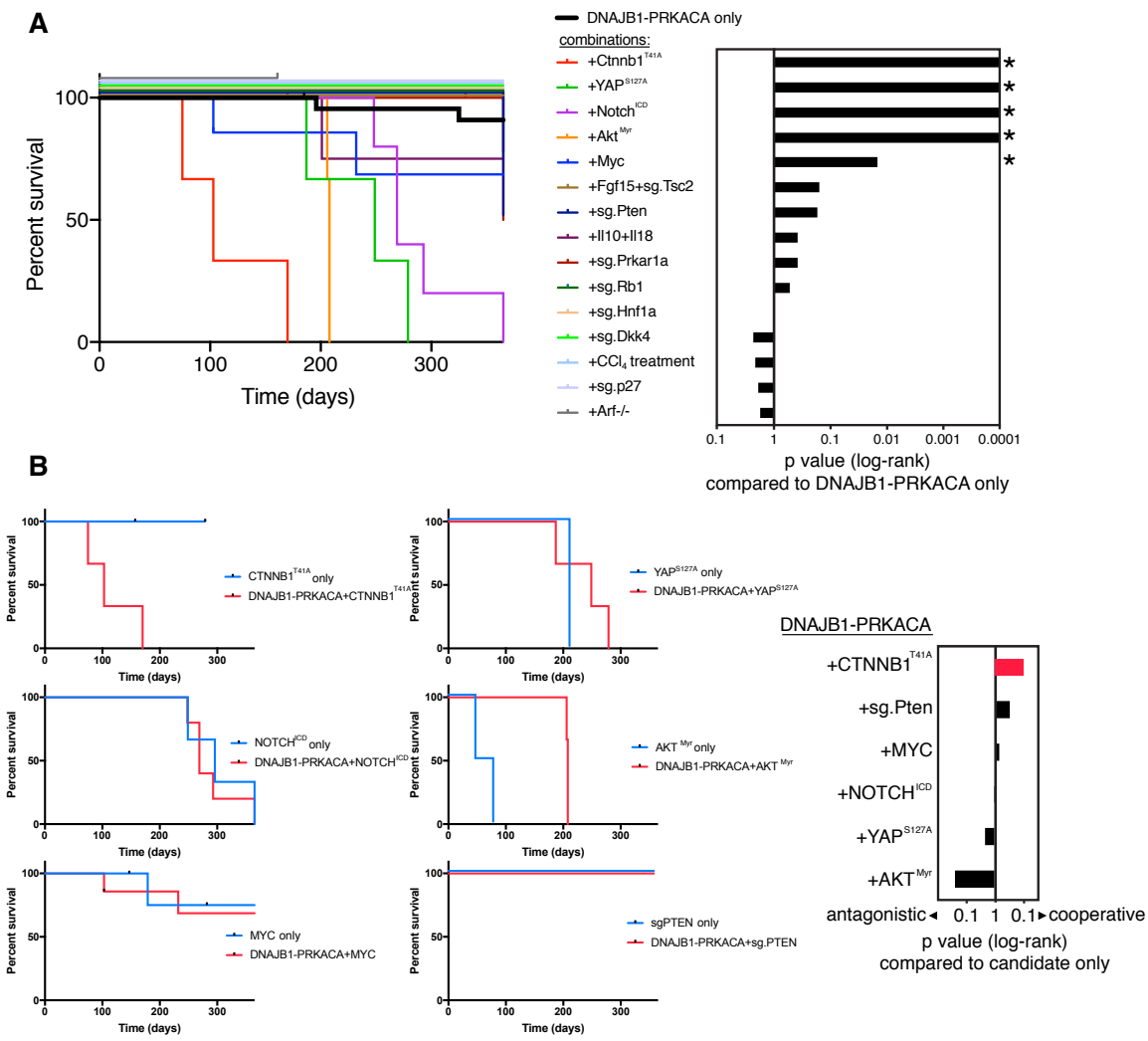

Figure 1.15. Screen for cooperating mutations.

(**A**) Survival data of cohorts injected with DNAJB1-PRKACA fusion cDNA alone or in combination with the indicated genotypes. (**B**) Survival data of cohorts injected with candidate genes from Figure 1.15A alone or in combination with the fusion. CTNNB1^{T41A} cooperates with DNAJB1-PRKACA resulting in higher lethality than either gene alone.

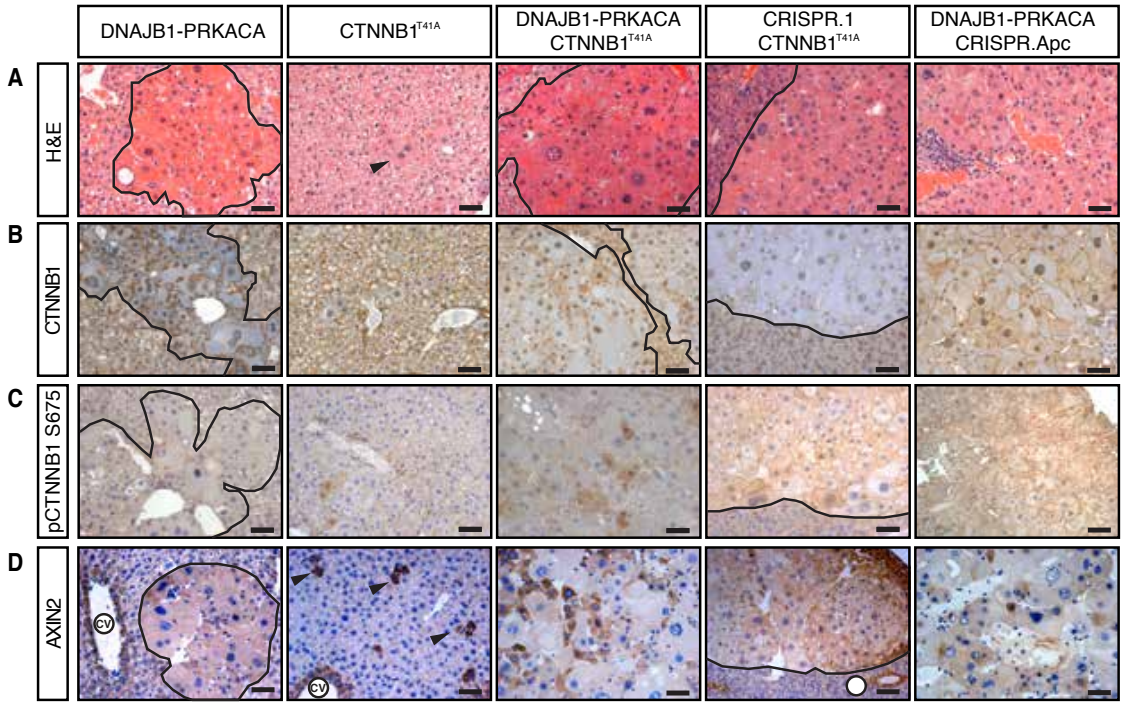

Figure 1.16. Wnt pathway in murine FL-HCC.

Livers injected with *DNAJB1-PRKACA*, *CTNNB1*^{T41A}, *DNAJB1-PRKACA* + *CTNNB1*^{T41A}, CRISPR.1 + *CTNNB1*^{T41A}, or *DNAJB1-PRKACA* + CRISPR.Apc. (**A**) H&E or immunohistochemistry with antibodies against (**B**) CTNNB1, (**C**) p-CTNNB1 S675 (PKA target site), and (**D**) AXIN2 (Wnt target gene). CV=Central vein (AXIN2 internal positive control). All scale bars are 50 μm.

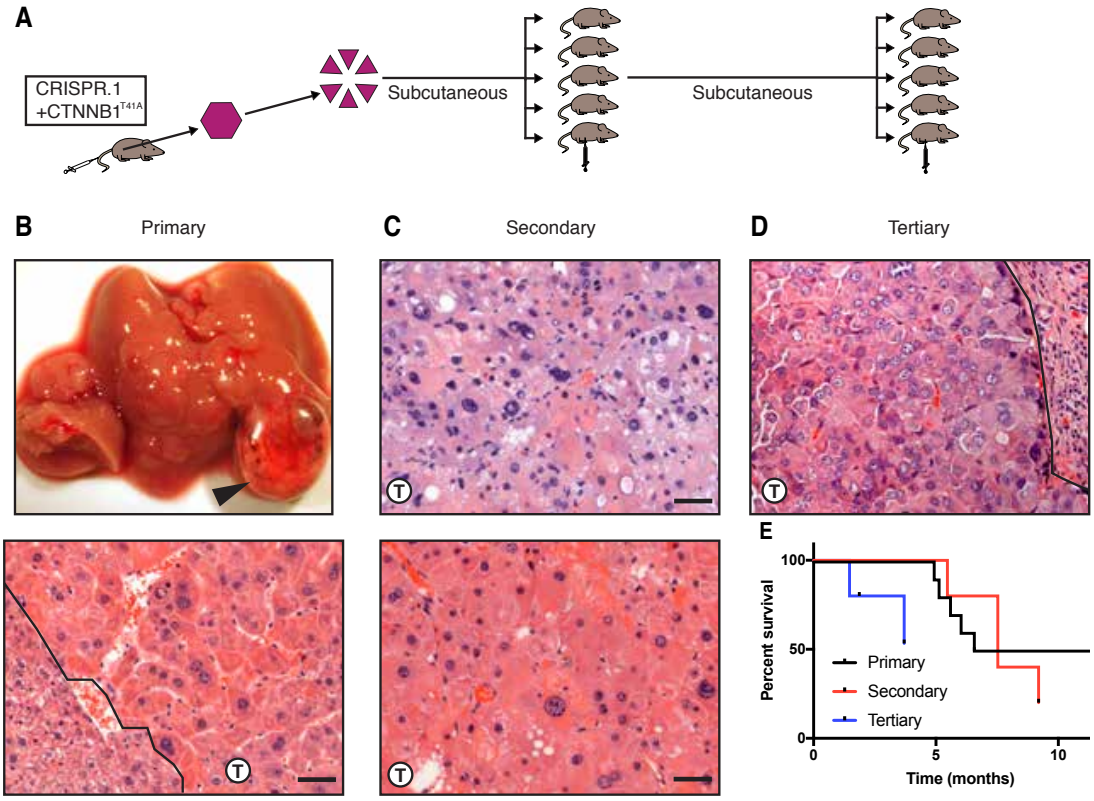

Figure 1.17. Serial transplantation of murine FL-HCC.

(**A**) Primary tumor driven by CRISPR.1 fusion and CTNNB1^{T41A} was minced and transplanted subcutaneously into syngeneic C57/BL6 mice. (**B**) Macroscopic view (top) and histology (bottom) of primary tumor, (**C**) transplanted tumors (first generation) (**D**) and second serial transplantation of a tumor from Figure 1.16C (tertiary) (**E**) Survival of cohorts of primary tumors (from Figure 1.13C) and tumors from two rounds of serial transplantation.

Inflammatory and fibrotic agent DDC enhances FL-HCC tumorigenesis

FL-HCC typically occurs in young patients that do not have the overt chronic liver diseases that often promote fibrosis and contribute to the emergence of classic HCC (Torbenson, 2012). Nevertheless, since the tumors arising in our mouse model lacked the eponymous fibrosis characteristic of human FL-HCC, we wondered whether experimental strategies to induce fibrosis might accelerate murine tumors. The hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) causes oxidative liver damage, cell death in periportal hepatocytes, atypical ductal expansion of progenitor cells, and ultimately, fibrosis (Preisegger et al., 1999), and can accelerate HCC tumorigenesis by specific oncogenic events (Beer et al., 2008; Matter et al., 2016).

Consistent with published results, mice treated with a 0.1% DDC-containing diet develop hepatomegaly, inflammation, and fibrosis with portal bridging by 8 weeks of treatment, but did not develop tumors (Fickert et al., 2007; Preisegger et al., 1999) (**Figure 1.18A-C, 1.19A**). Although DDC diet showed some increase in the onset of tumors following expression of mutant *CTNNB1* alone, the effects on the combination of mutant *CTNNB1* and *DNAJB1-PRKACA* were dramatic: in fact, 6-month survival was decreased from 60-70% with *CTNNB1*^{T41A}/*DNAJB1-PRKACA* to 0% observed with the same combination in DDC-treated mice (**Figure 1.18A,B**). The histology of the tumor cells themselves remained largely unchanged by DDC treatment, but as expected, the surrounding tissue acquired DDC-associated phenotypes associated with tissue regeneration following injury (**Figure 1.18C, 1.19B, 1.20**). Surprisingly, the morbidity of the combination often preceded the establishment of significant fibrosis (**Figure 1.18C,**

1.19). Therefore, these data suggest that one or more factors associated with the DDC-induced regenerative response can fuel murine FL-HCC. Furthermore, the combination of our non-germline genetic approaches and a DDC diet produces FL-HCC like tumors at high penetrance and with a short latency.

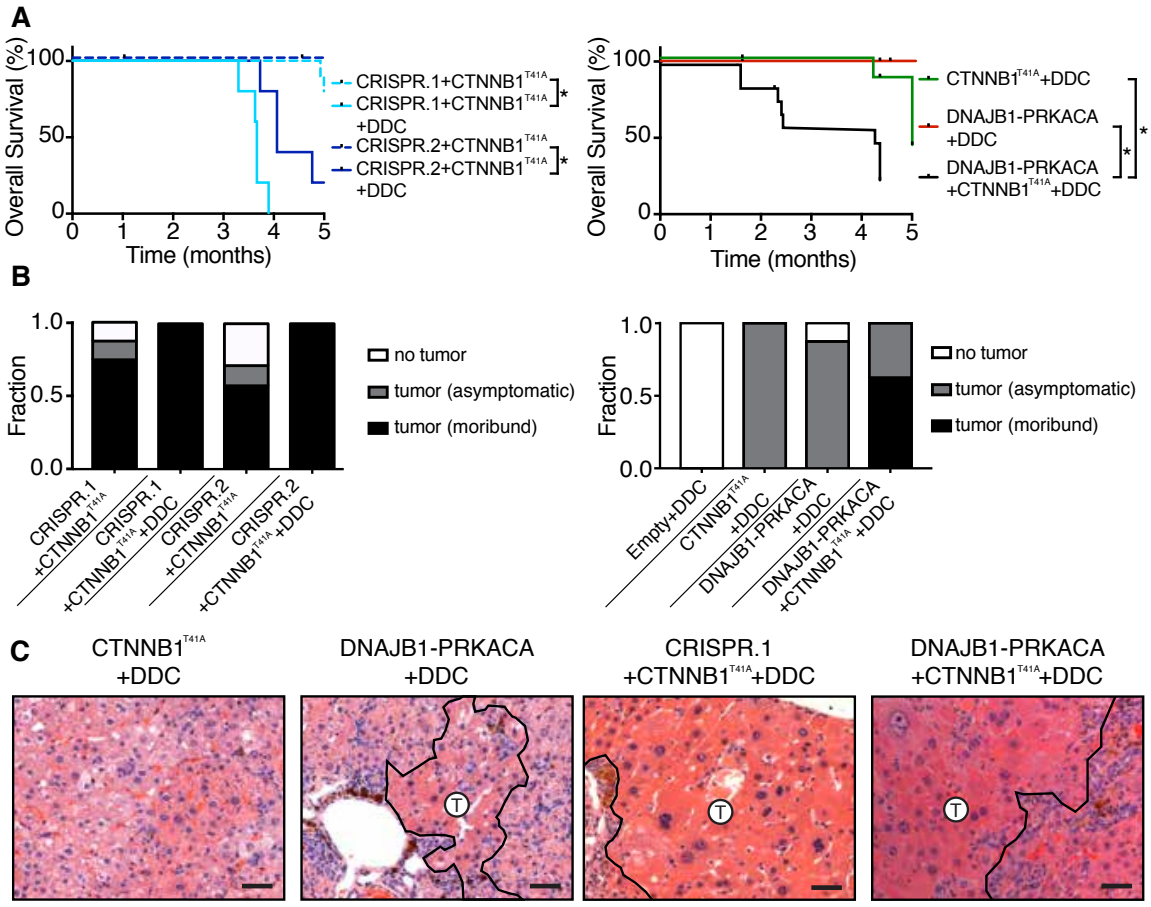

Figure 1.18. Inflammatory and fibrotic agent DDC enhances FL-HCC tumorigenesis.

(**A**) Overall survival of mice injected with indicated genotypes +/- DDC diet: CRISPR.1+ *CTNNB1*^{T41A} (n=5, log rank p<0.001 +/- DDC), CRISPR.2+ *CTNNB1*^{T41A} (n=5, log rank p<0.001 +/- DDC), *DNAJB1-PRKACA*+ *CTNNB1*^{T41A} (n=15, log rank p<0.001 +/- DDC). Survival data of mice without DDC treatment repeated from (**Figs. 1,2,5**). (**B**) Fraction of mice harvested with no detectable tumor, asymptomatic mice with histologically detectable disease (asymptomatic), or moribund mice with tumors (moribund) for each indicated genotype with or without DDC diet. (**C**) H&E images depicting histology of mice of the indicated genotypes and fed DDC diet.

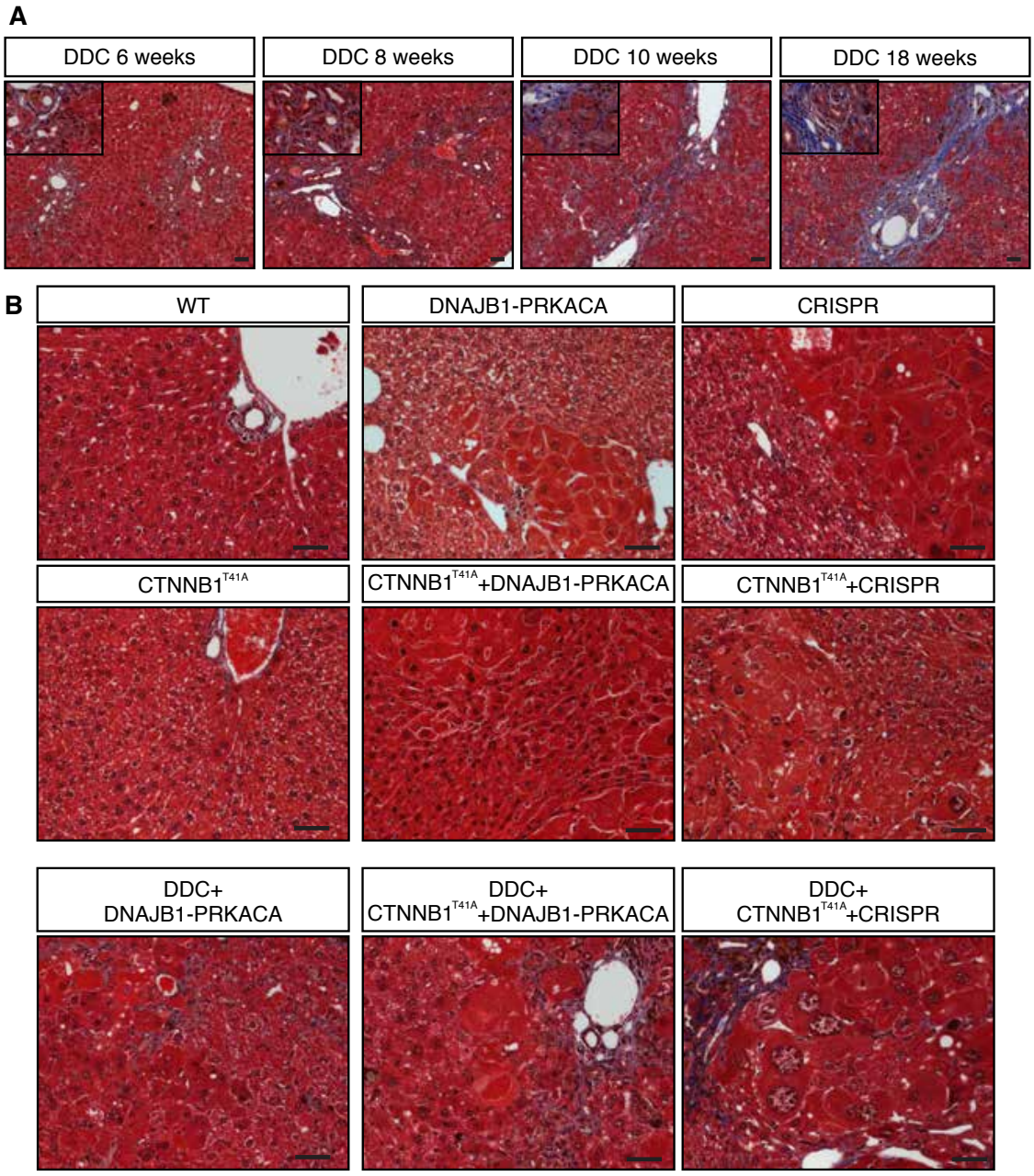

Figure 1.19. DDC-induced changes in liver pathology.

Masson's Trichrome staining (**A**) indicating progressive development of fibrosis (scale bars: 50 μm) and atypical ductal proliferation (inset). (**B**) No indication of fibrosis was detected in mice with any combination of genetic perturbations, when the mice were fed a normal diet. Mild fibrosis stained positive (blue) in some areas adjacent to tumors following DDC treatment and injection with the indicated constructs.

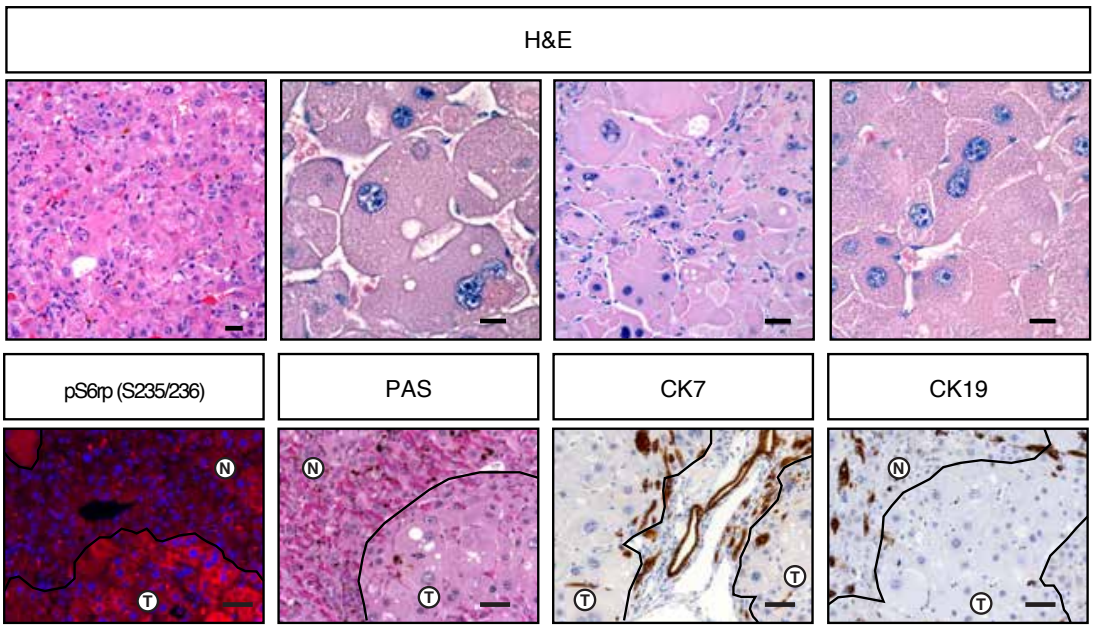

Figure 1.20. Stable phenotype in DDC-treated tumors.

The indicated histological features were found in tumors expressing *DNAJB1-PRKACA* and *CTNNB1*^{T41A} in DDC-treated mice (top). Tumors had recognizable inflammation. Pale bodies, atypical nuclei, and abundant rough endoplasmic reticulum were present. *DNAJB1-PRKACA*/*CTNNB1*^{T41A}/DDC tumors were pS6rp$^{S235/236}$+,PAS-, CK17-, and CK19- (bottom).

Tumorigenicity of DNAJB1-PRKACA is dependent on kinase domain

To illustrate the potential of a rapid and robust model of FL-HCC, we used the above methods to address whether the kinase activity of the *DNAJB1-PRKACA* fusion is essential for its ability to drive tumorigenesis—a prerequisite for rationalizing the use of small molecule inhibitors targeting the PRKACA kinase for treatment of FL-HCC. To this end, we produced a kinase dead version of the *DNAJB1-PRKACA* fusion harboring a mutation in the PRKACA component, equivalent to the previously described K72H mutation (Iyer et al., 2005), and compared its oncogenic potential to the intact fusion cDNA when combined with DDC and *CTNNB1*^{T41A}. A cohort of mice was produced and sacrificed at 9-10 weeks to examine the presence or absence of liver lesions. While clusters of neoplastic hepatocytes with classic FL-HCC morphology were observed in samples with an intact kinase domain, no such atypical hepatocytes were identified in samples expressing the kinase dead (KD) fusion cDNA (**Figure 1.21A**). Furthermore, expression of the kinase intact fusion led to a significantly elevated Ki67 positive fraction of GFP+ cells, while the GFP+ cells in samples expressing the kinase dead fusion cDNA did not show a significantly higher Ki76 positive fraction compared to adjacent normal liver or DDC-treated empty vector controls (**Figure 1.21C**). Thus, the PRKACA kinase domain is required for FL-HCC tumor initiation.

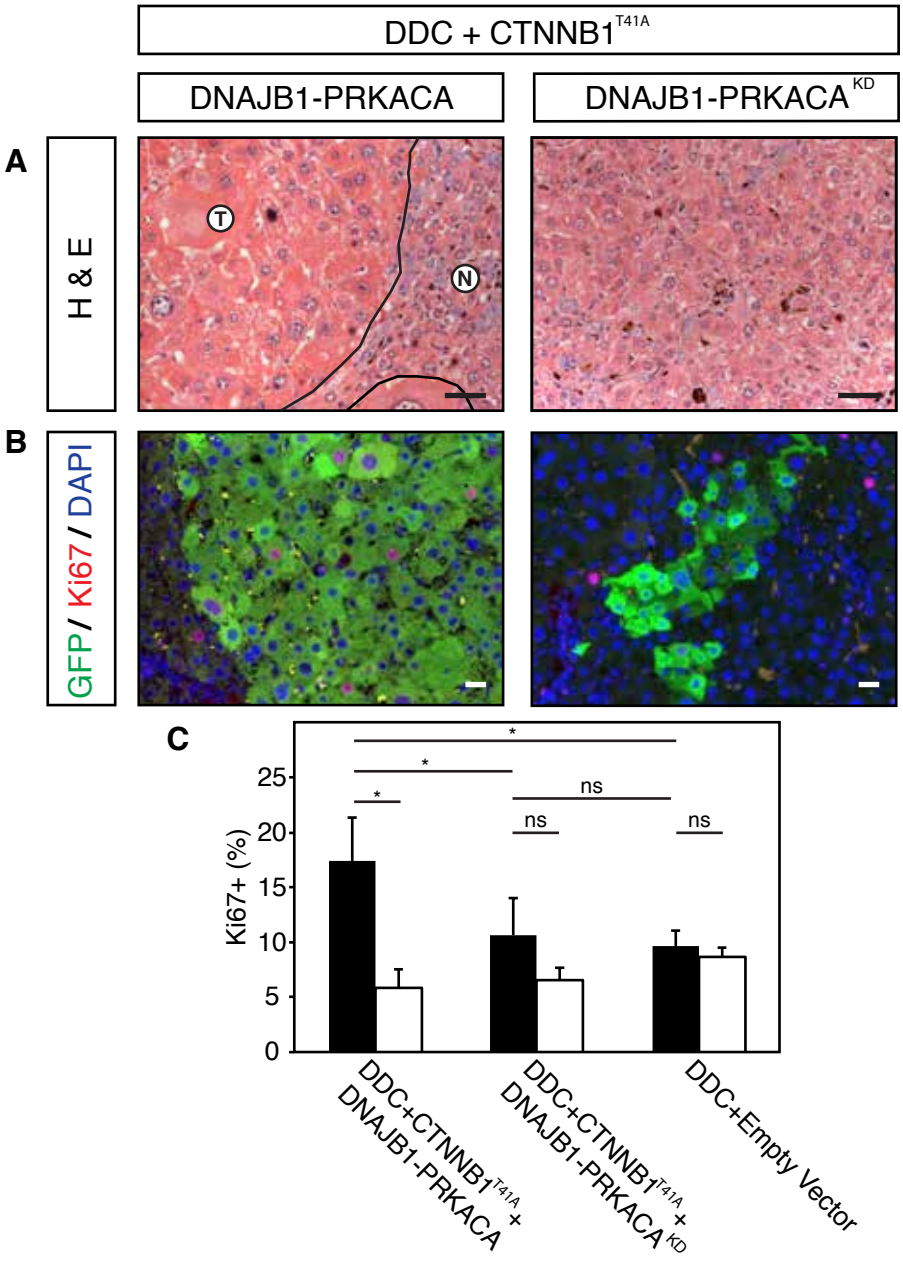

Figure 1.21. Tumorigenicity of DNAJB1-PRKACA is dependent on kinase domain.

(**A**) H&E staining of DDC-treated mouse livers transfected with *CTNNB1*^{T41A} combined with either *DNAJB1-PRKACA* or kinase-dead *DNAJB1-PRKACA* (KD, right) (**B**) Immunofluorescence co-staining for Ki67 (red) and GFP (green). (**C**) Quantification of Ki67 positive cells. Error bars represent standard deviation. Two-tailed t-test $p<0.05$ (*).

Discussion/Future Directions

Using a combination of hydrodynamic transfection, somatic genome editing, and transposon-mediated gene delivery, we demonstrate that the *DNAJB1-PRKACA* fusion is a *bona fide* oncogene that drives FL-HCC. Since tumors were produced by both the endogenous fusion and ectopic expression of the fusion cDNA, it appears that the loss of the intervening 400kb deleted region, encompassing 7 additional genes, is dispensable for tumorigenesis. The gene fusion appears to be functionally distinct from the mere overexpression of wild type *PRKACA*, which is insufficient to drive tumor progression, at least when expressed in adult hepatocytes. On the other hand, the kinase domain of PRKACA is required for the FL-HCC phenotype. Importantly, this indicates the potentially druggable enzymatic activity of the chimera is important for tumorigenesis.

Model

Numerous features of the FL-HCC models described here mimic the human disease. Histologically, our methods generate tumors whose morphology is strikingly reminiscent of human FL-HCC. Dramatic accumulation of mitochondria appears to be a consistent consequence of DNAJB1-PRKACA activity, further linking our model to the human disease. Globally, we observe an expression signature significantly enriched with the genes differentially expressed in human FL-HCC. Nonetheless, DNAJB1-PRKACA expression in the murine hepatocytes does not appear to directly drive all molecular features of FL-HCC (fibrosis, expression of biliary markers, CD68). Additionally, while human FL-HCC often metastasizes, no metastases were observed in the mouse model. Perhaps other biological factors, or time, are needed for metastatic progression. These

differences notwithstanding, the mouse model allows for insight into the phenotype of protein kinase A fusion activity in the liver in a controlled setting. We observe that induction of the fusion results in tumors with activated mTOR signaling (a druggable pathway) and detectable effects of oxidative stress (a potential vulnerability), validated by multiple previous reports describing human clinical samples (Cornella et al., 2015; Kannangai et al., 2007; Riehle et al., 2015; Simon et al., 2015).

In human FL-HCC, we observed recurrent mutations that hyperactivate the Wnt pathway together with the DNAJB1-PRKACA fusion. Furthermore, genetic alteration of this pathway – but not several other oncogenes or tumor suppressors – synergized with DNAJB1-PRKACA-driven tumorigenesis in the mouse. While the basis for this genetic interaction remains to be determined, it is possible that modification of β-catenin may be one downstream output of DNAJB1-PRKACA and stabilization of β-catenin may amplify its functional consequences. PKA has been described to regulate β-catenin through a variety of mechanisms, including direct modification via C-terminal phosphorylation (Fang et al., 2000; Hino et al., 2005; Kang et al., 2002); accordingly, phospho-β-catenin is elevated in human FL-HCC (Cieply et al., 2009; van Veelen et al., 2011). Alternatively, β-catenin may protect cells from oxidative stress imposed by DNAJB1-PRKACA (Tao et al., 2013).

DDC dramatically shortened tumor latency, unexpectedly preceding extensive fibrosis. By causing oxidative stress-induced cell death in a subset of periportal hepatocytes, DDC sets in motion a liver regenerative response that is associated with compensatory expansion of liver progenitor cells, activation of myofibroblasts, fibrosis,

and massive immune cell infiltration (Fickert et al., 2007) in a process supported by β-catenin expression in hepatocytes (Tao et al., 2013). Any or all of the activated and recruited cell types could contribute paracrine signals that protect and stimulate growth in the spatially separated (largely pericentral) population of hepatocytes that are transfected by hydrodynamic injection (Chen and Calvisi, 2014). While chronic liver damage is not considered to be necessary for human FL-HCC, our data raise the possibility that some environmental factor, perhaps in a susceptible population, may be relevant in the etiology of the disease. Alternatively, it is possible that β-catenin and/or DDC facilitated a change in cell state that supports or expands a susceptible progenitor cell population more prevalent in adolescents.

Animal models set a gold standard for assessing the oncogenic potential of aberrations observed in human cancer and provide experimental systems to study disease mechanisms or test novel therapeutic strategies (Kersten et al., 2017). While one patient-derived xenograft (PDX) of FL-HCC has been described (Oikawa et al., 2015), our models introduce the ability to examine the entire process of tumor initiation in immune competent organisms. The somatic engineering methods described here involve only delivery of plasmid DNA to hepatocytes and do not require expensive and time-consuming generation or breeding of germline mouse strains, cell transplantation, or stable expression of Cas9. The model is easily implemented, reproducible, and genetically defined. As such, these systems are a powerful platform to further understand the biology of FL-HCC and facilitate drug discovery for a disease that disproportionately affects young patients and has limited treatment options.

Whereas PDX samples bear closest resemblance to biliary tree stem cells (Oikawa et al., 2015), the CRISPR-induced tumors by hydrodynamic transfection of adult hepatocytes show that, in principle, fibrolamellar hepatocellular carcinoma could develop from a hepatocyte cell-of-origin. This may not be surprising, since a significant amount of cellular plasticity and transdifferentiation is seen in the liver in the settings of regeneration and oncogenesis, consistent with the possibility that the plentiful reserve of hepatocytes can serve as the cell-of-origin for liver tumors of either hepatocyte or biliary identity under certain conditions (Merrell and Stanger, 2016; Tschaharganeh et al., 2014; Yanger et al., 2013). However, exercising greater control over the cell type in which the oncogenic driver is induced could reveal a greater understanding of the origins and nature of the disease (He et al., 2017). Defining the cell of origin could also instruct the optimization of the most predictive disease models possible. Metastasis is a significant challenge in the clinical management of FL-HCC, which has not been adequately represented in existing *in vivo* models; novel or optimized models will be needed to study this aspect of the disease.

One glaring deficiency is the lack of any available FL-HCC cancer cell lines. If a panel of stable, proliferating cancer cell lines were to be isolated and culture conditions optimized, it would enable a more relevant context to explore the basic biology of the pathway and also open the door to high-throughput chemical and genetic screens. Hits of candidate vulnerabilities identified in cell lines could be further validated in more physiologically relevant *in vivo* models.

In addition to mouse models and cell lines, development of experimental systems in other smaller model organisms could provide a unique set of opportunities by balancing advantages of scalability and latency, while potentially maintaining some important aspects of the tumor microenvironment. Along with the possibility of advancing more complex organoid cultures, such platforms may help bridge the gap between high throughput *in vitro* assays and physiologically relevant *in vivo* experiments (Dar et al., 2012).

Tumor microenvironment and immunosurveillance (immunotherapy)

One major area of interest is in the field is the tumor microenvironment of FL-HCC, including its potentially exploitable interaction with the immune system. For instance, the abundance and roles of immunosuppressive cell types such as Tregs and MDSCs have not yet been investigated. The concept of escape from immunosurveillance as a fundamental step to the progression of many cancers is coming to maturity in parallel with novel pharmaceutical tools to manipulate tumor-immune interaction (Dunn et al., 2002). Tumors interact and exchange signals with innate and adaptive immune cells, as well as the regulatory immune system, which keeps reaction to self-antigens in check. Certain pro-tumorigenic stromal cells could be depleted or their state modified with agents such as CSF1R inhibitors (Pyonteck et al., 2013).

There is considerable enthusiasm concerning the exploration of immunotherapy, specifically immune checkpoint blockade (CTLA4, PD-1, and others), based largely on the achievement of impressive durable responses in a subset of lung cancer and melanoma patients (Brahmer et al., 2015; Larkin et al., 2015). One prerequisite question

remains to be answered: to what extent is FL-HCC immunogenic? Immune checkpoint blockades relies on infiltrating tumor-specific cytotoxic T cells that are otherwise inhibited by immune regulatory interactions, which have not yet been observed in FL-HCC. Cancers linked to mutagenic etiologies (smoking and UV-damage) or defects in DNA damage repair harbor high numbers of mutations, offer opportunities for the immune system to distinguish cancer cells from corresponding normal tissue through the recognition of tumor neoantigens. Indeed, exceptionally high mutation rate is one predictor of response to immune checkpoint blockade (Le et al., 2017). Other factors also correlate response to immune checkpoint blockade within these tumor types, such as the abundance of infiltrating T cells, T cell exhaustion, and tumor cell PDL1 expression (Topalian et al., 2016), but notably, the predictive power of these markers have been evaluated only in settings where the potential for mutant neoantigens is far greater than FL-HCC (Darcy et al., 2015; Lawrence et al., 2013). As a tumor type with an ultra-low mutational load, FL-HCC is substantially different than most successful applications of immune checkpoint blockade reported to date (Snyder et al., 2014). On the other hand, neoantigens can arise in the absence of mutation through ectopic expression of wild type proteins, abnormal post-translational modification, or the presentation of viral antigens (in virus-associated cancers). Further investigation will be needed to determine the balance of potential benefit with the risk of severe immune-related adverse events associated with inhibiting immune regulatory processes (Brahmer et al., 2015; Larkin et al., 2015).

Other strategies do not rely on endogenous anti-tumor immune recognition, but instead induce new immunity against a specific tumor antigen. These include peptide

vaccination, drug- or radionucleotide-conjugated antibodies, dendritic cell vaccines (which can also be loaded by bulk tumor lysates), adoptive T-cell transfer, bispecific T-cell engager antibodies (BiTEs) and CAR-T therapies (Okada et al., 2009). Each has a unique set of advantages and disadvantages, but the challenge of defining a generalizable and specific tumor-associated antigen remains, despite recent advances (Gee et al., 2018). Even different products targeting the same tumor antigen with different affinity can have remarkably different efficacy and toxicity profiles (Lim and June, 2017). Currently, no known FL-HCC-associated antigens have been described. Although targeting fusion oncogenes has been proposed (Powell et al., 2003), it bears remarking that of the universe of intracellular peptides, only a minority are effectively processed, MHC bound, and are ultimately immunogenic (Bassani-Sternberg et al., 2017; Jappe et al., 2018). The peculiar immature differentiation state, incorporating components of hepatic, biliary, and neuroendocrine cell fate (Torbenson, 2012) leave researchers hopeful that ectopic expression or PTM may be exploited in immunotherapy.

Drugging FL-HCC/ Target identification and Pharmacology

Driving fusion oncogenes often serve as "smoking gun" evidence of dependency. A prototype of targeted therapy is the success of inhibiting another fusion kinase, *BCR-ABL*, in chronic myeloid leukemia (CML). This frequently resulted in clinical responses and radically increased the long-term prognosis for CML patients (Druker et al., 2001b). Targeting PML-RARA fusion for degradation induces differentiation and commonly cures acute promyelocytic leukemia (de The and Chen, 2010). Targeted kinase inhibition of recurrent oncogenes has also induced responses in heavily pre-treated lung cancers

harboring *ALK* and *ROS* rearrangements, or cancers harboring point mutations in *BRAF*, *EGFR*, or *KIT* (Pagliarini et al., 2015).

Even upon identification of a target, developing the right drug is important. Taking a lesson from the development EGFR inhibitors, distinct small molecule inhibitors of the same target can have distinct optimal clinical applications (Vivanco et al., 2012). One on the most commonly used compounds for PKA inhibition in the literature is H89, a tool compound that is unlikely to be a suitable clinical drug, due to multiple potent off-target effects (Lochner and Moolman, 2006). Small molecule inhibitors of PKA are in preclinical development by Blueprint Medicines and others. The active site of PKAcα is closely related to Akt, and many Akt inhibitors have off-target effects against PKAcα; medicinal chemistry may prove useful to modify existing Akt inhibitors in order to generate novel PKA inhibitors (Pearce et al., 2010). Given the importance of PKA in a wide array of biological setting, including cardiac contractility (Marx et al., 2000), defining a therapeutic window of PKA inhibition will be a critical next step. If inhibition of wild type PKA appears toxic, it may be worthwhile to attempt to derive strategies that specifically interfere with the *DNAJB1-PRKACA* fusion, a possibility indicated by the substantial flexibility that the J domain fusion confers to the N terminus of the protein revealed by molecular dynamics studies (Tomasini et al., 2018). However, a number of experiments using PKA inhibitors in mice provide some reassurance that PKA inhibition remains a priority to test in the treatment of FL-HCC (Lochner and Moolman, 2006).

Alternative disease vulnerabilities

By experimentally manipulating AKAPs in future studies, the relevant downstream targets for FL-HCC tumorigenesis could potentially be narrowed down and exploring the regulators of specific AKAPs, alternative means of blocking oncogenic signaling could be developed. Although drugging protein-protein interaction is notoriously difficult, peptides disrupting PKA-AKAP interaction has been explored experimentally, with additional modifications to enhance cell membrane permeability and stability (Nygren and Scott, 2015).

The availability of tools and the vindication of new paradigms in cancer research have fundamentally changed the study of rare cancers over the last decades. Herein, we focus on how a diversified and coordinated effort to study fibrolamellar hepatocellular carcinoma can be best executed in the present research environment, in a manner that could be generalized to and draws from the study of other malignancies.

Outlook

On the basis of clinical experience and the detailed molecular characterization of patient tumor samples, the fundamental understanding of the Protein Kinase A pathway biochemistry continues to expand, where a recently generated collection of physiologically relevant experimental models will complement the clinical efforts. Such studies will evaluate proposed therapeutic targets as well as provide opportunities to uncover additional candidate genes for intervention. Medicinal chemistry and pharmacology will be required to exploit potential dependencies, which, if successful, will culminate in clinical trials. In this iterative process, the cross-fertilization of each

discipline can inform further experiments to enhance the understanding of basic biology and to optimize therapeutic and diagnostic strategies.

Relatively few clinical trials have been conducted specifically for FL-HCC, given the challenge of accruing a large number of patients with this rare disease. Retrospective epidemiological data does not support the conclusive superiority of any particular chemotherapeutic regimen (Lim et al., 2014). A trial for the multi-kinase inhibitor Sutent was terminated for lack of inclusion (NCT01215565). Clinical trials for an Aurora Kinase inhibitor (NCT02234986) and mTOR inhibitor with or without estrogen blockade (NCT01642186) remain open but have not yielded promising results. The fact that multi-center clinical trials can be conducted with sufficient enrollment is, in itself, an accomplishment, and the limited throughput to clinical adaptation requires the careful prioritization of therapeutic strategies with the strongest rationale and significant preclinical data.

While there is optimism that PKA inhibition could yield substantial clinical benefit, it is appropriate to anticipate the emergence of resistance analogous to single agent treatments in other contexts. Whereas both retinoic acid and arsenic trioxide as single agents can degrade PML-RARA and induce remissions in acute promyelocytic leukaemia, the combination cures the vast majority of patients (de The and Chen, 2010). Innate resistance, where release of feedback inhibition reactivates a signaling pathway may lead to transient pathway inhibition followed by adaptation by tumors cells, as is seen in the application of MEK and AKT inhibitors (Chandarlapaty et al., 2011; Manchado et al., 2016). Feedback regulation has also been seen in the PKA pathway

(Rapacciuolo et al., 2003; Zamah et al., 2002) , this phenomenon may necessitate pulsatile dosing or targeting multiple nodes in the PKA signaling pathway. Also, a defining feature of oncogene addiction is the positive selection of drug resistant point mutations in the targeted oncogene (Gorre et al., 2001). This can be anticipated in vitro and ameliorated by further optimizing drug design or by combining multiple drugs which non-overlapping mechanisms of resistance (Glickman and Sawyers, 2012).

We are optimistic that a multidisciplinary and collaborative approach to improving outcomes in fibrolamellar hepatocellular carcinoma will be fueled by new technologies, comprehensive characterization of patient samples, advances in basic science and the opportunities for clinical adaptation and partnership with industry. Wide distribution of new insights and resources such as PDXs and cell lines will help accelerate the discovery process. Many challenges still lie ahead, but new technologies, strong patient engagement, and a community of interested researchers are actively investigating for a path forward.

CHAPTER II: p53.

Summary

Although a long pedigree of biological studies have explored the instigation of genetic abnormalities as a result of chromosomal instability and mutational processes, very little is known about the process of competition, persistence, and extinction of alleles that arise in populations of tumor cells. Given the role of pre-existent minorities of drug-resistant subclones in acquired resistance, a better understanding of diversification and maintenance of diversity in tumor cell populations will be absolutely essential in achieving a higher rate of durable cures in lymphoma and other cancers using rationally designed therapies.

Introduction

TP53 is the most frequently mutated gene in human cancer. Functionally, p53 is activated by a host of stress stimuli and, in turn, governs an exquisitely complex anti-proliferative transcriptional program that touches upon a bewildering array of biological responses. Despite the many unveiled facets of the p53 network, a clear appreciation of how and in what contexts p53 exerts its diverse effects remains unclear. How can we interpret p53's disparate activities and the consequences of its dysfunction to understand how cell type, mutation profile, and epigenetic cell state dictate outcomes, and how might we restore its tumor suppressive activities in cancer?

p53: the textbook view

p53 was discovered during the peak of tumor virus research as a 53 kD host protein bound to simian virus 40 large T antigen in virally-transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). First classified as an oncogene, subsequent work established that wild-type p53, encoded by the *TP53* gene, suppresses growth and oncogenic transformation in cell culture (Finlay et al., 1989) and that inactivating *TP53* mutations are common in human tumors (Baker et al., 1990) and in many cancers linked to poor patient prognosis (Olivier et al., 2010). Consistent with its action as a tumor suppressor, *TP53* mutations are a hallmark of a hereditary cancer predisposition disorder known as Li-Fraumeni Syndrome (Malkin et al., 1990), and *Trp53* knockout mice develop tumors at high penetrance (Donehower et al., 1992).

p53 is a sequence-specific DNA binding protein that regulates transcription (reviewed in Laptenko and Prives, 2006). The p53 protein consists of two N-terminal transactivation domains followed by a conserved proline rich domain, a central DNA binding domain, and a C-terminus encoding its nuclear localization signals and an oligomerization domain needed for transcriptional activity. Consistent with the importance of p53-mediated transcription in tumor suppression, the vast majority of tumor-derived *TP53* mutations occur in the region encoding p53's DNA binding domain. In normal cells, p53 protein is maintained at low levels by a series of regulators including *MDM2*, which functions as a p53 ubiquitin ligase to facilitate its degradation (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). However, p53 is stabilized in response to various cellular stresses, including DNA damage and replication stress

produced by deregulated oncogenes. How p53 activation occurs can be stimulus-dependent: for example, DNA damage promotes p53 phosphorylation, blocking MDM2-mediated degradation (Shieh et al., 1997), whereas oncogenic signaling induces the ARF tumor suppressor to inhibit MDM2 (Pomerantz et al., 1998; Quelle et al., 1995; Zhang et al., 1998).

The best understood functions of p53 focus on its ability to promote cell cycle arrest and apoptosis. Indeed, seminal studies from the early 1990s showed that p53 is crucial for a reversible DNA damage-induced G1 phase checkpoint (Kastan et al., 1991) that is mediated, in part, by its ability to transcriptionally activate the p21 cyclin-dependent kinase inhibitor gene (el-Deiry et al., 1993; Harper et al., 1993), presumably facilitating DNA repair prior to further cell division. In some circumstances, p53 induces cellular senescence, a stable if not permanent cell cycle arrest program that also involves the retinoblastoma (RB) gene product (Serrano et al., 1997; Shay et al., 1991). p53 can also promote apoptosis (Clarke et al., 1993; Lowe et al., 1993; Yonish-Rouach et al., 1991), relying on the induction of pro-apoptotic BCL-2 family members whose action facilitates caspase activation and cell death (Miyashita et al., 1994). Why p53 promotes cell cycle arrest in some cell types and apoptosis in others is incompletely understood (see below).

The settings in which p53 can be activated to arrest or eliminate pre-malignant cells have guided current thinking as to why p53 is such a potent tumor suppressor. On one hand, its ability to arrest or eliminate cells following DNA damage suggests that it might prevent cancer by preventing the *accumulation* of oncogenic mutations

(Livingstone et al., 1992; Yin et al., 1992). In this model, p53 loss indirectly promotes cancer by increasing the number of mutations in surviving daughter cells. On the other hand, the ability of p53 to halt the proliferation in response to aberrant oncogene expression suggests a role in limiting the *consequences* of oncogenic mutations. Here, p53 loss directly enables cancer development by allowing oncogene-expressing cells to proliferate unabated, explaining why *TP53* mutations cooperate with oncogenes in transformation (Lowe et al., 1994; Serrano et al., 1997). In both models, p53 acts as the "guardian of the genome" to limit the deleterious consequences of mutation (Lane, 1992). Although this historic view provides a basic conceptual framework as to why *TP53* mutations are so common in human tumors, more recent work paints a much more nuanced picture of p53 action that highlights its context-dependent regulation and the broadly diverse consequences of its activation.

p53 controls a broad and flexible network

As if regulating genome integrity, cell cycle arrest, and apoptosis were not enough functions for a single gene, an ever-growing body of work suggests that p53 also controls additional "non–canonical" programs that contribute to its effects (**Figure 2.1**). As examples, p53 can modulate autophagy, alter metabolism, repress pluripotency and cellular plasticity, and facilitate an iron-dependent form of cell death known as ferroptosis (reviewed in Aylon and Oren, 2016). Even basal levels of p53 can reinforce multiple other tumor suppressive networks (Pappas et al., 2017). Given extensive past

research, it is surprising that there is no clear and simple answer to the question of what exactly p53 does and how. Nevertheless, a take-home message is that the p53 response is remarkably flexible and depends on the cell type, its differentiation state, stress conditions, and collaborating environmental signals.

The varied functions of p53 are anchored in its ability to control distinct sets of its many target genes (**Figure 2.1**). For example, observations that cell cycle arrest and apoptosis are associated with upregulation of p21 or pro-apoptotic Bcl-2 proteins, respectively, obscure the fact that the global transcriptional response to p53 activation includes many other potential modifiers of outcome. Historically, genes have been implicated as p53 targets if p53 binds the locus and the mRNA is induced. More recently, Global Run-On Sequencing has improved specificity by enabling detection of nascent transcripts induced upon p53 activation (Allen et al., 2014). The nature of p53 targets identified in this analysis provides strong confirmation that non-canonical processes including ROS control, tissue remodeling, autophagy, and metabolism are *bona fide* processes controlled by p53 (**Figure 2.1**).

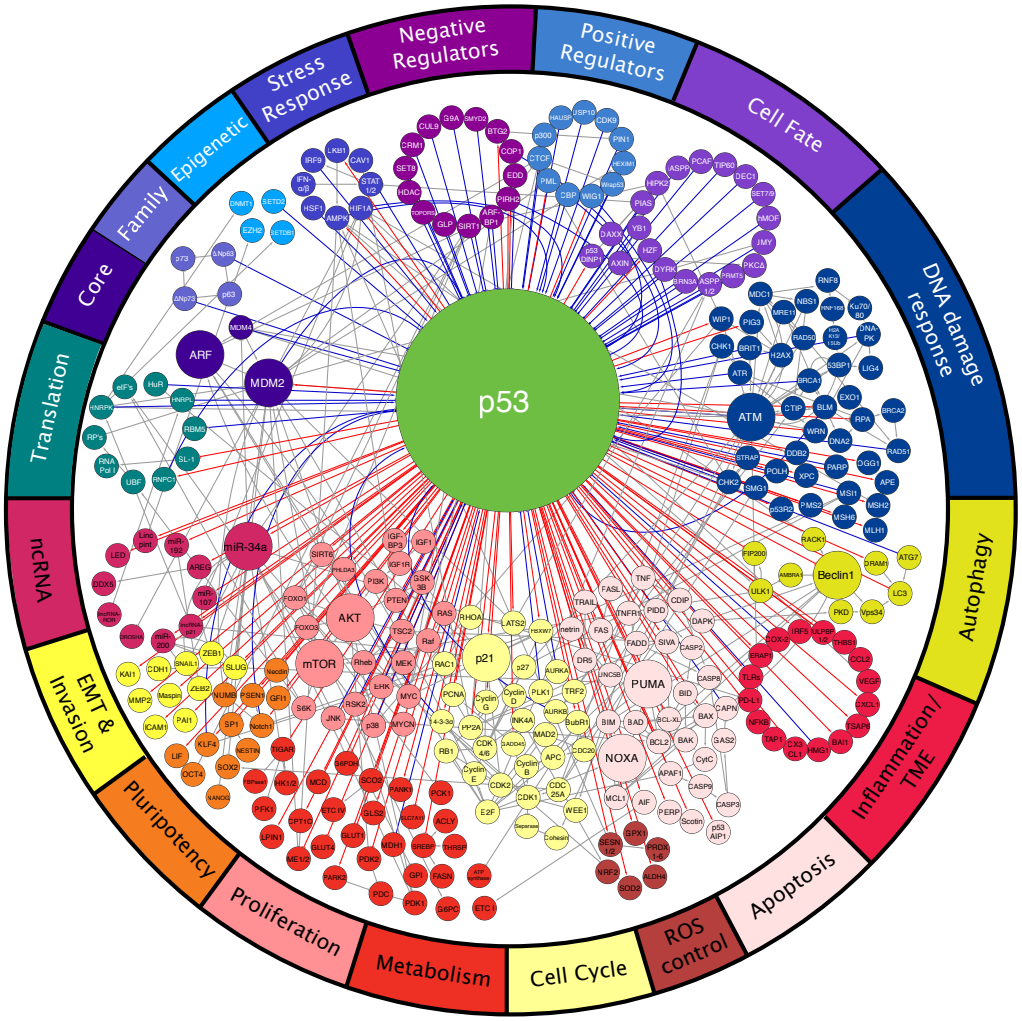

Figure 2.1. The p53 network

A wide variety of regulators govern the activity of p53 (top), which, in turn, controls many distinct biological processes (bottom). Each node represents a gene and each line represents an interaction. Direct p53 inputs are indicated as blue lines and direct p53 outputs are indicated as red lines. Noticeably, p53 controls effector processes by activating multiple target genes. Downstream pathways are highly interconnected (gray lines). Interactions are annotated as positive (arrow), negative (T-bar), or modifying (solid circle).

Efforts to identify a universal set of p53 target genes have invariably failed. Meta-analyses from 16 genome-wide datasets revealed that only about 60 genes were implicated as common targets (Fischer, 2017). It is noteworthy that these surveys involved a restricted number of different cell types and employed distinct methods for p53 induction. However, a central theme is that cellular context and various stimuli incite transcription of qualitatively different sets of genes, not just different levels of the same set of genes. It seems naive to expect that oncogene activation in different tissues (for example, KRAS activation in colon, pancreas, and lung) would precipitate an identical p53 transcriptional response. Moreover, one would not presume *a priori* that the p53 output generated by DNA damage would exactly mirror the gene expression signature elicited by oncogene activation, even in a single cell type. Despite data indicating that p53 can, in principle, control a wide variety of biological processes (reviewed in Olivos and Mayo, 2016), the physiological settings in which one or more processes predominate are incompletely understood and deserve more systematic study.

Cellular metabolism is one non-canonical p53-controlled process that has received much attention (reviewed in Kruiswijk et al., 2015). The collection of metabolic target genes controlled by p53 affect many individual processes: p53 is reported to increase glutamine catabolism, support anti-oxidant activity, downregulate lipid synthesis, increase fatty acid oxidation, or stimulate gluconeogenesis (Kruiswijk et al., 2015). Depending on the cell type, p53 can also have opposing effects on the same metabolic processes. For example, in breast and lung cancer cells, p53 inhibits glycolysis by attenuating glucose uptake (Zhang et al., 2013) or repressing the expression of glycolytic enzymes (Kim et al., 2013). By contrast, in muscle cells, p53 induces

glycolytic enzymes (Kruiswijk et al., 2015). Likewise, p53 typically increases (Stambolsky et al., 2006) but can also restrict flux through the tricarboxylic acid (TCA) cycle (Jiang et al., 2013; Wang et al., 2013). Taken at face value, these results imply that p53 can regulate different aspects of metabolism that produce distinct, or even opposite, biochemical and phenotypic outcomes. Here again, precise contextual factors have yet to be identified.

While it is often assumed that each p53 effector function is a standalone process, there is increasing evidence that cross-talk between separate input and output pathways is more important than previously recognized (Figure 1, gray lines). Complex interactions amongst multiple p53-controlled processes undoubtedly contribute to cell fate. For example, p53-driven cellular senescence may be supported by activation of autophagy (Young et al., 2009). In some settings, p53-mediated processes can apparently be antagonistic: autophagy has the potential to delay apoptosis by reducing levels of PUMA (Thorburn et al., 2014). However, in contexts where p53 fails to repress glycolysis, autophagy is not efficiently engaged and apoptosis is favored (Duan et al., 2015). In these examples, interaction between distinct *biochemical* processes controlled by p53 elicits different *biological* outcomes.

The mechanistic basis underlying the ability of p53 to induce different biological outputs remains unclear. On one hand, p53 can induce *qualitatively* different programs that produce different biological outcomes depending on cell type and stimulus. One proposed mechanism for qualitatively modulating biological p53's effects involves stimulus-dependent post-translational modifications (PTMs) that can alter p53 affinity for

different target genes; for example, phospho-p53 (S46) or acetyl-p53 (K120) stimulates apoptosis, whereas PRMT5-methylated p53 activates p21 more readily than apoptotic genes (reviewed in Kumari et al., 2014). A broad array of other PTMs at many different sites in the p53 protein have been described to not only modify protein stability, but also influence target gene bias, such as SUMOylation, glycosylation, and prolyl isomerization (Kumari et al., 2014). Moreover, one post-translational modification may enhance acquisition of another, unlocking additional layers of regulation of protein stability, protein-protein interaction, and biasing DNA-binding toward select target genes. Another contextual factor governing p53 output tissue/substrate stiffness, which through signaling dependent on a mevalonate-Rho axis, reinforces stabilization of p53 (Ebata et al., 2017; Wagstaff et al., 2016).

p53 induction can yield either a steady signaling output or one that can oscillate in discrete waves; remarkably, the kinetics of its expression, independent of maximal p53 protein levels, can determine cell fate in response to genotoxic stress (reviewed in Stewart-Ornstein and Lahav, 2017). p53 activation kinetics can be translated into target gene bias owing to differences in p53 binding and dissociation rates at distinct target loci. Here, the p21 promoter is sensitive to short pulses of p53 activity whereas the pro-apoptotic p53 target FAS is not; consequently, a short pulse drives proliferative arrest but a sustained signal induces apoptosis (Espinosa et al., 2003; Gomes and Espinosa, 2010a; Morachis et al., 2010). Perhaps certain p53-driven stress responses instigate a short-term repair and salvage program that, if necessary, reaches a tipping point that progresses to cellular self-destruction.

On the other hand, several factors influence how the cell *interprets* p53 activation. For instance, cell lineage may be a large determinant in the nature of a hypothetical tipping point between alternative cell fates. First, cell type- and state-specific chromatin modifications may make particular genes more or less accessible to p53 transactivation (Su et al., 2015). For instance, CTCF insulates the *PUMA* locus from repressive histone modifications under certain conditions, governing whether PUMA is expressed and apoptosis occurs (Gomes and Espinosa, 2010b). In embryonic stem cells (ESCs), p53 binds efficiently to the p21 promoter, but p21 and cell cycle arrest is not induced, dependent on cell-type-specific repressive histone H3K27me3 marks at the locus (Itahana et al., 2016). Second, the p53 target spectrum can be altered by cooperation or antagonism with other transcription factors, such as FOXO and NF-κB, whose levels and occupancy are also context-dependent (Cooks et al., 2014; Eijkelenboom and Burgering, 2013). Finally, the same transcriptional output may have different effects depending on the state of the cell. ATM signaling protects cells from p53-mediated apoptosis, not by changing p53-driven transcriptional output, but by blocking autophagy, thus maintaining mitochondrial homeostasis and suppressing ROS levels (Sullivan et al., 2015).

Collectively, these observations imply that p53 response is not merely an "on-off" switch; to the contrary, cell fate is a result of a rich palette of p53-driven stress responses. Clearly, p53 is embedded in a densely populated and interconnected network of regulators and effectors (**Figure 2.1**) that permit a flexible p53 response coordinated to fit cell type and conditions at the time of activation. In short, cellular context (cell type, epigenetic state, tissue microenvironment, activating signal) is central to both the biochemical aspects of p53 activity as well as the biological outcome of a p53 response.

Putting Tumor Suppression in Context

By definition, tumor suppressor genes regulate processes that limit inappropriate cell expansion and whose inactivation facilitates tumor initiation or progression. Given the many processes that p53 controls, which of its effector functions are critical for tumor suppression has been the topic of much debate. Senescence and apoptosis can clearly be detected in tumors and when these processes are activated, they are certainly tumor suppressive. Still, a recent body of work suggests that apoptosis and senescence can be dispensable for tumor suppression and that, in some settings, other non-canonical p53 functions may be more critical (Valente et al., 2016). There is no consensus view on which p53-dependent process is most important.

The only relevant metric of "tumor suppression" is whether a gene impairs the onset or progression of tumors arising in vivo. In this regard, the p53 knockout mouse is a powerful model that develops thymic lymphoma (and sometimes sarcoma) at complete penetrance (Donehower et al., 1992). To address which p53 function(s) is crucial for tumor suppression, mutant strains have been produced in an attempt to isolate specific p53 functions, and the resulting animal cohorts monitored for tumors over time. If the ablation of a p53-driven function allows for tumorigenesis, the underlying process is crucial for the tumor suppressive activity of p53. If it does not, it is deemed dispensable. However, it bears consideration that thymic lymphoma rarely occurs in people, including Li-Fraumeni patients, so the requirements for suppressing this unusual cancer do not necessarily extend to other systems.

One line of investigation has compared tumor onset and pathology between mice harboring knockouts of p53 target genes versus p53 itself (**Figure 2.2A**). For example, p21 and Puma-deficient mice do not develop thymic lymphoma, hinting that p53-mediated cell cycle arrest and apoptosis might be dispensable for tumor suppression (Valente et al., 2016). Still, p53 target genes may already expressed at basal levels so, for example, p53-null cells are by no means p21-null. Consequently, this approach could *overestimate* the contribution of a particular p53 effector to the null phenotype. Conversely, since multiple effectors mediate most p53 outputs, mouse strains deficient for individual p53 effector genes do not fully disable the associated p53 effector program (e.g. p21 loss does not completely disable p53-mediated cell cycle arrest). Hence, this approach may *underestimate* the contribution of the targeted process to tumor suppression. Changes in feedback loops and compensatory mechanisms arising as a consequence of manipulating the pathway may further complicate the interpretation of such studies (Sullivan et al., 2012).

Another approach isolates p53 effects through separation-of-function mutants that selectively retain or lose the ability to regulate certain subsets of p53 target genes and activities (**Figure 2.2B**). For example, the tumor-derived $p53^{R175P}$ and $p53^{E180R}$ alleles show defects in apoptosis while retaining the capability to provoke cell cycle arrest, so that mice harboring the equivalent mutations display extended tumor-free survival compared to p53-null animals (Liu et al., 2004). Furthermore, the tumors that do arise in these mice exhibit far less CIN than p53-null tumors, indicating that different p53 mutants may impinge selectively on downstream effector pathways (Liu et al., 2004). Alternatively, engineered structure-function mutants that disrupt p53 transcriptional

domains or are defective in being acetylated can separate key p53 functions, at least in vitro (Jiang et al., 2015). While these studies reinforce the importance of p53-mediated transcription for tumor suppression (Brady et al., 2011; Jiang et al., 2011), they do not pinpoint a single key process (Jiang et al., 2015).

Although such structure-function approaches are compelling, they also have caveats. Mutant p53 proteins can be more or less stable than the wild-type protein (Brady et al., 2011) and thus differential phenotypes may reflect quantitative as well as qualitative effects. Most structure-function mutants have only been characterized in a limited number of cell types, and given context dependencies, it cannot be assumed that these results extrapolate to tumorigenesis in all tissues. Perhaps these caveats explain why technically sound studies have failed to converge on a common mechanism or theme.

Several studies have circumvented the issues surrounding the manipulation of individual functions peripheral to p53: rather than measuring tumor onset upon p53 loss, they instead take advantage of mouse strains harboring "switchable" p53 alleles to reawaken endogenous p53 in established tumors (**Figure 2.2C**). In all situations examined, p53 restoration produces a marked anti-tumor response, the nature of which depends on the model employed (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). In Myc-expressing B cell lymphomas, this response is massive apoptosis; in liver carcinomas and sarcomas, the response is senescence. In other contexts, p53 reactivation can trigger cellular differentiation and a loss of self-renewal (Messina et al., 2012). Although the consequences of p53 reactivation in an established cancer may not reflect

the same processes lost during tumorigenesis, they reinforce the notion that the p53 response is context dependent.

So then, what are the most important p53 activities needed for tumor suppression? Certainly, the above caveats preclude generalities without considering context-specificity. Indeed, the importance of context is readily observed in studies demonstrating that Puma loss does not promote thymic lymphoma, but does cooperates with Myc to drive B cell lymphoma (Garrison et al., 2008; Hemann et al., 2004). Embracing this notion should enable the identification of tumor-specific modes of tumor suppression and pave the way for restoring the most relevant p53 functions in individual tumors.

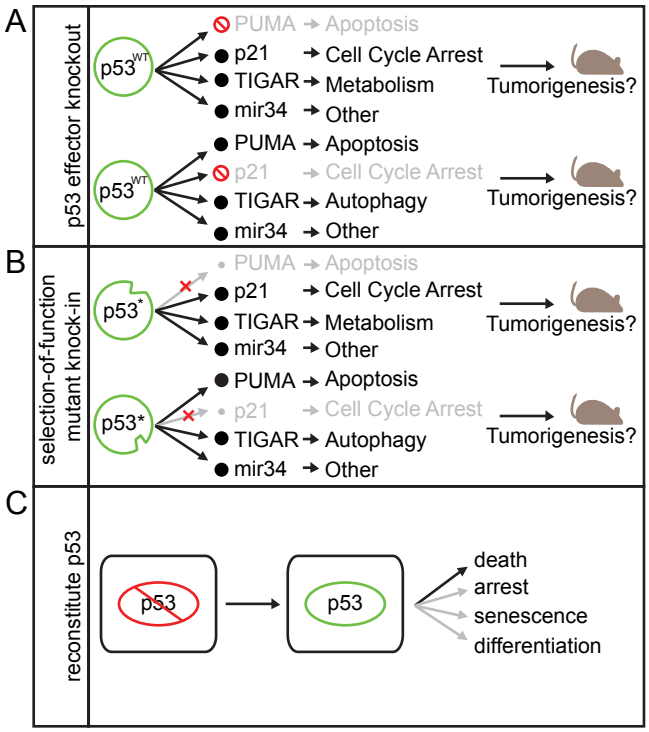

Figure 2.2. Investigating mechanisms of tumor suppression

Defining the mechanism of p53-mediated tumor suppression has been interrogated in several ways: (A) knocking out p53 target genes and assessing tumor formation, (B) mutating p53 itself, such that it can activate some targets but not others, and (C) reconstituting p53 in p53 deficient cancer and determining the cell fate.

The Origins of p53

How and why did the p53 network evolve? Most tumors arise after reproductive age, implying that the *TP53* gene did not evolve to prevent cancer. Moreover, given the diverse outputs of the p53 network, it seems surprising that neonatal p53-null mice seem initially normal. Genes that resemble *TP53* by sequence similarity and induction by DNA damage are found in simple invertebrates (including choanoflagellates, sea anemone and worms) that are not susceptible to cancer (Lane et al., 2010a; Lane et al., 2010b; Pearson and Sanchez Alvarado, 2010). Like mammalian p53, these genes induce apoptosis in response to stress but, in contrast, are expressed principally in germline stem cells. Perhaps protection of the germline is central and evolved further to suppress tumors in the soma at advanced age (Wylie et al., 2014).

Beyond the germline, a closer look at p53 and the consequences of its disruption indicate it has a number of important roles in embryonic development. Fundamentally, multicellularity is a compromise among the cells of complex organisms. The most proliferative individuals outcompete populations of single cell organisms, while multicellular organisms require cellular cooperation, at the expense of competition, to maintain coordinated, specialized functions. The need for cooperation starts in embryonic development, where p53 restricts expansion of individual "cheater" cells, observed in chimeric blastocysts upon p53 knockdown (Dejosez et al., 2013) and following positive selection of spontaneous *TP53* mutations detected in commonly used human ES lines (Merkle et al., 2017). Tight regulation of DNA methylation by DNMT and TET family enzymes requires p53 and it appears that epigenetic disorder contributes to this clonal

heterogeneity in p53-deficient ESC colonies (Tovy et al., 2017). Inactivation of p53 rescues cultured cells from apoptosis caused by DMNT1 deficiency and subsequent genomic demethylation, supporting the notion of p53 as the guardian of the epigenome (Jackson-Grusby et al., 2001). Other than its familiar role in restricting inappropriate clonal outgrowth, p53 also regulates target genes that fulfill specific biological requirements in development, such as LIF, which is required for efficient mammalian embryo implantation (Feng et al., 2011). *Trp53* knockout mice exhibit a variety of low-penetrance tissue-specific developmental abnormalities in the neural tube, eyes and testes (Danilova et al., 2008). p53 orthologs in more primitive species can also exhibit conserved non-canonical p53 functions, such as promoting redox control and survival (reviewed in Aylon and Oren, 2016). Moreover, the ortholog Lvp53 is expressed in the soma in shrimp, where cross-talk with NF-kB eliminates virally infected cells and activates innate immunity (Li et al., 2017). Such observations are consistent with a role for the p53 family in promoting cell survival or fighting infection. Hence, the p53 network evolved diverse physiological roles prior to its implementation for tumor suppression.

TP53 is a member of a broader gene family that includes the *TP63* and *TP73* genes that have diverse and complementary roles. *TP53* of higher eukaryotes diverged from *TP63/TP73* sometime before the appearance of sharks (Lane et al., 2011). Since its split from its homologs, the *TP53* network has acquired tumor suppressive capabilities not shared by *TP63*/*TP73*, which display even clearer ties to embryonic development (reviewed in Belyi et al., 2010). Triple p53/p63/p73 knockout mice demonstrate that the p53 family is required for mesendodermal differentiation (Wang et al., 2017),

exemplifying how p53 can interact with p63/p73 in redundant or cooperative ways. It seems likely that compensation between p53 family members has masked other roles for p53 during development.

Although the p53 protein sequence itself is relatively conserved in higher eukaryotes, domains involved in p53 regulation (on the N- and C-termini)(Lane et al., 2011) as well as the downstream p53 response are under continued evolutionary pressure. Indeed, many p53 response elements exhibit surprisingly low conservation with respect to other transcription factor recognition sites (Horvath et al., 2007; Su et al., 2015). Another way in which the p53 network has evolved is by increasing gene dosage. That elephants have acquired up to 20 *TP53* retrogenes may explain, at least in part, how an animal with such a large body size and relative longevity is not subject to high cancer risk (Abegglen et al., 2015; Sulak et al., 2016). A more detailed exploration of the factors selected and counter-selected in p53 biology over evolutionary time has the potential to provide insight into the biological processes critical for tumor suppression.

There is substantial evidence that p53 has additional functions in non-pathological tissue homeostasis. One illustrative example is that p53 function appears to be intertwined with stem cell biology and differentiation in the soma of higher organisms. In stem and progenitor cells of the hematopoietic system, liver, brain, and breast, p53 restricts cellular self-renewal (Friedmann-Morvinski et al., 2012; Tosoni et al., 2015; Tschaharganeh et al., 2014). *Trp53*-null mice consequently have expanded numbers of tissue specific stem cells, highlighting its importance in maintaining tissue homeostasis (Olivos and Mayo, 2016). At its extreme, p53 limits cellular plasticity (governing

transition between cell states) and restricts dedifferentiation and, ultimately, the ability of normal cells to undergo epigenetic reprogramming into induced pluripotent stem cells (Olivos and Mayo, 2016). The iPS-promoting factors KLF4 and Oct4 repress p53, and conversely, p53 activity antagonizes the efficiency of iPS cell reprogramming (Menendez et al., 2010).

An application of these principles can be seen in tissue regeneration and the wound-healing response, which is a complex process involving waves of inflammation, angiogenesis, tissue regeneration, extracellular matrix (ECM) remodeling, and fibrosis to prevent infection and resolve tissue damage. During an initial proliferative phase of regeneration, mitogens are activated and p53 must be suppressed to allow tissue remodeling (Charni et al., 2017). By triggering cellular senescence, p53 promotes the release of secretory factors that allow resolution of fibrosis (Krizhanovsky et al., 2008) and coordinate ECM remodeling (Ritschka et al., 2017). Of note, the requirement for p53 to regulate plasticity appears to be evolutionarily conserved, which requires the coordinate suppression and derepression of p53 during salamander limb regeneration (Yun et al., 2013).

It is intriguing that the physiological and developmental functions of p53 are intertwined with the cancer-associated phenotype of p53 loss. Evading terminal differentiation is an essential step in malignant transformation and p53 loss may be one route to weaken this innate barrier to tumorigenesis. Consistent with this notion, an embryonic stem cell-like gene signature is observed in p53-mutant breast cancer (Mizuno et al., 2010). By affecting differentiation, incipient *TP53* mutations facilitate the

expansion of hematopoietic stem cell (HSC) clones in otherwise healthy individuals, occasionally overtaking the entire hematopoietic system (Steensma et al., 2015; Xie et al., 2014). The competitive expansion of pre-treatment p53 mutant HSC clones is accentuated by genotoxic chemotherapy, fostering therapy-related AML (t-AML) (Wong et al., 2015). At its extreme, p53 loss can even facilitate lineage switching as a mechanism of resistance to anti-androgen therapy in prostate cancer (Mu et al., 2017). Furthermore, p53 action in wound healing also shapes the tumor microenvironment. For example, the p53-driven senescence associated secretory phenotype (SASP) in tumor stroma can create a tumor suppressive immune milieu that influences the incidence of cancer (Lujambio et al., 2013; Xue et al., 2007). In other settings, the SASP can be tumor promoting, by inducing epithelial–mesenchymal transition (EMT) (Canino et al., 2012; Laberge et al., 2012; Ritschka et al., 2017; Scheel et al., 2011).

It appears that evolution has selected for a delicate balance of p53 activity, since too little p53 leads to early onset cancer and too much p53 exacerbates aging. Regardless, the dangers of excess p53 are evident in pathologies beyond cancer, including aging, ischemic injury, and degeneration (reviewed in Gudkov and Komarova, 2010). As animals age, the cost of eliminating potentially dangerous cells is the attrition of stem cells required for tissue homeostasis. In an accelerated process, those who suffer from the heritable DNA repair deficiency syndrome Fanconi anemia hyperactivate p53 in response to unresolved DNA damage and experience bone marrow failure characterized by progressive HSC loss (Ceccaldi et al., 2012). Excessive p53-dependent apoptosis can also drive developmental disorders of the brain (Houlihan and Feng, 2014) and aging-associated neurodegenerative diseases, namely Alzheimer's and Parkinson's diseases

(Checler and Alves da Costa, 2014). As a regulator of cell death, p53 has been implicated in the pathological response to cerebral and cardiac ischemia; p53 inhibition has been proposed as a protective strategy in the acute phase following injury (Gudkov and Komarova, 2010). Also, excessive p53-mediated ferroptosis can trigger lethal kidney ischemia (Friedmann Angeli et al., 2014). Collectively, the characteristics of p53 action in normal physiology and non-cancer pathologies can shed new light on additional regulatory mechanisms and downstream functions, insight into how to dissect these various roles, and potentially reveal new drug targets.

The diversity of TP53 mutational events produces distinct functional consequences

Just as advances in our understanding of p53 biology have complicated, rather than simplified, our views on how *TP53* mutations promote cancer, so has our appreciation of surprising range of ways in which the *TP53* locus is altered in tumors. The most common and well-characterized *TP53* mutations are missense mutations in the DNA binding domain, implying that this feature of p53 is crucial for tumor suppression. Current dogma tends to classify p53 as either wild-type or mutant, but *TP53* mutations occur with different patterns, distinct co-mutations, and in many allelic configurations that lead to remarkably interesting functional and phenotypic ramifications.

Genome sequencing of thousands of tumors has confirmed that approximately half of all cancers harbor a *TP53* mutation, though the frequency and the distribution of mutations can vary dramatically between tumor types (**Figure 2.3A**). Most of the single nucleotide variants (SNVs) observed across cancers are missense mutations, with 25% of those falling into 5 "hotspot" mutations (Shirole et al., 2016). Unexpectedly, nearly 25% of *TP53* mutations are nonsense or frameshift mutations predicted to encode truncated proteins, whereas the remainder consists of splice site SNVs and in-frame indels of unclear biological significance (Shirole et al., 2016). While several modes to disable the second *TP53* allele are possible, this typically occurs through "loss of heterozygosity" (LOH) by segmental deletion (**Figure 2.3B**). These deletions vary widely in size and occur at a frequency that is similar to p53 SNVs (Liu et al., 2016). Nearly all possible allelic combinations are observed in tumors such that, in reality, only ~25% of them harbor the canonical p53 missense mutation/deletion combination (Liu et al., 2016).

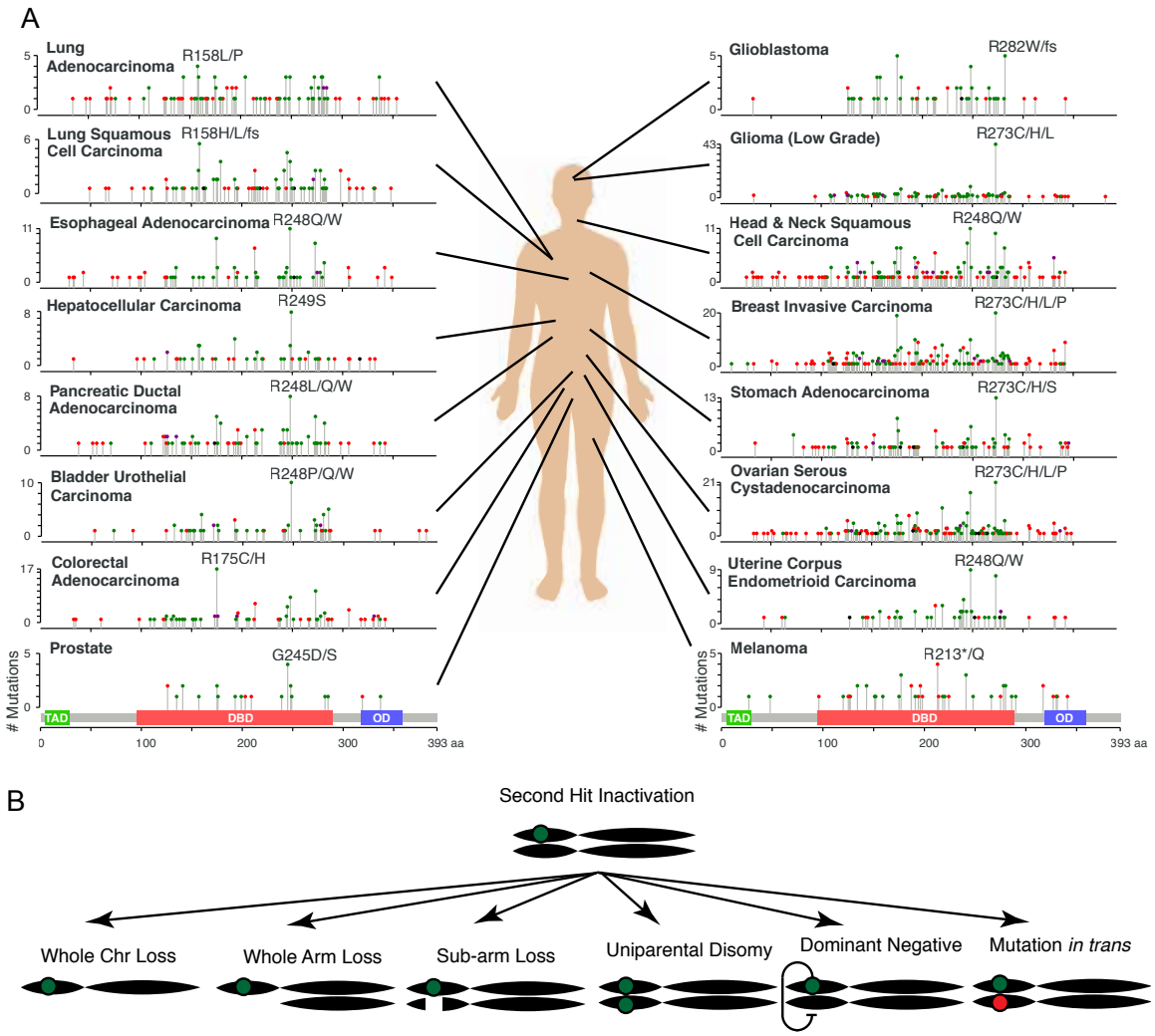

Figure 2.3. p53 alteration spectrum

(A) The *TP53* mutation distribution for 16 cancer types with sufficient available data and frequency of *TP53* alteration. Each histogram depicts the number of mutations found at each position along the p53 protein coding sequence, with the transactivation domain (TAD), DNA-binding domain (DBD), and oligomerization domain (OD) illustrated below. Symbol color indicates mutation type, including missense (green), nonsense (red), inframe indels (black), or multiple mutation types (purple). Data source: MSKCC cbio portal (Gao, Schultz, Sci Signal, 2013). (B) Multiple avenues to inactivating the second allele of *TP53*.

Cancer genome projects have also produced interesting insights into the spectrum of *TP53* mutation and its association with other somatic events. In some cancers, *TP53* mutations often co-occur with activating *KRAS* mutations or *MYC* amplification, an observation reminiscent of age-old functional studies demonstrating the ability of p53 loss to cooperate with oncogenes to transform primary cells. And, as mentioned earlier, *TP53* mutations are frequently associated with high rates of CNV, for example, as occurs in ovarian carcinoma and complex karyotype AML (Ciriello et al., 2013).

The extensive cataloging of *TP53* alterations in different settings allows one to consider whether distinct alterations reflect functional selection or simply different mutagenic processes present during tumorigenesis. Distinct mutational signatures in *TP53* and other genes can be attributed, in part, to the specific source of mutagenesis (Alexandrov et al., 2016). For instance, the R249S mutation prevalent in hepatocellular carcimoma arises from G-to-T transversions linked to aflatoxin exposure and R213* mutations in melanoma are associated with the C-to-T transition signature of UV mutagenesis (Alexandrov et al., 2016). Characterization of mutagenic signatures has revealed recurrent C-to-T mutation patterns attributed to cytidine deaminases, such as AID and APOBEC, which are an intrinsic source of mutagenesis with a physiological role in antibody diversification (Alexandrov et al., 2013). Curiously, APOBECs are a target gene that can in fact be activated by either wild type or mutant p53 (Menendez et al., 2017), though a clear link between p53 status and APOBEC-mediated mutagenesis has not yet been described (Burns et al., 2013; Shinmura et al., 2011). Still, while epidemiology and genome sequencing can implicate environmental or endogenous

mutagens as responsible for particular *TP53* mutations, it is difficult, if not impossible, to assess individual alleles without direct functional studies.

In fact, experimental data emerging over the last 25 years have hinted that certain mutant *TP53* alleles have "gain of function" properties that produce phenotypes distinct from the null. The most prominent phenotype produced by such mutant proteins is their ability to enhance invasion and metastasis, though in some settings particular mutants enhance drug resistance, epigenetic reprogramming or angiogenesis (reviewed in Aschauer and Muller, 2016). While proposed activities are diverse, an emerging "rule of thumb" is that tumor-derived p53 mutants oppose wild-type p53 functions or, more explicitly, exacerbate the consequences of p53 loss. In any case, the notion that not all p53 mutations are functionally equivalent is further supported by the fact that the onset and pathology of tumors in genetically engineered mouse models and in Li-Frameni patients varies by the type of mutant allele (Achatz and Zambetti, 2016; Olive et al., 2004; Xu et al., 2014).

A p53 mutant that has distinct phenotypes from the p53 null is not sufficient to define a mutant as "neomorphic." Theoretically, p53 mutant alleles may reflect attenuation of function, separation of function, or neomorphic function. Attenuation of wildtype function (**Figure 2.4A**) can produce hypomorphs that can also yield produce unpredictable and qualitatively different phenotypes depending on the level of p53 suppression (**Figure 2.4B**). For instance, a series of p53-targeting shRNAs with varying knockdown efficiency display different abilities to disrupt p53 effector functions and drive lymphomagenesis in mice, with only complete p53 deletion capable of instigating

chromosomal instability (Hemann et al., 2003). Loss of function is a common characteristic across all cancer-associated p53 mutants, given the failure of most mutants to induce apoptosis (Freed-Pastor and Prives, 2012). Separation of function – whereby a p53 mutant can retain some but not all interactions (reviewed in Muller and Vousden, 2014) – is also possible (**Figure 2.4C**), as exemplified by the aforementioned apoptosis-deficient $p53^{R175P}$ allele (Liu et al., 2004). Finally, neomorphic mutant activities (**Figure 2.4D**) have also been described (discussed below). The mutations encountered in cancer acquire some combination of these independent characteristics; thus, hotspot mutants undoubtedly reflect a combination of both attenuation of wildtype function and gain-of-function. Although p53 mutants are generally classified by their effect on structure -- i.e. those that perturb DNA binding and those that lose proper folding (modifying a key residue for folding or a zinc-coordinating site) -- we lack comprehensive data that allow for the unambiguous categorization of cancer- associated mutations by how exactly they impact function.

The diversity of proposed mechanisms by which mutant p53 alleles elicit their pro-oncogenic effects are a source of much confusion in the field (Aschauer and Muller, 2016). First, some p53 mutant proteins retain residual transactivation activity and activate novel targets. For instance, mutant p53 is proposed to impact chromatin states by inducing MLL1/2 and MOZ (Zhu et al., 2015). Second, certain unstructured p53 mutants sequester other proteins that, in some settings, enable mutant p53 to bind p63 or p73, leading to changes in transcriptional profiles that alter receptor tyrosine kinase signaling to promote invasion and metastasis (Muller et al., 2013; Weissmueller et al., 2014). Finally, in an instance of gain-of-function protein-protein interaction, mutant p53 can

cooperate with the SWI/SNF complex to upregulate the angiogenesis regulator VEGFR2 (Pfister et al., 2015). It remains difficult to reconcile how so many distinct yet selective protein-protein interactions can occur for disparate mutant proteins (reviewed in Freed-Pastor and Prives, 2012).

Although it is generally assumed that *TP53* truncating mutations are null alleles, there are emerging data that even these alleles can have neomorphic activity. Implying some selective advantage, the frequency of *TP53* nonsense mutations, particularly targeting exon 6, is greater than expected by chance (Shirole et al., 2016). At least some of these are not subject to nonsense-mediated decay, allowing certain truncated p53 mutants to promote invasion, metastasis, and sustain tumor maintenance in a manner that mirrors established gain of function missense mutants (**Figure 2.5)** (Shirole et al., 2016). Provocatively, exon 6 truncated proteins mimic the structure and function of a naturally-occurring p53 splice variant (p53psi) that promotes cell invasion and is transiently expressed during certain wound healing responses (Senturk et al., 2014), suggesting that these mutants may represent "separation of function" alleles. Expression or mimicry of alternative splice variants may contribute to the phenotype of other common mutations as well (Candeias et al., 2016).

Beyond the heterogeneity produced by different p53 SNVs, the variable extent of human chromosome 17p deletions can produce heterogeneity in the nature and number of p53-linked genes subject to reduced dosage during tumorigenesis. Loss of these neighboring genes could well reflect a "passenger" event of no functional consequence; however, 17p deletions observed in human cancer often include other genes now

functionally validated as tumor suppressors. Deletions engineered to be syntenic to 17p13 drive more aggressive cancers than simple p53 deficiency in mice by virtue of single copy loss of multiple haploinsufficient tumor suppressors, consistent with the negative prognostic association of 17p deletion independent of p53 mutation that is evident in AML (Liu et al., 2016). These observations and others underscore the unique biology underlying CNVs and highlight the importance of dissecting these understudied events (Tschaharganeh et al., 2016).

Collectively, our emerging understanding of the complexities of the gamut of*TP53* alterations is changing our views on how "the most frequent event in human cancer" promotes tumorigenesis. While there is little doubt that the most substantial biological consequence results from inactivation of p53, it is now clear that both *TP53* mutations and 17p deletions contribute phenotypes to cancer that go beyond p53 loss. Thus, as clinical decision-making in the future becomes increasing based on genomic data, the current classification of tumors as simply "p53 wild-type" or "p53 mutant" must be replaced.

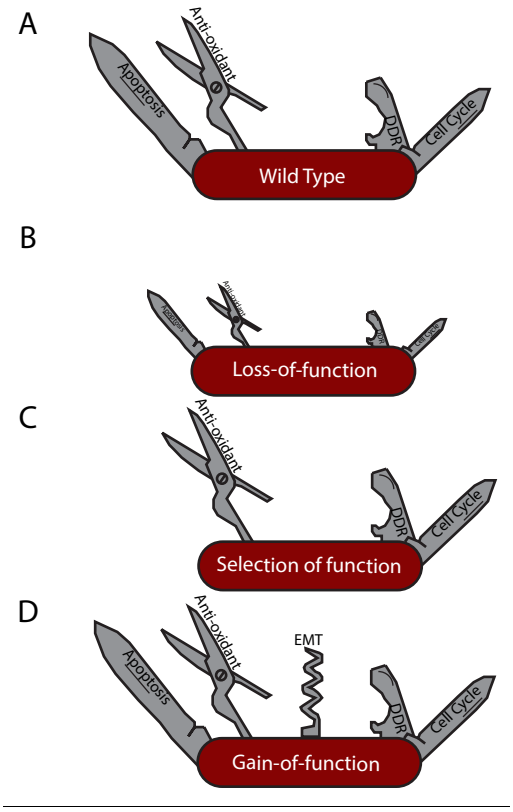

Figure 2.4. Mutant p53 gain-of-function

Several alternative mechanisms can lead to divergent phenotypes depending on p53 status, including (A) wild type, (B) loss or partial loss of function, (C) selection of function, or (D) neomorphic/gain-of-function.

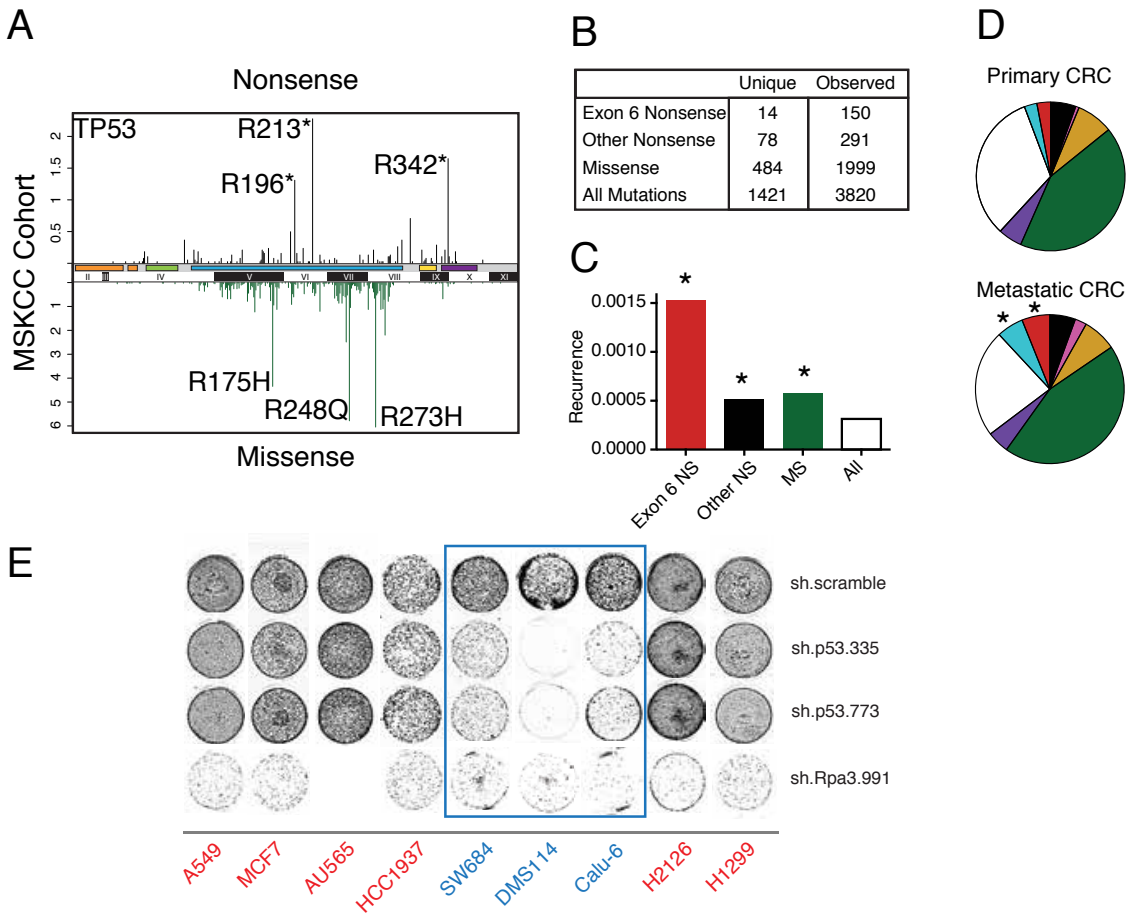

Figure 2.5 TP53 exon-6 truncating mutations produce gain-of-function.

(A) Distribution of *TP53* nonsense (top, black) and missense (bottom, green) mutations in the MSK-IMPACT cohort (n=8,557 tumors). Domains are demarcated on the upper baseline as follows: trans-activating domains (orange), proline rich domain (green), DNA binding domain (light blue), nuclear localization sequence (yellow), and oligomerization domain (purple). The lower baseline and Roman numerals below indicate *TP53* exon location relative to the p53 coding sequence. (B) Count of unique reported amino acid changes and observed instances of exon 6 nonsense, other nonsense, missense, or all mutations. (C) Frequency of recurrence of each mutation type per unique change per sample. (D) Relative frequency of *TP53* mutation type for colorectal cancer primary tumors (top left, n=403) and metastases (top right, n=395). Splice site mutations (Splice, light blue, p=0.035, Fisher's exact test), exon 6 nonsense mutation (Ex6 NS, red, p=0.041), other nonsense mutation (Other NS, black), inframe insertion/deletion (IF indel, pink), frameshift insertion/deletion (FS indel, gold), missense mutation (MS, green), multiple mutations (Multiple, dark blue), or no *TP53* mutation (None, white). (E) Crystal violet staining of the indicated cell lines upon p53 knockdown with two independent p53 shRNAs. A scramble shRNA was used as a negative control while shRNA targeting the essential gene *RPA3* was used as a positive control. Reproduced from (Shirole et al., 2016).

Revisiting the guardian of the genome

Upon DNA damage, p53 is activated to either promote the elimination or repair of damaged cells, ultimately reducing their risk of propagating mutations. DNA damage response (DDR) kinases phosphorylate p53, driving cell-cycle arrest, senescence, or apoptosis (reviewed in Williams and Schumacher, 2016). Additionally, p53 stimulates DNA repair by activating target genes that encode components of the DNA repair machinery, and p53-null cells are defective in certain DNA repair activities in vitro (Williams and Schumacher, 2016).

While *TP53* mutation can be correlated with patterns of single nucleotide variants and specific co-mutated genes, what is striking is that the association between *TP53* mutation and copy number variation (CNVs) is strong and universal across CNV-associated clusters of tumors in pan-cancer analysis (Ciriello et al., 2013). Also, cancers harboring *TP53* mutations are typically aneuploid, with gross changes in numbers of whole chromosomes (Ciriello et al., 2013). Various biological explanations for this association have been proposed, but one mechanism contributing to this relationship is the ability of p53 to regulate processes in G2/M transitions (reviewed in Vitre and Cleveland, 2012). For example, p53 loss dysregulates the spindle assembly checkpoint by derepressing MAD2, leading to an increased rate of chromosome missegregation and tetraploidization (Schvartzman et al., 2011). In the context of tetraploid cells, p53 loss leads to an increased rate of multipolar mitoses and subsequent chromosome missegregation (Vitale et al., 2010).

In an alternative but non-mutually exclusive explanation, p53 can restrict chromosomal instability through its ability to cull cells at risk of aberrant mitoses, particularly following centrosome amplification and/or telomere dysfunction (Dewhurst et al., 2014a; Eischen, 2016; Lanni and Jacks, 1998). Extra centrosomes lead to Hippo pathway up-regulation that, in turn, activates p53 by inhibiting MDM2 (Aylon et al., 2006; Ganem et al., 2014). It remains counterintuitive that the human body contains physiologically normal polyploid cells, including tissues that rely on p53 for tumor suppression. Naturally occurring tetraploid hepatocytes exhibit reduced but not abolished proliferation (Ganem et al., 2014)and isolates of rare tetraploid cells from a colon cancer cell line proliferate with genetically intact p53 (Dewhurst et al., 2014b). Additional studies suggest that p53-deficient cells are better at tolerating proteomic (or other) stress produced by aberrant gene dosage (Tang et al., 2011). Others suggest that p53-mediated culling of aneuploid cells is more efficient against structural aneuploidy than whole chromosome imbalances, implicating the role of DDR in response to chromosome shearing (Santaguida et al., 2017; Soto et al., 2017). Accordingly, *TP53* mutations are also associated with whole genome doubling events in human tumors (Cancer Genome Atlas Research, 2013). Hence, it appears that the absence of p53 both facilitates the emergence and permits the survival of aneuploid cells.

p53 also appears to suppress a particular type of chromosome shattering and rearrangement event known as chromothripsis. Cells that bypass replicative senescence after p53 and RB inactivation can proliferate despite telomere erosion (Hayashi et al., 2012). Failing this checkpoint, telomere dysfunction initiates chromosome breakage-fusion-bridge cycles that contribute to chromothripsis (Maciejowski et al., 2015).

Although the extent to which chromothripsis fosters tumorigenesis remains an open question, the phenomenon is significantly more prevalent in tumors harboring *TP53* mutations (Rausch et al., 2012).

An unanticipated way in which p53 helps maintain genomic integrity is by suppressing retrotransposons, which are latent virus-derived genomic elements whose aberrant expression can lead to mutagenesis through their mobilization and re-insertion throughout the genome (reviewed in Levine et al., 2016). Experimental activation of mobile elements in drosophila induces DNA double strand breaks and p53-mediated apoptosis (Wylie et al., 2014) that could, in principle, reduce their mutagenic effects. However, recent evidence demonstrates that the association between p53 mutation and retrotransposon expression is more than simply a culling effect: indeed, p53 binding to target sites within LINE elements and other transposon sequences are associated with their downregulation (Chang et al., 2007). p53-mediated repression is dependent on epigenetic silencing of retrotransposon loci and not apoptosis, and derepressed retrotransposons are competent for reintegration into the genome (Leonova et al., 2013; Wylie et al., 2016), promoting mutagenesis (Tubio et al., 2014). Genomic analyses have revealed that retrotransposon mobilization is common in human cancers (Ting et al., 2011; Tubio et al., 2014). While the precise impact remains to be determined, there is a significant association between repetitive element expression and p53 status in mouse and human tumors (Wylie et al., 2016).

The immediacy with which p53 cooperates with oncogenes to transform cells indicates that genomic instability is not absolutely required for tumor initiation (Lowe et

al., 1994). Still, the genomic instability fueled by p53 loss enables acquisition of additional driver events with the potential to accelerate transformation, metastasis and drug resistance (reviewed in McGranahan and Swanton, 2017). Just as species diversity in an ecosystem is associated with its robustness, subclonal diversity, not the total number of mutations in a tumor, dictates the resilience of a cancer cell population to changing conditions and challenges. In this regard, p53 inactivation may be unique in its ability to both promote genomic instability (by increasing the rate of new variants) and permit the survival of a wider pool of genetic configurations (decreasing the likelihood of extinction of variants). Together, these observations raise the possibility that p53 inactivation contributes to intratumoral heterogeneity.

Heterogeneity and tumor evolution

We are interested in better understanding factors that influence the extent and nature of intratumoral heterogeneity, and how diversity affects the robustness of a population. Our hypothesis is that p53 deficiency tends to permit the accumulation of greater subclonal diversity than in equivalent p53 wild type cancer cell populations.

The high complexity of mixed genotypes and genetically distinct tumor regions in solid tumors (Gerlinger et al.; Navin et al., 2011; Yachida et al.) suggest that tumors can progress as polyclonal entities with neighboring cells undergoing parallel evolution. The importance of tumor heterogeneity has long been acknowledged (Greaves and Maley, 2012; Nowell, 1976), but most studies have been limited in scope, primarily due to the inadequacies of the existing technologies.

Predicted model of heterogeneity:

Alterations that promote instability may contribute to heterogeneity, but also could increase the rate of "jackpot" clones by randomly generating a clone with elevated fitness. Alterations that promote survival may contribute to increased representation over generations of cell divisions, but have minimal genetic drift per generation. TP53 alteration may incorporate elements of both, thus promoting intratumoral heterogeneity (**Figure 2.6**).

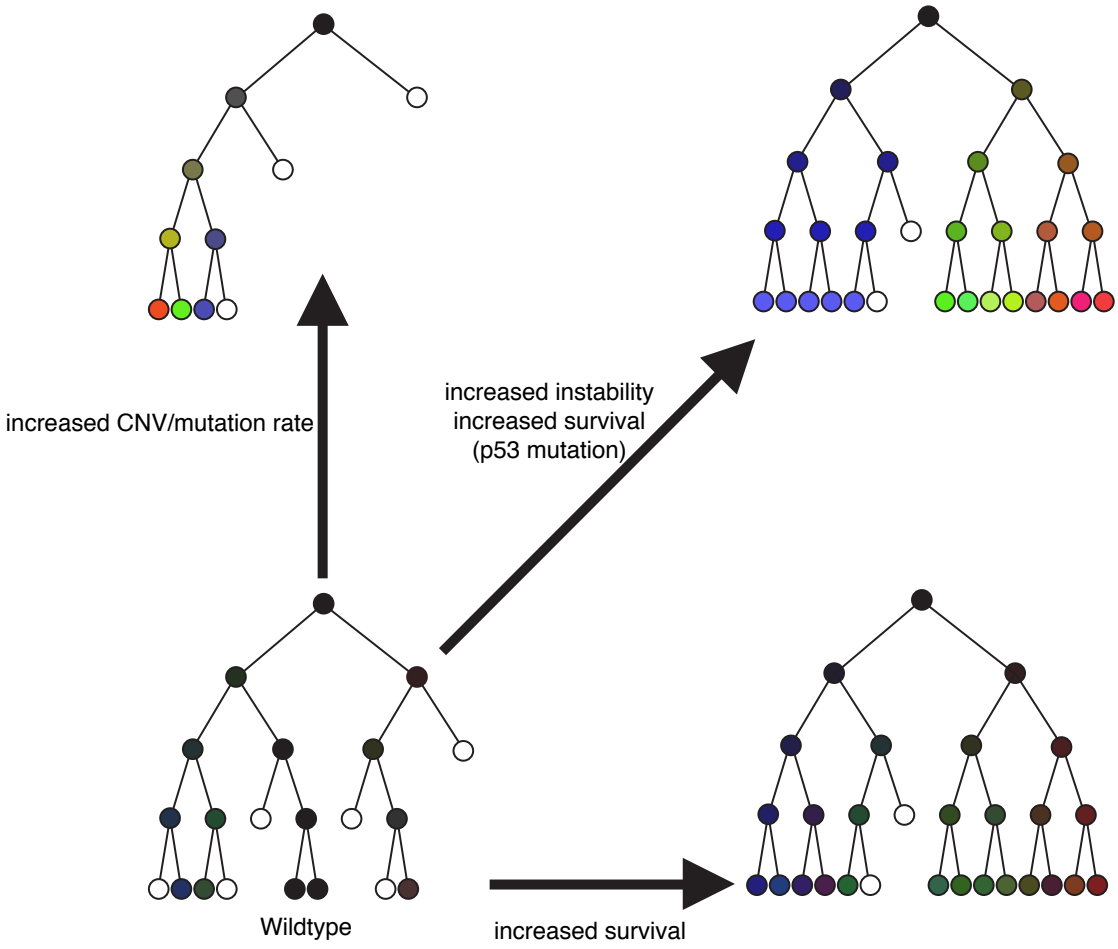

Figure 2.6. Predicted model of heterogeneity.

Alterations that promote instability (e.g. MAD2) may contribute to heterogeneity, but also likely increase the rate of "jackpot" clones. Alterations that promote survival (e.g. BCL2) may contribute to increased representation over generations of cell divisions, but have minimal genetic drift per generation. TP53 alteration may incorporate elements of both, thus promoting intratumoral heterogeneity.

Eμ-myc Lymphoma

The Eμ-myc model of lymphoma is a transgenic mouse model in which expression of the oncogene Myc is driven by an immunoglobulin enhancer, leading to enforced overexpression in all immature B cells in the organism. This model is not an ideal representation of human B-cell lymphomas, but it is one of the best-characterized mouse models of cancer (Adams et al., 1985) and has successfully allowed dissection of gene function *in vivo* in countless studies over the last 30 years. Mouse models can recapitulate the instability and clonal evolution observed in human tumors. Induction of chromosomal instability can also support recurrence of genetic oncogene withdrawal in mouse models of cancer (Sotillo et al.). The p53 pathway plays an important role to block Eμ-myc tumorigenesis. Myc overexpression will activate p19/ARF, leading to p53-dependent apoptosis. It has been shown that there is a high frequency of somatic p19 or p53 deletion in established disease (Eischen et al., 1999). Breeding Eμ-myc transgenic mice to p19 or p53 knockout mice rapidly accelerates disease progression. In many ways, p53 deficiency can be substituted by p19/ARF deficiency. However, p53 activation occurs can be stimulus-dependent: for example, oncogenic signaling induces the p19/ARF tumor suppressor to inhibit MDM2 (inducing a p53 response), whereas DNA damage promotes p53 phosphorylation and activation, independent of p19/ARF. Does p53 loss allow the outgrowth of a single aneuploid "jackpot" clone? Alternatively, does it allow the long-term survival of a wider field of sub-optimally fit clones, which could serve a diverse reservoir of potential resistance to changing conditions, as in metastasis and treatment?

Materials and Methods

Clonal analysis of TCGA data

Estimations of distinct subclones was extracted from Andor et al (Andor et al., 2016) and Raynaud et al (Raynaud et al., 2018), which were calculated as described previously. Briefly, the *EXPANDS* algorithm works by clustering the variant allele frequencies of somatic mutations to perform cell frequency estimation. These are filtered and individual mutations are assigned to clusters of subclones. The approach by Raynaud et al works by a similar concept, where cell purity estimation and copy number correction allow for co-clustering of mutations of different zygosity into the same cluster. Furthermore Raynuad et el estimate a phylogenetic relationship between the various estimated subclones. Linear modeling was performed using *R* version 3.4.0 (http://cran.r-project.org).

Mouse model of lymphoma

As mentioned above, Eμ-myc mice develop spontaneous lymphomas that are accelerated by crossing with p19 or p53 knockout mice. This yields progeny that are Eμ-myc;$p19^{+/-}$ or Eμ-myc;$p53^{+/-}$. The remaining wild type allele of the indicated tumor suppressor gene is typically lost though LOH. Alternatively, we have also generated equivalent disease by breeding Eμ-myc; vav-cre; $p53^{loxP/+}$ mice, where p53 deletion is restricted to B cells, reducing complications associated with the shortened life span of $p53^{+/-}$ breeders.

Mouse model of hepatocellular carcinoma

Tumors were generated by hydrodynamic injection of Myc-expressing Sleeping Beauty transposons as described in **Chapter I**. In this case recipient mice were either $p19^{-/-}$ or $p53^{-/-}$. Livers of moribund mice were harvested, minced, and enzymatically dissociated in Collagenase/Dispase solution (37°C, 30 min, shaking 250 rpm). HCC cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1 mM l-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin.

Cell culture

Murine B cell lymphoma lines were generated by mechanically dissociating the spleens of moribund lymphoma-bearing animals and passing through a 100um cell strainer. Single cell suspensions were centrifuged (5 min, 4°C, 300g) and resuspended in 1mL ACK buffer for red blood cell lysis for 1 minute, then washed in media. Cells were cultured in 50:50 DMEM:IMDM supplemented with 10% FBS, 50 μm β-mercaptoethanol, 2 mM l-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. The cells grow in suspension over a layer of irradiated feeder cells. We used S17 bone marrow stromal cells irradiated at 25 Gy.

Single cell RNA-sequencing

We utilized 10x genomics single cell RNA sequencing technology (Zheng et al., 2017). A microfluidic chip is used to co-capture single cells, enzymes, and oligo-containing gel beads in oil droplets. Each bead contains primers, adaptors, and unique indentifiers used to oligo-dT prime the 3' end of mRNA molecules. This material is reverse transcribed and amplified such that each read can be assigned to the individual cell and molecule from which it came.

Single cell sequencing data is typically affected by a high degree of noise, which could be both technical and biological. We normalize the expression data for each cell to the median library size (van Dijk et al., 2018). This removes substantial cell-to-cell variation in the number of molecules identified associated with inefficient mRNA capture and other technical challenges (van Dijk et al., 2018). The normalized data is log-transformed adding a pseudocount of 0.1.

We remove cells with low molecular counts, since low capture efficiency and insufficient sampling will like introduce additional noise and possibly distort the data and interpretation. For this, we remove the lower mode from the distribution of library size (**Figure 2.7A**). Likewise, we attempt to reduce noise by removing lowly expressed and inconsistently captured genes, excluding genes that are expressed in less than 64 cells (**Figure 2.7B**).

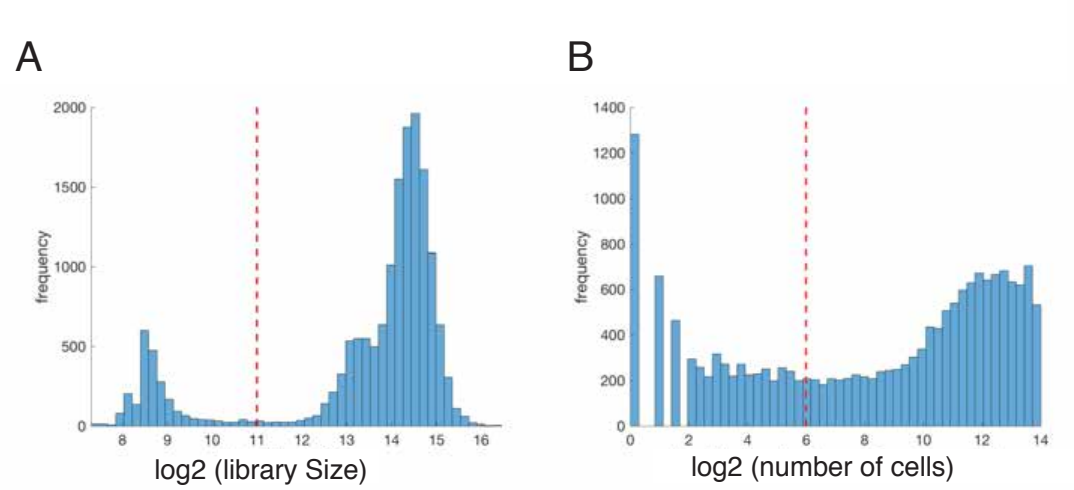

Figure 2.7. Single cell RNA-seq data pre-processing.

(A) Cells with less than 2048 read counts are removed from scRNAseq data.

(B) Genes expressed in less than 64 cells are removed from scRNAseq data.

In order to quantify the entropy, we clustered the combined data using PhenoGraph (Levine et al., 2015), which is a well-established and robust clustering method especially suited for single cell data analysis (Paul et al., 2015; Shekhar et al., 2016; Wei et al., 2017). The parameter *k* used in this approach defines how may nearest neighbors are grouped together into neighborhoods in the initial step of the process, where these constitute a functional unit to map a weighted graph. Ultimately categorical bins of similar cells are generated based on the structure of the data.

Following this, we compute the sample specific entropy (H) as follows:

$$H(j) = -\sum_{i} p_{i,j} \log\left(p_{i,j}\right),$$

where,

$$p_{i,j} = \frac{number\ of\ cells\ in\ cluster\ i\ belonging\ to\ sample\ j}{number\ of\ cells\ in\ sample\ j}$$

We also sought to understand the phenotypic volume occupied by each sample. One approach would be to consider the determinant of the covariance matrix of the data. Given genes $\{\vec{g}_1, \vec{g}_2, \ldots, \vec{g}_m\}$, the $(i,j)^{th}$ entry of the covariance matrix C is given by $\left(\vec{g}_i \cdot \vec{g}_j\right)$, where $\cdot$ indicates the dot product of the two vectors. Intuitively, the determinant $\det(C)$ gives a measure of variability in the data; a larger determinant should indicate more dispersed data. $\det(C)$ is computed from the product of all eigenvalues of C. While the eigenvectors of C give the directions of maximal variance in the data, the

eigenvalues quantify the variance along the corresponding eigenvector. Therefore, the larger an eigenvalue, the more the dispersed the data is along the corresponding eigenvector. By computing the product of the eigenvalues, we effectively compute the volume occupied by the data in the phenotypic space. We note that in the context of biological data sets where we expect co-regulation of many genes, the covariance matrix will be singular (that is will have determinant 0, that is at least one eigenvalue will be 0). This is a direct result of the interdependencies between the genes. To circumvent this, we compute the pseudo-determinant, which is the product of only the non-zero eigenvalues. We also wanted to avoid over estimating volume by contributing empty space within the boundaries of the expression data of a given sample. To address this we calculate volume on individual clusters first and then take the sum of the volume of each cluster as a representative of the volume of the whole data.

Metaphase Spreads

Cultured cells were treated with 100ng/ml Nocadazole for 1hr 15min. Cells were washed in PBS and resuspended in a residual 200ul of PBS. Then, 8-10mL of .56% KCl and10mM HEPES was added dropwise while vortexing on 2/10 speed. After 6 min incubation in 37°C water bath, cells were centrifuged (800 rpm, 5 min, 4°C) and resuspended in a residual 200ul. Carnoy Solution (3:1 methanol:acetic acid, 5-8 mL) was added dropwise while vortexing on 2/10 speed. Cells were centrifuged (800 rpm, 5 min, 4°C) and the procedure was repeated with 2 mL Carnoy Solution. Cells are resuspended in 100-200ul Carnoy plus 1 ug/ml DAPI and incubated on ice 5 minutes. Per slide, 50uL of cell suspension is dropped onto steamed slides (over 65°C water bath) from

approximately 50cm in height. Slides are dried and coverslipped. Images were acquired on a Zeiss Axioscope Imager Z.1 and analyzed using the *Cell Counter* tool in ImageJ.

Lymphoma Transplants

Lymphoma cells were washed twice in PBS and injected in 200ul PBS into the tail vein of syngeneic C57/BL6 recipient mice (Envigo). One million cells per recipient was used unless otherwise indicated.

Results

Deficiency of p53 permits greater intratumoral phenotypic diversity

We investigated intratumoral heterogeneity in murine primary Eμ-Myc lymphoma cell lines that were either p19-deficient or p53-deficient. Single cell RNA sequencing (scRNAseq) was selected as a tool that allows for comprehensive characterization of single cells, yielding an understanding of population structure and diversity. Accumulation of genetic alterations (by tolerance of DNA damage and aneuploidy) and non-genetic variation (such as epigenetic and lineage plasticity) have been linked to p53. We expected the genetic drift, widespread epigenetic modification, and changes in cell state would all have consequences on individual cellular transcriptional profiles. Single cell resolution is essential to examine not just the abnormalities at the end of malignant transformation, but also the population-level diversity of those abnormalities maintained following tumorigenesis. Thus, we expected that measuring transcriptional heterogeneity by scRNAseq would enable us to assess the effect of p53 pathway alterations on intratumoral diversity in cancer cell populations.

High dimensional scRNAseq data can be visualized effectively on t-distributed stochastic neighbor embedding (t-SNE) plots, as in **Figure 2.8A**, whereby high dimension data can be projected non-linearly into a low-dimensional (2D) space such that similar points are depicted as closer neighbors than dissimilar points. All four cell lines analyzed were distinctly well-separated in t-SNE projections (**Figure 2.8A**). Normal tissues and non-cancer cells in the tumor microenvironment, even if highly dysregulated, tend to co-cluster by cell type when samples from multiple patients are merged (Azizi et

al., 2018; Filbin et al., 2018). On the other hand, previous scRNAseq studies of cohorts of human tumors have consistently revealed that independent tumors generally diverge significantly and do not commonly occupy common transcriptional clusters or trajectories (Azizi et al., 2018; Filbin et al., 2018). By cursory visual inspection, p53-null lines appear to be more diffuse in their distribution on t-SNE projections. This can be potentially misleading, so we pursued a number of strategies to quantitatively measure intra-sample heterogeneity.

We sought to quantify this diversity by computing the entropy of each sample. Higher entropy indicates that there is a more intratumoral transcriptional heterogeneity in a given sample, which would indicate that the sample is more diverse, at least by molecular phenotype. First we clustered the dataset using (Levine et al., 2015), which is robust clustering method well-suited for single cell data analysis (Paul et al., 2015; Shekhar et al., 2016; Wei et al., 2017). A number of interesting patterns are revealed by this approach (**Figure 2.8B**). Most (16/18, 88.9%) of clusters are comprised of cells from only one sample. The p53-null samples contain members of a greater number of distinct clusters than their p19-null counterparts (6-10 vs. 4, respectively, see **Figure 2.8C**).

Two hypothetical populations with the same number of subtypes could contain very different levels of diversity. For example, a *population A* contains 1000 individuals, divided equally amongst 10 subgroups with 100 members each. Another *population B* contains 1000 individuals of which 991 individuals were similar and constituted a single subgroup and the remaining 9 individuals were each unique and are classified as 9 distinct single-member subgroups. Although each population has 10 subgroups,

population A can be considered well-mixed and *population B* is nearly a monopoly. From the standpoint of ecology, species diversity can be informative about the robustness of an ecosystem (Estrada, 2007). Understanding of diversity in population structures is generalizable to many fields; in economics, the diversity of a marketplace or a national economy can be indicative of its robustness to changing conditions. Such determinations require information about the frequency of membership in each subclass and not only a quantification of subclasses.

One broadly applied method is the use of Shannon's diversity index, also known as Shannon entropy (Magurran, 2005). For example, multi-color fluorescent in situ hybridization (FISH) has been used to define copy number-based classes of single cells in breast cancer samples in order to measure the extent of genetic intratumoral heterogeneity (Maley et al., 2006; Park et al., 2010). Based on the number of cells assigned to each PhenoGraph cluster, we calculated the entropy of each sample (see methods). This approach confirmed that p53-null samples were significantly more diverse than p19-null samples (**Figure 2.8D**). We ensured that the results are robust to changing the input parameter 'k' to PhenoGraph (data not shown, see methods). To rule out effects of sample size, we confirmed the result by subsampling a random 1500 or 2000 cells from each sample (data not shown).

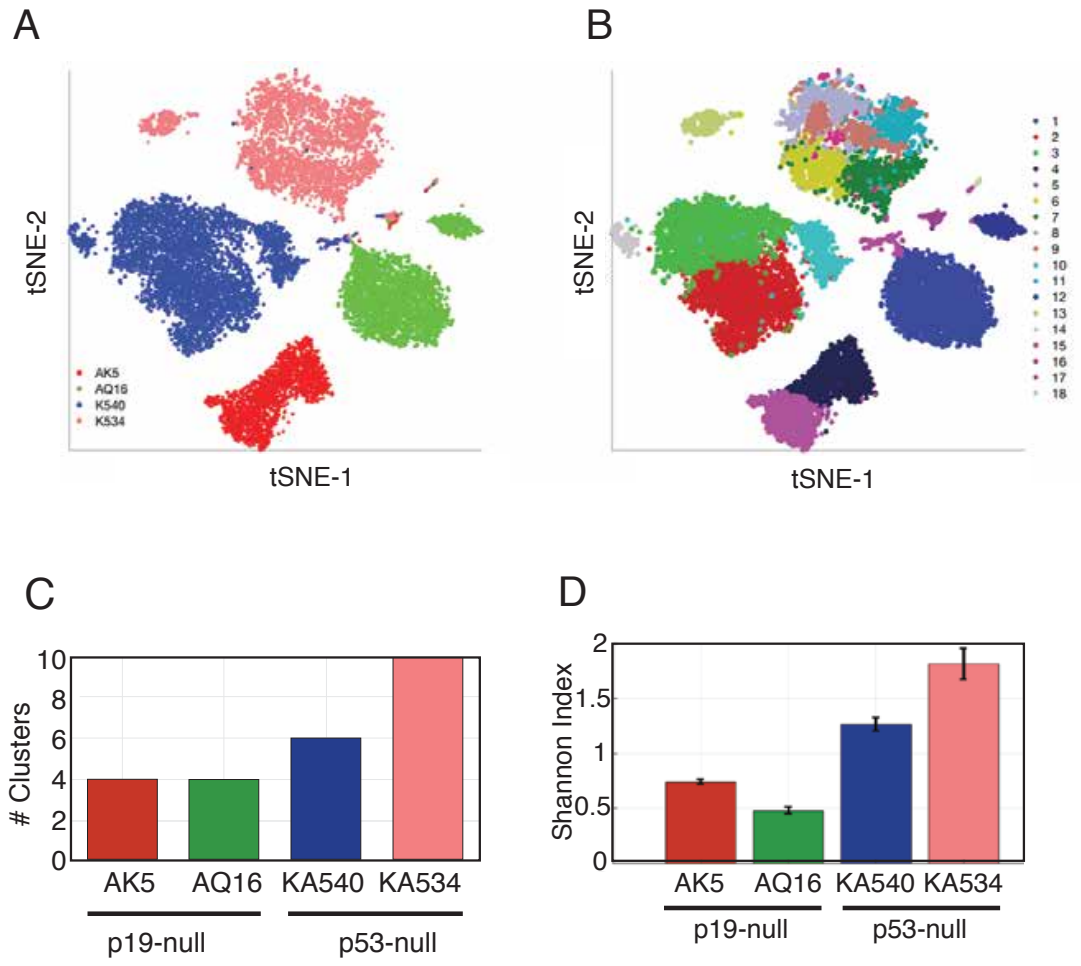

Figure 2.8. Clustering Analysis

(A) tSNE plots of scRNAseq data. (B) PhenoGraph Clustering (C) Number of distinct PhenoGraph clusters for which each sample contains members (D) Entropy of each sample for 50 random iterations of PhenoGraph. * indicates p-value of two-sided U-test (*: p-value <= 0.05, **: p-value <= 0.01, ***: p-value <= 0.0001).

The preceding analyses of intratumoral heterogeneity in scRNAseq data rely on the construction of discrete subclasses. The cell clusters in murine lymphoma data are relatively less divergent from one another than some other existing scRNAseq datasets, such as that of the distinct cell types of the normal hematopoietic system. Misannotation or the annotation of cells into nearby adjacent clusters unstable with changes in parameters of the analysis could be problematic. We demonstrated that the result is robust to changing parameter in the clustering algorithm and subsampling the number cells per line (see above). Nevertheless, we reasoned that relaxing the requirement for discrete categories might allow for a more robust, or at least an independent, method of measuring intratumoral heterogeneity. Therefore, we developed a metric herein referred to as phenotypic volume. Briefly, the determinant of the covariance matrix is used to condense single cell expression data into a single measure of dispersion. Unoccupied space is excluded from introducing artifactual variation by calculating the volume of non-adjacent regions individually (see methods). Confirming our results using PhenoGraph clustering and Shannon entropy, this approach demonstrated that p53-null samples occupy greater phenotypic volume than p19-null samples (**Figure 2.9A**). Again, these results are robust to changing the parameter *k* of PhenoGraph and to subsampling 1500 or 2000 cells per sample (**Figure 2.9B**). Altogether this provides evidence that p53 restrains intratumoral heterogeneity of transcriptional output. Even when compared to the effect of attenuating the p53 response to oncogenic and replicative stress by deficiency in p19, deletion of p53 allows for the maintenance of a more divergent set of abnormal cell states.

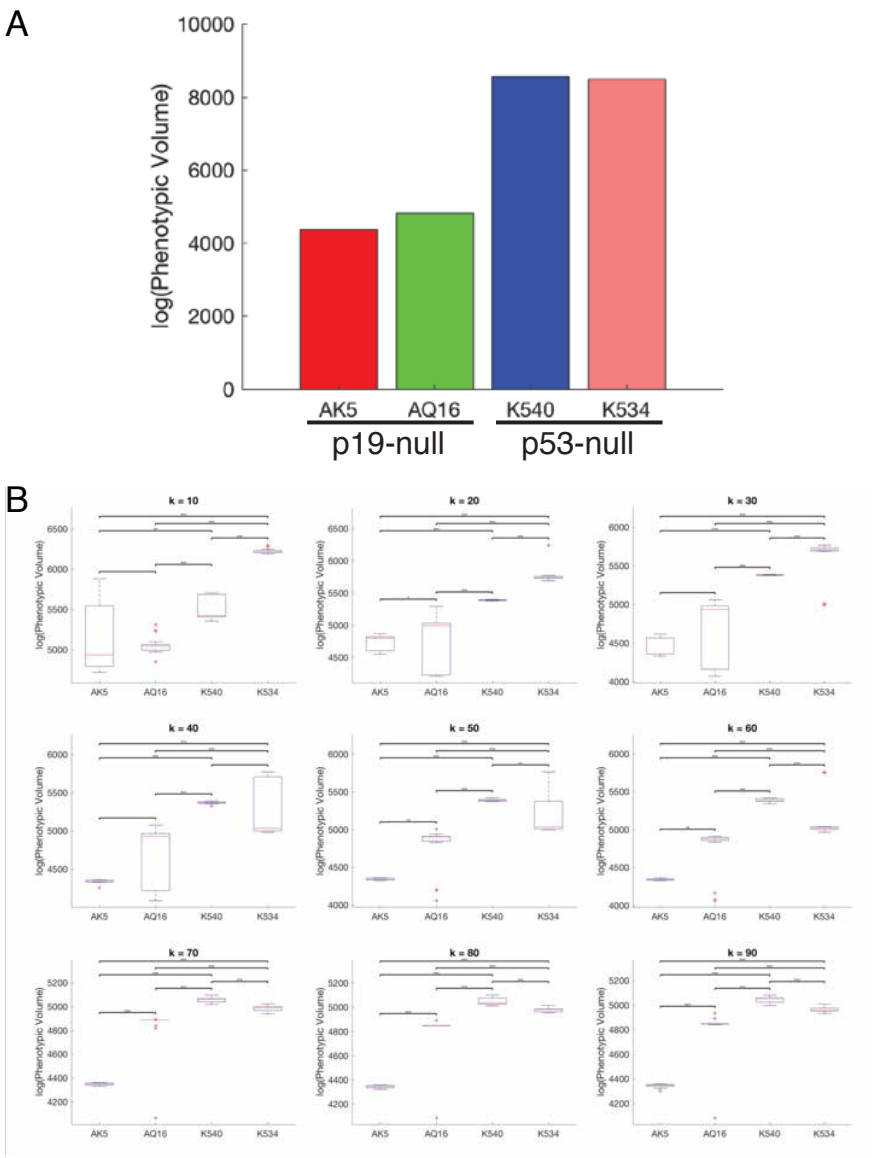

Figure 2.9. Phenotypic volume

(A) log(Phenotypic Volume) occupied by each sample (parameter *k*=30). (B) The procedure is repeated after randomly subsampling 2000 cells from each sample and varying parameter *k*, which adjusts the granularity of individual regions whose volumes are summed.

CIN and heterogeneity

Given the numerous characterized sources of genetic instability and non-genetic plasticity attributable to p53 loss, we were interested in exploring what types of abnormalities were contributing to cellular diversity. Classically, p53 loss is associated with chromosomal instability (CIN) by permitting tolerance of aneuploid states that would otherwise lead to arrest or death (Santaguida et al., 2017). In short term experiments, p53 deficiency allows for isolated clones to acquire copy number alterations in a semi-random manner (Cahill et al., 1999). The question remains if this typically leads the emergence and selection of one (or few) dominant aneuploid clone(s), which occasionally are overtaken and outcompeted by a newly emergent, randomly occurring aneuploid clone with a selective fitness advantage (Bozic et al., 2010). Alternatively, p53 nullizygosity could permit meaningful intratumoral heterogeneity, where the population evolves as a dynamic polyclonal mixture of multiple configurations of aneuploid clones.

To address whether p53-associated CIN would manifest in intratumoral heterogeneity in this setting, we examined counted chromosomes at metaphase from a panel of murine lymphoma lines. Primary Eμ-Myc lymphoma cell lines that were p19 deficient were euploid with very little variation. Primary Eμ-Myc lymphoma cell lines that were p53 deficient were not only aneuploid, but contained a polyclonal mixture of karyotypic states (**Figure 2.10A**). To address the question of whether these populations are vulnerable to clonal sweeps by a dominant clone, we transplanted the cells to syngeneic recipient mice, re-established the cells *in vitro* and examined the resulting populations. Lines starting from an aneuploid and diverse p53-null parental line

maintained representation of diverse aneuploid states, while p19-null lines remained chromosomally stable following serial transplant (**Figure 2.10B**). The transcriptional output and cell fate consequences of p53 are context-dependent (Kastenhuber and Lowe, 2017), so we examined primary cell lines from another cell type of origin, namely Myc-driven hepatocellular carcinoma in both p19- and p53- deficient backgrounds. With the same initial drivers, hepatocellular lines showed the same pattern as lymphoma lines: p19-null HCC remain diploid with minimal variability and p53-null HCC is both aneuploid and diverse (**Figure 2.10C**).

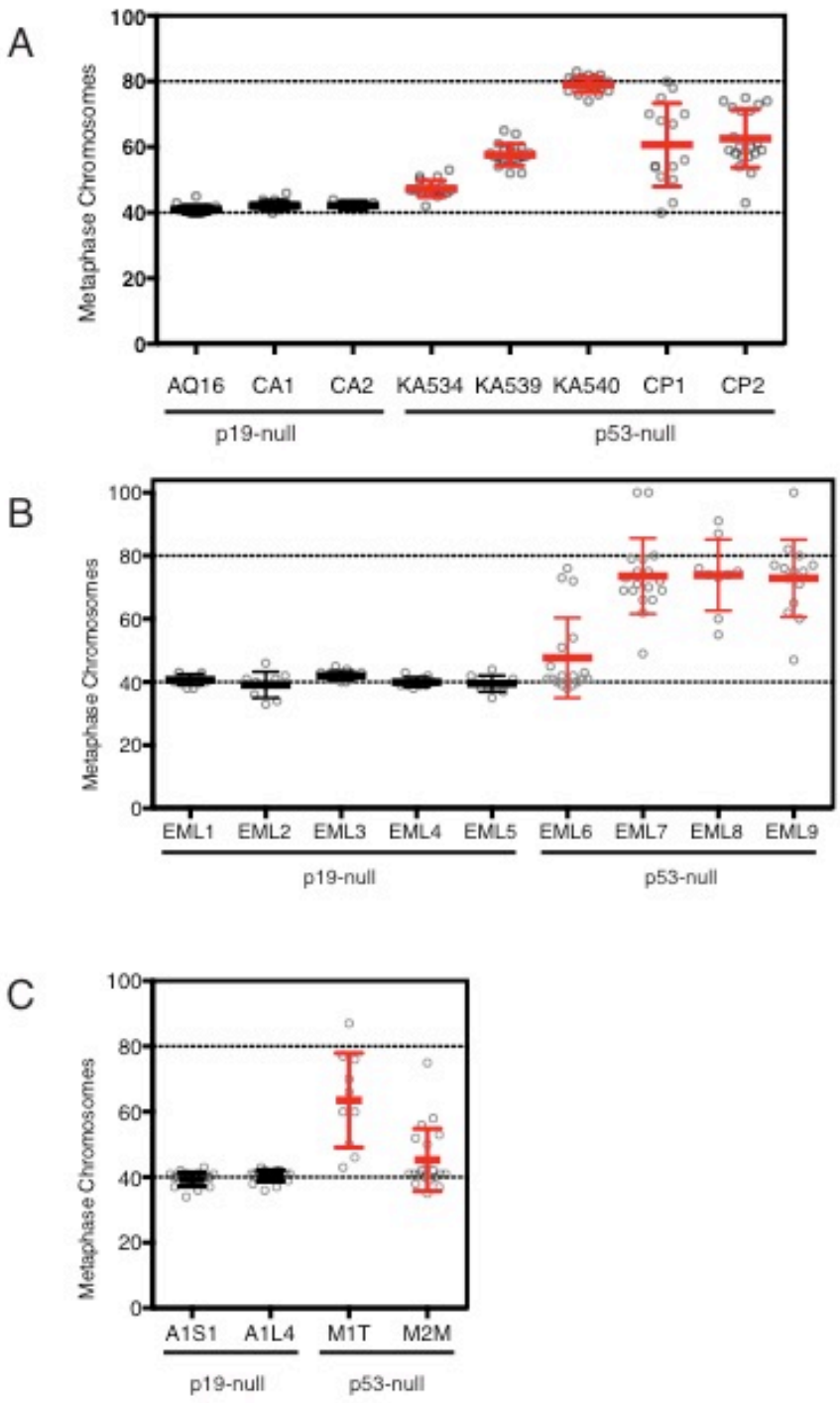

Figure 2.10. Karyotypic diversity in murine lymphoma is associated with p53 status

Chromosome count at metaphase for (A) Primary Eμ-Myc lymphoma cell lines (B) Eμ-Myc lymphoma cells following secondary transplant to syngeneic recipients (C) Myc-driven p19-null and p53-null murine HCC cell lines.

Differentiation state heterogeneity

Another key phenotype of p53 is its ability to reinforce differentiation and to limit cellular plasticity (Mizuno et al., 2010; Tschaharganeh et al., 2014). We interrogated the possibility that a stable differentiation hierarchy in B cell lymphoma populations is a source of transcriptional heterogeneity. Many malignancies have been described as having an intrinsic hierarchy where multipotency and tumor initiating capacity are characteristics that are enhanced in a subpopulation of cancer cells, sometimes referred to as cancer stem cells (Bonnet and Dick, 1997). In this model, a minority of cells can both self renew and can divide asymmetrically, giving rise to progeny that are less likely to propagate indefinitely. Even in a model without a well-defined class of tumor initiating cells with more stem-like characteristics, one could envision that heterogeneity in cell maturation state could be present or that a pathogenic epigenetic state could yield lower resistance to dedifferentiation or transdifferentiation. Thus, we aimed to evaluate if heterogeneous differentiation states could be observed in B cell lymphoma and if this was effected by p53 status.

The Immunological Genome Consortium (ImmGen) has generated bulk RNAseq data collected from various sorted immune cell populations. We utilized this data to extract prototypes of B cell maturation states (BioProject PRJNA429735/GEO GSE109125) from $Lin^{-}AA4^{+}Kit^{+}IL7Ra^{+}B220^{-}$ Common Lymphoid Progenitor (CLPs) to more mature $CD19^{+}$ $B220^{+}$ IgM^{+} $AA4^{-}$ $CD23^{+}$ follicular or $CD19^{+}$ $B220^{+}$ IgM^{+} $AA4^{-}$ CD23- $CD21/35^{+}$ marginal zone B cells (**Figure 2.11A)**. Twelve B cell stage references were plotted by principle component analysis (PCA) to reduce dimensionality (**Figure**

2.11A-B). Since scRNAseq data is sparse and generates a matrix containing many zero values, we decided to use clusters of single cells to assess B cell stage (see methods). We then mapped the centroid of each cluster onto the established principle component space (**Figure 2.11B**). When this procedure is repeated for each sample, all clusters closely resemble $AA4^{+}IgM^{-}CD19^{+}CD43^{+}HSA^{+}$ Fraction B/C pro-B cells.

To quantify the similarity of the lymphoma clusters to B cell stages, we simply took the Spearman correlation of each clusters expression values to the signature of each B cell stage reference. All clusters in all cell lines most closely correlated to pro-B cells (**Figure 2.11B**).

Again, we wanted to consider the possibility that this approach might mask important intra-cluster variability, so we took a complementary approach by imputing the scRNAseq data in order to reduce the sparseness of the data while minimizing information loss (van Dijk et al., 2018). When comparing imputed single cell data to B cell stage references, the highest correlation was again to pro-B cells with no detectable subpopulations matching an alternative differentiation state (**Figure 2.12**). In sum, there is no appreciable heterogeneity of lymphoma cells in differentiation space.

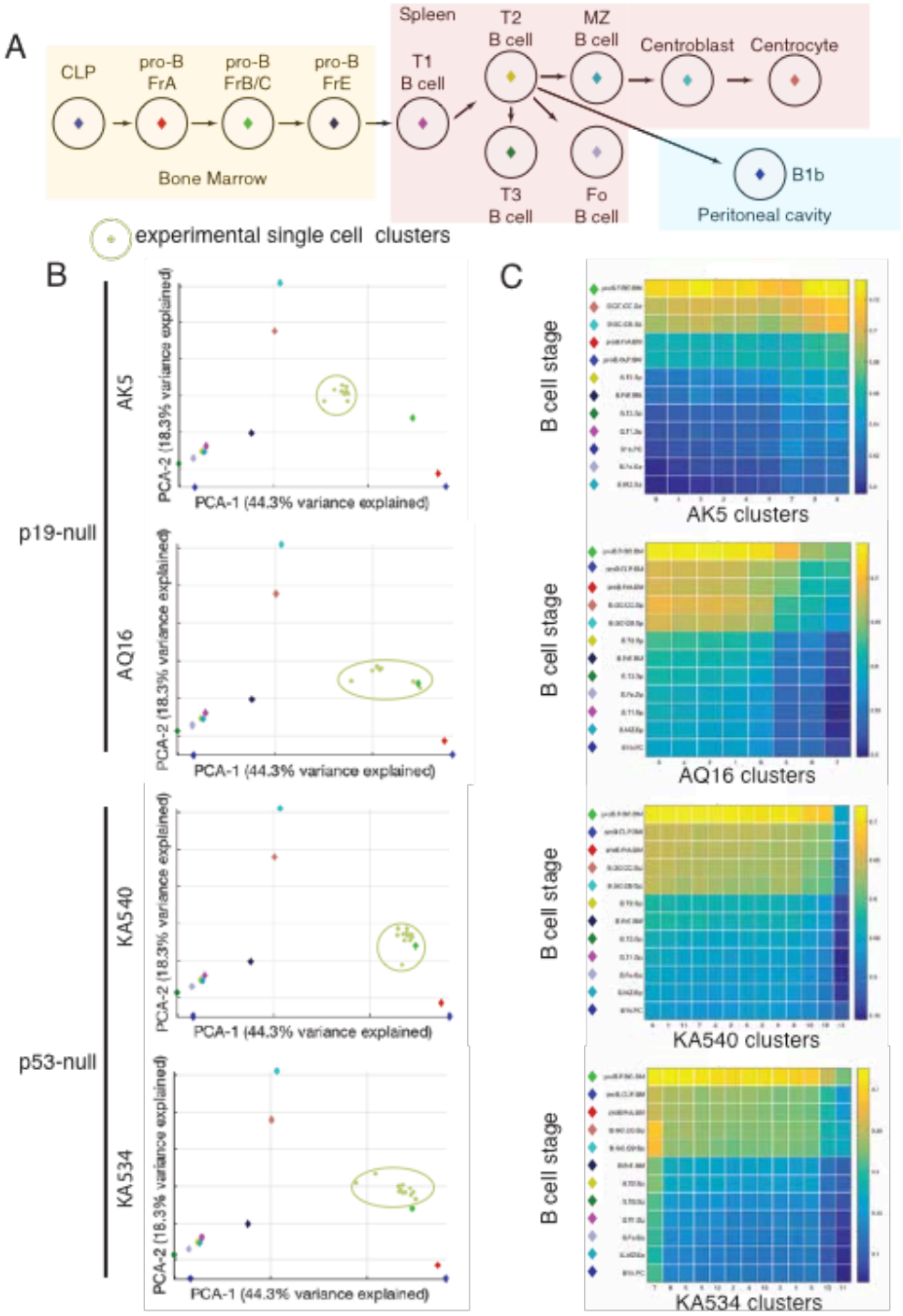

Figure 2.11 transcriptional heterogeneity and differentiation state (cluster level)

(A) Schematic of stages of B cell differentiation (Nagasawa, 2006; Samitas et al., 2010). (B) Clusters of experimental cells from the indicated sample superimposed on a PCA map of prototypical B cell stages. (C) Expression correlation of experimental single cell clusters with prototypical B cell stages.

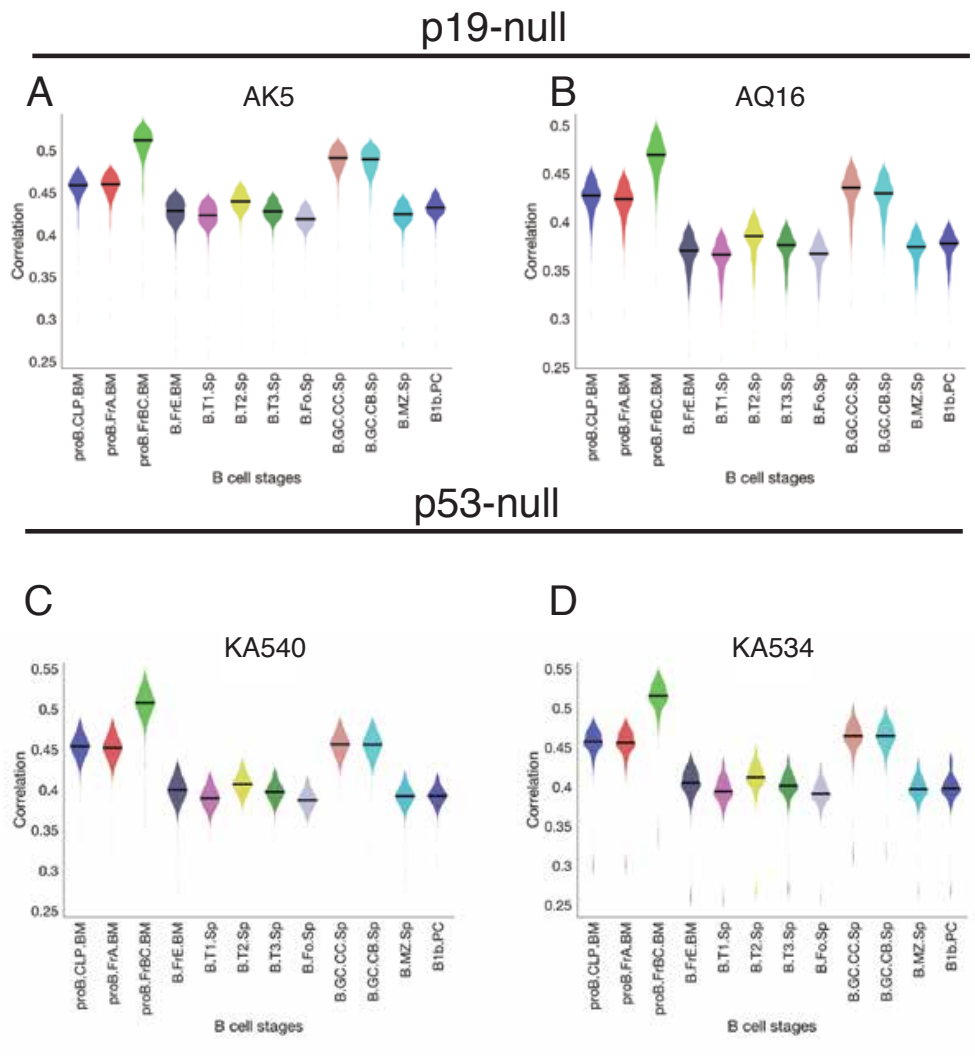

Figure 2.12: transcriptional heterogeneity and differentiation state (single cell level)

Expression correlation of prototypical B cell stages with imputed single cells from the indicated samples.

Genetic alteration of TP53 is associated with subclonal diversity in human cancer.

Statistical inference based of the allelic frequencies of variants in whole exome or whole genome sequencing data have been used to estimate the number of distinct genetic subclones present within a given population (**Figure 2.13A**) (Roth et al., 2014). This approach assumes that (1) any two mutations arising in independent cells at different times since the most recent common ancestor will be detected, by chance, at different allelic frequencies and (2) multiple mutations typically occur before major clonal expansion and thus clusters of mutations should exist at similar allelic frequency to independently mark a genetically distinct subclone.

The *EXPANDS* algorithm, developed by Andor *et al*, takes the variant allele frequencies to perform cell frequency estimation, clustering, filtering, assignment of mutations to clusters of estimated subclones (Andor et al., 2014). Based on *EXPANDS* analysis (Andor et al., 2016) of publically available pan-cancer data generated by the Cancer Genome Atlas (TCGA), we examined the relationship between p53 status and clonality. Consistent with the hypothesis, p53-altered tumors harbor an estimated 45% greater estimated number of subclones per tumor (**Figure 2.13B**). Raynaud and colleagues also analyzed the TCGA database using a combination of *ABSOLUTE* copy number correction and *phyloWGS* clonal estimation (Raynaud et al., 2018). Similarly, *TP53* mutant tumors were found to contain significantly more estimated subclones than *TP53* wild type tumors by *phyloWGS* (**Figure 2.13C**). Both the tissue of origin and the total number of genetic alterations may serve as lurking variables that confound this association, so we examined these factors in more detail.

Both the cell type and the physical niche it occupies are a strong determinant of clonal composition. If there is strict spatial, oxygen, nutrient, or paracrine signaling requirements for a cancer cell population to thrive, competition is presumably intensified and populations tend toward a restricted monoclonal or oligoclonal state with a high probability that frequent clonal sweeping is possible. On the other hand, more invasive tumors or those that are less dependent on environmental cues have presumably less local clonal competition, resulting in the possibility of a broader, more egalitarian representation of diverse clones. Indeed, the tissue of origin is a strong predictor of the number of estimated subclones in the TCGA dataset (**Figure 2.13D**), as previously reported (Raynaud et al., 2018).

Technically, if a subclone is operationally defined a detectable population of cells with a distinct collection of mutations, clearly a tumor with more mutations has the opportunity to discriminate more subclones. Biologically, a tumor with high mutational burden or a highly rearranged genome is expected to be more likely to be unstable and may be able to continuously generate new, emerging subclones. For extreme outliers, hypermutated cancers, the link between mutation number and rate is illustrated in cases where high mutational burden can be attributed to ongoing processes such as DNA mismatch repair deficiency (Cancer Genome Atlas, 2012). Although terms describing mutagenic states and processes are often used interchangeably, this oversimplification masks the effects of time. Mutation load is a function of the accumulation over time of acquisition and extinction of mutations. For example, normal cells, as they acquire somatic mutations at some basal mutation rate, will harbor a greater number of somatic mutations in the elderly than in children, without necessarily indicating a different

mutation rate. Likewise, aneuploid cells often but not necessarily undergoing continued high chromosomally instability. Examination of the pan-cancer TCGA dataset confirms reports that both the number of SNVs (**Figure 2.13E**) and CNVs (**Figure 2.13F**) are correlated to the number of subclones (Raynaud et al., 2018).

We wanted to evaluate if the association between p53 status and clonality (**Figure 2.13B-C**) could be explained by coincidental association with lineage, mutation rate, and copy number alterations. To do so, we constructed a linear model to predict the expected number of subclones for any given tumor based on these three variables. This prediction performed reasonably well, although these factors explain only part of the variance in subclonal diversity (**Figure 2.13G**). In order to determine if p53 status remained informative, we compared the ratio of observe/expected subclones in TP53 wildtype and TP53 mutant tumors. Indeed, p53 mutation was significantly associated with a greater than expected level of clonality (**Figure 2.13H**). Lastly, an alternative linear model was constructed, now including p53 status along with tumor type number of SNVs, and number of CNVs as inputs. In this integrated model, p53 is a significant independent predictor of subclonal content (**Figure 2.13I**).

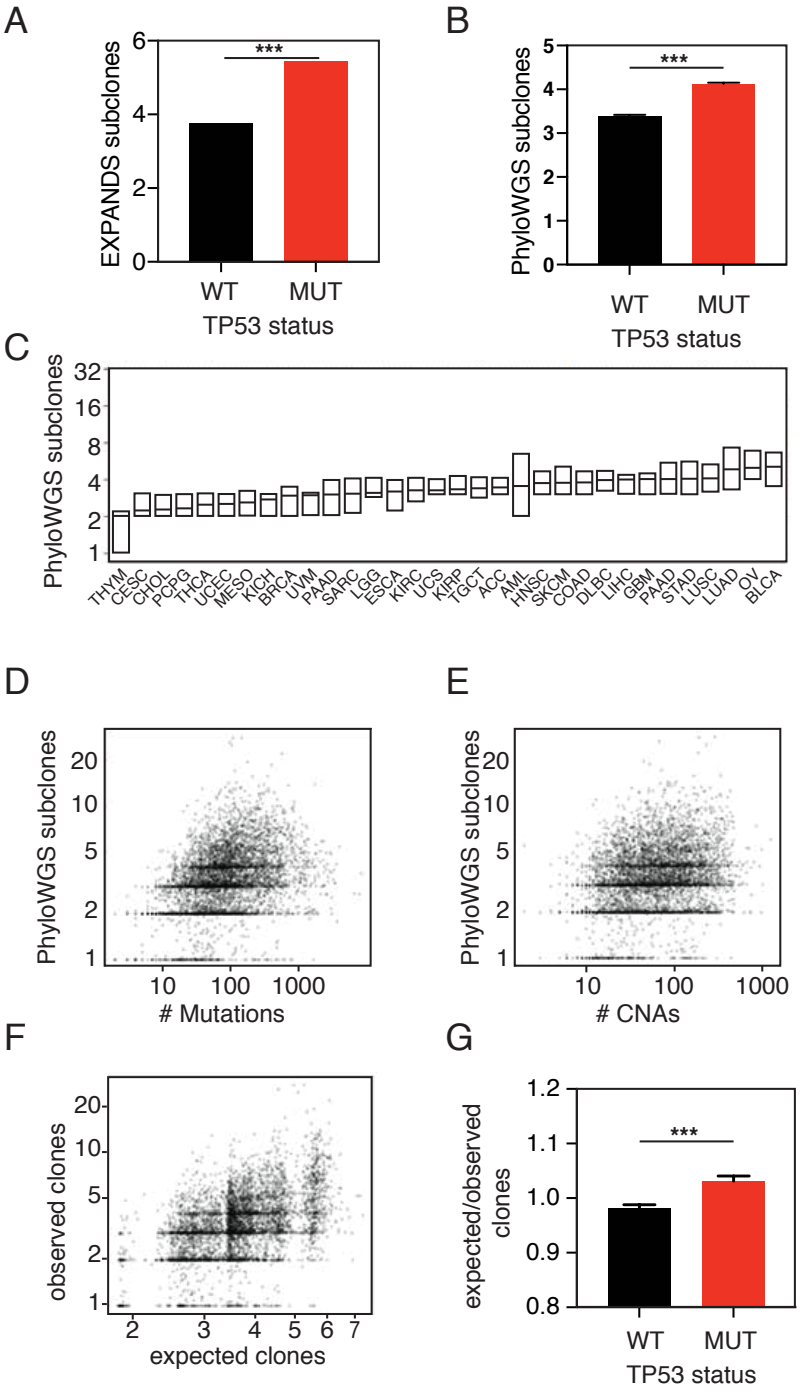

Figure 2.13. p53 status is associated with clonality in human tumors.

(see next page)

(A) *EXPANDS* estimated subclones per sample segregated by *TP53* mutation. (B) *PhyloWGS e*stimated subclones per sample segregated by *TP53* mutation. p<2.2e-16, Welch's t-test . (C) *PhyloWGS e*stimated subclones per sample segregated by cancer type. Central line: mean, box: interquartile range. p<2.2e-16, ANOVA. (D) *PhyloWGS* estimated subclones per sample as a function of number of mutations. Spearman r_s=0.39, p<2.2e-16 (E) *PhyloWGS* estimated subclones per sample as a function of number of copy number alterations. Spearman r_s=0.26, p<2.2e-16 (F) Observed vs. expected number of clones based on a linear model with inputs: cancer type, number of mutations, and number of copy number alterations. Spearman r_s=0.26, p<2.2e-16 (G) Ratio of observed/expected clones from F, segregated by *TP53* mutation. P=1.21e-5, Welch's t-test (C-E) extracted from (Raynaud et al., 2018).

Discussion/Future Directions

Conventional understanding (Fearon and Vogelstein) and computational modeling (Bozic et al., 2010) predict that cancers evolve through repeated cycles of diversification and selection. Clonal dynamics are a largely stochastic process, but may be fundamentally linked to characteristics of the driving genetics of the tumor. Driver genes are selected for their ability to alter cellular fitness by enhancing cell-autonomous biological processes that make up the hallmarks of cancer. However as tumors expand, how these genetic drivers shape evolutionary dynamics could be relevant to the robustness of the tumor in unexpected ways. Many p53 molecular consequences have been described, but population-level tumor evolution is an emerging field enabled by advances in technology. Our results support the hypothesis that loss of p53 function is a significant and consistent determinant of the level of accumulated diversity within tumors.

We confirmed this observation extends robustly to genetic intratumoral heterogeneity in human cancer. Statistically estimating the clonal content of resected tumors samples has limitations. These static samples can yield important insight into the history of a tumor but information about lost clones and the ebb and flow of clonal diversity over time are lost without meaningful longitudinal sampling of the same tumors. The analysis of human tumors presented here represent a sampling of snapshots of many different unsynchronized tumors. These data collectively can characterize the range of diversity different classes of tumors contain. Indirectly, this provides an impression of the frequency and extent of clonal sweeps. The elevated clonality in p53 may indicate (1) a

tendency towards longer periods of time between clonal sweeps by emerging fitter clones or (2) that a leading dominant subclone may be less likely to completely overtake and fix in a population of *TP53* mutant cells. Further experimentation in a controlled, experimental system could begin to unravel some of these open questions.

Molecular mechanisms of p53-restricted intratumoral heterogeneity

It is not yet clear which p53 target gene(s) need to be disabled to recapitulate the effect of p53 deficiency. It is our prediction that a collection of functions, rather than a single target gene. The tumor suppressor p53 uniquely sits at the nexus of CIN (Ganem et al., 2007; Hayashi and Karlseder, 2013; Thompson and Compton, 2010), apoptosis (Lowe et al., 1993) and differentiation (Lutzker and Levine, 1996; Mizuno et al., 2010). Disabling the induction of apoptosis and cell cycle arrest will prevent the elimination of otherwise unviable cells from the population, reducing the loss of subclonal alleles from the system. Loss of p53 can lead to dysregulation of the spindle assembly checkpoint and increase the likelihood of aberrant mitoses and chromosomal imbalances (Schvartzman et al., 2011). While aneuploidy typically lowers fitness of normal cells, strong evidence explains that p53 deficiency drives tolerance of chromosomal rearrangement largely by disabling the DNA damage response associated with a CIN. This CIN-associated DNA damage can stem from DNA replication defects and fork stalling, chromosome shearing, telomere deprotection induced by prolonged prometaphase, or by compromise of the nuclear envelope (Hayashi et al., 2015; Santaguida et al., 2017; Yang et al., 2017).

Interestingly, the accumulation of heterogeneity is specific to the node at which the p53 pathway is disabled. Loss of p19/ARF disables blocks the instigation of a p53

response to replicative stress and Myc-induced apoptosis, fueling tumorigenesis. We observed p19-null lymphomas are apparently diploid and monoclonal, unlike their aneuploid and polyclonal p53-null counterparts. At least in murine B cell lymphoma, this reinforces the concept that a major source of heterogeneity is chromosomal missegregation, which activates p53 independent of the p19-MDM2 axis in p19-null cells. We find that inactivating p53 itself allows for not only aneuploidy, but also a heterogeneous population of divers configurations of aneuploidy. A caveat to this conclusion is that to some extent aneuploidy begets further CIN (Passerini et al., 2016; Sheltzer et al., 2011).

In B cell lymphoma, we found karyotypic diversity as a source of heterogeneity, but not diversity in differentiation state. This was not entirely surprising, since it has previously been shown that by limiting dilution of Eμ-myc cells that single cells can frequently inflict fatal lymphomas in recipient mice, contradicting the possibility that this disease contains a minority of stem-like cells with elevated tumor-initiating capacity (Kelly et al., 2007).

We have developed a set of retroviral vectors for the expression of Cas9 and sgRNAs in B cell lymphoma that are capable of knocking out p53 or other functionally related genes. We hope that future studies comparing knockout of p53 to inactivation of its regulators or relevant effectors will shed further light on the molecular mechanism of p53-restrained heterogeneity.

p53 as an evolutionary capacitor

Even if the molecular mechanism of p53-induced heterogeneity includes well-established p53 activities, the mechanism from a population dynamics perspective remains ambiguous. Over recent years, there has been a growing appreciation for tumorigenesis as a Darwinian evolutionary process (Greaves and Maley, 2012; McGranahan and Swanton, 2017). The adaptability of a cancer, in other words its robustness to change, is expected to be a function of phenotypic diversity independent of the prevailing genotypic drivers. Perhaps the ecological carrying capacity is determined by factors including p53 pathway function.

An apt analogy for tumor evolution is an electronic circuit, where current represents the birth-death dynamics of genetically distinct tumor subclones (**Figure 2.14A**). In this model, oncogenes fuel diversification in proportion to the increased rate of cell division and are analogous to a battery (voltage source). The gain-of-function activity of mutant p53 could contribute to the voltage source of the tumor circuit. Tumor suppressor genes, including p53, can be characterized as resistors. Capacitors build up charge to a given limit (capacitance) and then discharge (**Figure 2.14B**). Akin to the circuit, a tumor builds up an increasingly diverse collection of genotypically distinct cells until one exceedingly fit clone emerges and overtakes the population (**Figure 2.14C-D**). These components make up the boundaries in which tumorigenic clones emerge and pass through the system, analogous to charge in circuit. Among tumor suppressors, p53 has unique features of an evolutionary capacitor. The role of p53 in DNA damage repair indicates regulation of the rate of introduction of mutations into the population. The role

of p53 as a censor in response to dysregulated mitogenic signaling or aberrant mitoses indicates regulation of the rate of clonal extinction. Taken together, p53 permits the accumulation of a wider pool of genetic configurations of otherwise unviable progeny. Thus, one could envision p53 mutation as increasing the capacitance of the system, resulting in more diversity and more potentially drug resistant alleles in the population. Susan Lindquist pioneered the concept of HSP90 as a capacitor of phenotypic variation (Queitsch et al., 2002; Rutherford and Lindquist, 1998). Intact HSP90 allows for the exploration of genetic space by compensating for malformed mutant proteins with protein-folding chaperone activity, buffering against drift in phenotypic space. Attenuated p53 also increases tolerance for genetic drift, but unlike HSP90, this is accompanied by exploration of phenotypic space as well.

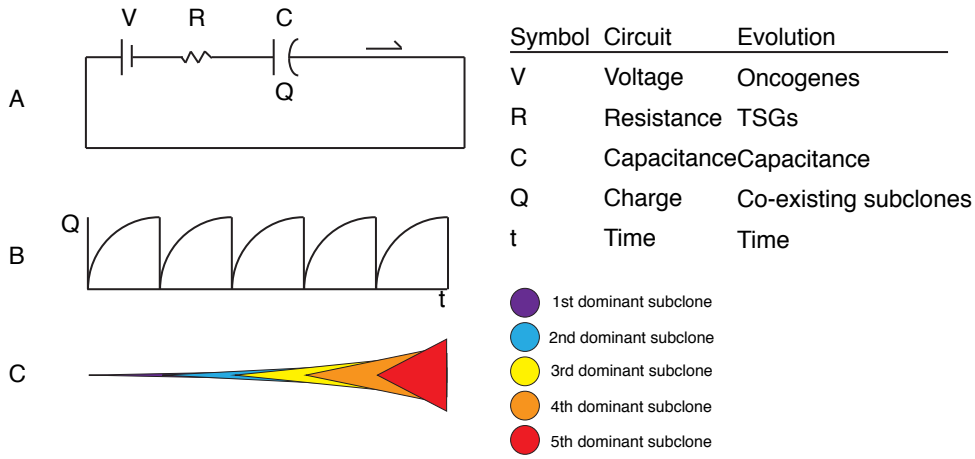

Figure 2.14. P53 as an evolutionary capacitor

(A) Hypothesis modeling the accumulation of tumor heterogeneity as an analogy to circuits. Electric potential (V) is supplied by oncogenes and resistance (R) is supplied by tumor suppressors. The activities of p53 uniquely contribute to the capacitance (C) of the tumor population circuit, as well as contributing to R. Build-up of charge (Q) represents the pool size of distinct subclones.

(B) Charge accumulates over time until discharged across the capacitor.

(C) Population content over time during repeated clonal sweeping of the population.

This model may be too simple to describe the relationship between p53 and diversity in a generalizable way. We envision that p53 deletion induces the chronic accumulation of heterogeneity over time, since our findings would argue against the increased frequency of jackpot clones overtaking the population. We have not, however ruled out the possibility that there are distinct phases in the relationship with p53. For instance, diversity could emerge quickly and saturate at a steady state or an escaper clone with a fitness advantage could stochastically sweep the population and reduce diversity. A high-fitness jackpot clone could be difficult to displace by interlopers. In this scenario, recent p53 mutations could be associated with elevated heterogeneity and long-standing p53 mutation could be associated with reduced heterogeneity. Gaining a greater appreciation of timing is particularly interesting, since *TP53* mutation has a tendency to be a relatively late stage event (Takeda et al., 2015). In any case, the duration of the history of p53 mutation is likely to effect cell populations independent of their genotype at the time of observation.

Furthermore, it is not yet clear whether the variation in p53-deficient disease is the mostly a result of a high rate of ongoing stochastic instability or if a wider variety of previously established subclones continue to persist long term in the population, even as new clones with additional mutations emerge. We hypothesize that p53 deficiency permits the accumulation of intratumoral heterogeneity, largely through the increased rate of clonal persistence. One way to investigate the relative persistence of cells in future studies is to use a barcode library (Wagenblast et al., 2015). DNA barcoding has proven to be a valuable strategy to longitudinally track clones in a manner analogous to viral insertion site identification of progeny from a common ancestor (Kreso et al., 2013;

Nguyen et al., 2014). Following the rate of barcode dropout over time compared between p53 knockout cell lines with respect to controls could help distinguish between ongoing clonal persistence as opposed to diversification followed by an accelerated clonal sweep.

The significance of p53-restrained intratumoral heterogeneity

Genetic diversity has been linked to disease progression and poor survival (Maley et al., 2006) and mutations with initially low pre-treatment allelic frequencies play a clear role in acquired resistance to therapy(Garcia-Caldentey et al., 2012; Turke et al., 2010). Thus, there exists a functionally relevant degree of genetic heterogeneity in tumors at the time of diagnosis that critically modifies clinical outcome.

We hypothesize that p53 deficiency permits the accumulation of intratumoral heterogeneity, largely through the increased rate of clonal persistence. Direct attribution of functional significance of heterogeneity *per se* is less well characterized. Does a longer duration of p53 deficiency lead to higher diversity a greater frequency of "escapers" after treatment? While many cell-autonomous activities of p53 are linked to drug sensitivity, we have the opportunity to compare not only the effects of genotype, but the genotypic history of cancer. A comparison could be made of the effects of recent or long-standing p53 mutations, using Eμ-myc;$Arf^{-/-}$ cells and Cas9/sg.p53 retroviral vectors.

If p53 mutation results in more diversity and more potentially drug resistant alleles in the population, conversely, p53 reactivation might reduce evolutionary capacitance, supporting a role for p53-focused strategies in synergistic combination therapy from an evolutionary perspective (Rosenberg, Science, 2014).

Harnessing the p53 Network

The potency of p53 in tumor suppression and the high rate of p53 alteration in cancers has spurred the development of strategies to target the p53 network in cancer therapy. Indeed, the potential value of engaging p53 in an anticancer response is clear from studies showing that, in some cases, robust responses to conventional chemotherapy can depend on p53, and studies in mice described above document massive tumor regressions in response to p53 reactivation in vivo. For instance, the dramatic cures achieved by retinoic acid and arsenic treatment of acute promylocytic leukemia is dependent on p53-mediated senescence (Ablain et al., 2014). Since *TP53* mutations inactivate wild-type p53 protein, they are widely considered undruggable and, consequently, efforts to rationally exploit p53 for therapeutic benefit have yet to reach fruition. Nonetheless, some strategies to target mutant p53 proteins, p53 regulators, or vulnerabilities created by *TP53* mutation in cancer and other indications show promise.

One of the most advanced efforts to exploit our understanding of p53 biology for cancer therapy involves efforts to inhibit MDM2 in tumors harboring wild-type p53 (**Figure 2.16A**). Led by the development of Nutlin (Vassilev et al., 2004), a panoply of small molecule and peptide inhibitors of MDM2 and MDMX have been developed aimed at improving the properties of first generation inhibitors that generally act by targeting the p53 binding site in MDM2 (reviewed in Cheok and Lane, 2017). A number of phase I trials for MDM2 antagonists have been completed in leukemia and liposarcoma, with neutropenia and thrombocytopenia being prominent dose-limiting toxicities (Andreeff et al., 2016). While these dose-escalation studies preclude conclusions about drug efficacy,

induction of p53 target gene expression was observed in most p53 wild-type samples. Moreover, a partial response occurred in 5-10% of patients, a promising result given many were heavily pre-treated. Counterintuitively, only some of these MDM2 inhibitor clinical trials stratify patients by *TP53* status (reviewed in Burgess et al., 2016; Wang et al., 2011).

Flipping the situation around, MDM2 inhibitors have also been used in efforts aimed at reducing the toxic side effects of chemotherapy. In a strategy termed *cyclotherapy*, these drugs are used to stabilize p53 and trigger a transient cell cycle arrest in normal cells, with the intention of having no effect on the cell cycle progression of p53 mutant tumor cells (**Figure 2.16B**). As many cytotoxic drugs target cells actively in cycle, this strategy is predicted to allow use of a higher tolerable dose of chemotherapy, enhancing efficacy against cancer cells that continue to cycle while reducing toxicity to normal cells (Cheok and Lane, 2017). In preclinical studies, cyclotherapy protects mice treated with Polo kinase inhibitor from dose-limiting neutropenia (Sur et al., 2009).

One attractive therapeutic approach involves identifying agents that cause mutant p53 to regain sufficient wild-type p53 activity for tumor suppression (**Figure 2.16C**). Although the thermodynamic requirements for achieving this seem daunting, structural studies and *in silico* predictions have propelled multiple strategies that supply proof-of-principle for this approach, including peptides and small molecules that stabilize unstructured mutants (Boeckler et al., 2008; Friedler et al., 2002; Yu et al., 2012). One drug, APR-246, which is purported to reactivate mutant p53 but also has off target effects, is currently in clinical trials (NCT03072043, NCT02999893)(Deneberg et al.,

2016). Other agents that directly stabilize the p53 DNA binding domain show promise in preclinical studies (Cheok and Lane, 2017). Through an indirect mechanism, agents known as metallochaperons can facilitate the reincorporation of zinc into unfolded p53 proteins leading to a more normal confirmation and an ability to bind DNA (reviewed in Blanden et al., 2015). Yet another approach exploits the unexpected observation that certain p53 mutant proteins have a penchant for aggregation into amyloid-like structures (de Oliveira et al., 2015) that, when disrupted, restore p53 function and can trigger tumor regression in xenograft models (Soragni et al., 2016).

While the above drugs all aim to coax native wild-type activity out of mutant proteins, instead neutralizing the function or knocking down the levels of mutant p53 in cancer cells represents an unexplored alternative direction that is justified by the observation that tumors can become "addicted" to mutant p53 (Alexandrova et al., 2015). Several indirect strategies have been proposed to destabilize mutant p53 protein including HSP90 inhibitors, HDAC inhibitors, or SIRT1 activators (reviewed in Parrales and Iwakuma, 2015). In the absence of readily available tools to directly inhibit mutant p53 function, the opportunity remains to apply existing drugs to target the underlying mechanism whereby mutant p53 promotes invasion and metastasis (e.g. via HMG CoA reductase, EGFR, or PDGFRb inhibitors) that enhance survival in mouse cancer models (Aschauer and Muller, 2016; Weissmueller et al., 2014).

Another way in which to attack mutant p53 directly is to harness its potential to serve as a tumor-specific neo-antigen. Mutant p53 proteins are typically expressed at high levels and can be antigenic (Crawford et al., 1982; DeLeo et al., 1979); furthermore,

mutant p53-based vaccination can protect mice from transplanted tumors (Roth et al., 1996). Based on this premise, peptide vaccines (Zeestraten et al., 2013), viral vectors (van der Burg et al., 2002), and dendritic cell vaccines (Ellebaek et al., 2012) have entered Phase I/II clinical trials. Regardless of platform, immunotherapy has been able to induce p53-specific immune reactions in humans, through clinical responses have yet to be observed. In theory, tumors that have escaped immunoediting are more likely to contain immunogenic neoantigens that portend a response to immune checkpoint inhibition. Therefore, there is interest in combining p53 immunotherapy with so-called checkpoint blockade to enhance T cell reactivity, which may be able to translate previously observed generation of p53-specific T-cells into the desired cytotoxicity and clinical responses (Hardwick et al., 2014). Indeed, p53 loss can shield cancer cells from $CD8^+$ T cells via PD-L1 derepression, an interaction that accelerates disease and is evident in human lung cancer (Cha et al., 2016; Cortez et al., 2016; Schuster et al., 2011), yet a positive association between p53 alteration and response to immunotherapy by PD-L1 inhibition has not been observed.

An attractive approach to targeting p53 mutant tumors is to exploit *synthetic lethality*, a term describing a situation in which gene mutation creates novel dependencies. Many previously characterized liabilities imposed by p53 mutation converge around the DNA damage response and metabolism. Although p53-deficient cells can evade apoptosis in the face of DNA damaging agents, further disabling the DDR leaves p53 mutant tumors hypersensitive to genotoxic damage (Ma et al., 2012). Accordingly, strategies combining DNA-damaging agents with inhibitors of DDR components ATM, CHK2, ATR, and CHK1 have been pursued (reviewed in Morandell

and Yaffe, 2012). In one proof-of-concept that has shown positive results in a phase II clinical trial of p53 mutant ovarian cancer, WEE1 inhibitor is used to weaken the G2 cell cycle checkpoint, exploiting the G1 checkpoint deficiency in p53 mutant cells and allowing the accumulation of catastrophic levels of DNA damage when combined with genotoxic chemotherapy (Leijen et al., 2016). Also, patients with *TP53* mutations have been reported to have higher response rates to extended cycles of the demethylating agent decitabine (Welch et al., 2016). While the mechanistic basis for this observation is not know, one plausible explanation is that wild type cells arrest in G2/M upon drug treatment, whereas p53-deficient cells pass through the cell cycle checkpoint, resulting in severe chromosomal damage and death (Nieto et al., 2004).

Although exacerbating instability may achieve therapeutic responses, the concern remains that mutagenesis associated with reducing the DDR likely fuels tumor evolution and perhaps even the emergence of treatment associated AML (t-AML). Additionally, the metabolic rewiring associated with p53 mutation also instills novel dependencies on druggable targets, including PIP4K2A/B, cholesterol biosynthesis, and IAPP (Emerling et al., 2013; Freed-Pastor et al., 2012; Venkatanarayan et al., 2015). Unlike synthetic lethal interactions related to p53 loss-of-function, a side-effect of single copy deletion of chromosome 17p deletions during LOH may be to expose cancers to heightened dependence on other essential genes in this region, such as POLR2A (Liu et al., 2015).

Beyond cancer, pharmacological modulation of p53 is a potentially useful and largely unexplored strategy to aid cell autonomous defense against infection. Pathogens evolved around mammalian cells, selected to keep the host alive despite DNA damage,

ROS induction, and activation of innate immunity through toll-like receptors that may follow infection, all of which can be mediated by p53 (Shatz et al., 2012). Hence, p53 can act as a suppressor of bacterial infection, leading to the concept of pharmacological p53 activation to mitigate severe infections (Siegl et al., 2014). Some pathogens encode components that inhibit p53, and nutlin-based stabilization of p53 can hinder their propagation (Kaushansky et al., 2013; Siegl et al., 2014). Consequently, it may be worth considering use of MDM2 inhibitors in cases of life-threatening multi-drug resistant infections with no other treatment options. However, induction of p53 is not universally conducive to combating infection, and defining its disease-specific immune interactions will be a prerequisite for clinical relevance of p53 in infectious diseases. *Trp53*$^{-/-}$ mice are actually more capable of recovering from bacterial pneumonia (Madenspacher et al., 2013).

Through restoring wild type function, inhibiting mutant function, or treating a dysregulated immune system, multiple avenues exist to target the p53 network in cancer. Given the obstacles that have been encountered using these strategies to date, further knowledge of basic p53 biology will be required for future successful clinical applications.

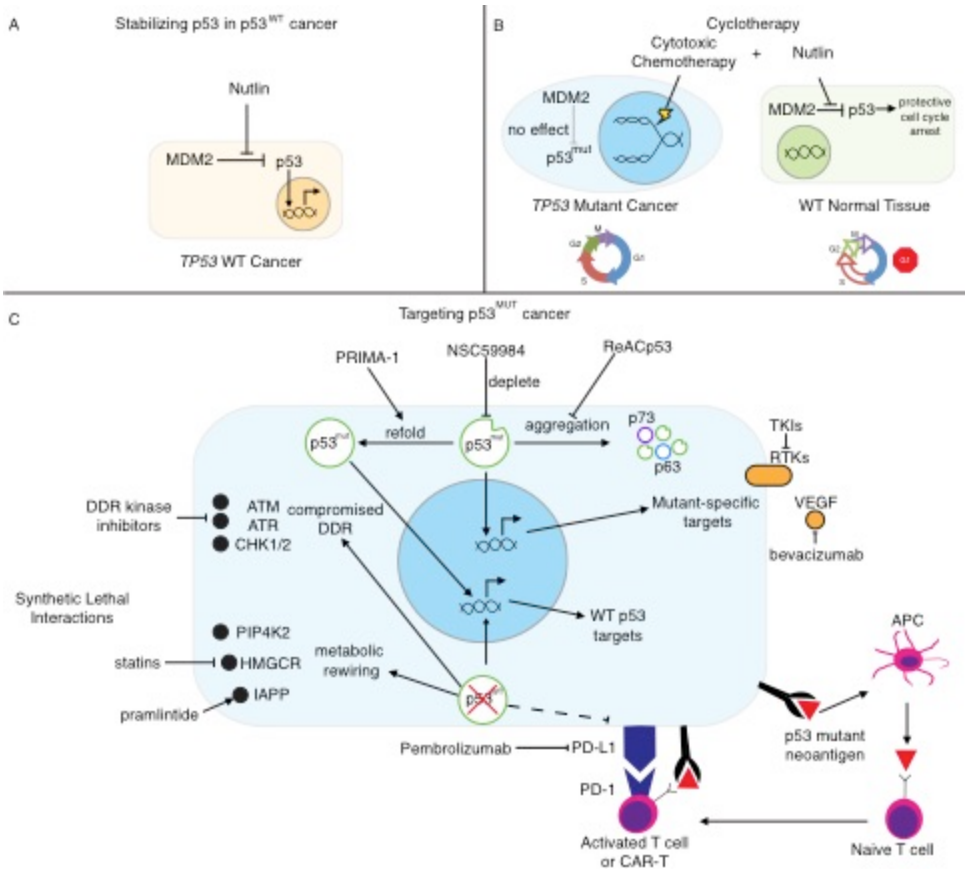

Figure 2.15. Harnessing p53

(A) Stabilizing p53 in $p53^{WT}$ cancer. Nutlin and other MDM2/MDMX inhibitors (RG7112, RO5503781, SAR405838, HDM201, MK4828, AMG232, and RG7388) allow for the accumulation and activity of p53 in cancer in which it is not mutated. (B) Cyclotherapy. Nutlin is used to transiently arrest $p53^{WT}$ normal cells, while $p53^{MUT}$ cancer cells continue to cycle and remain vulnerable to genotoxic chemotherapy. Sparing normal tissue allows for increased dosing and reduced toxicity. (C) Targeting $p53^{MUT}$ cancer. PRIMA-1 and other agents (APR-246, RITA, PK7088, p53R3, and ZMC1) are used to support proper folding of mutant p53 and restore wild type-like dtructure and activity. p53 mutant protein is depleted through a number of indirect mechanisms including inhibition of HSP90 (17-AAG), HDAC (SAHA), and SIRT1 (YK-3-237). The aggregation and inactivation of p53 mutant and its family members is inhibited by ReACp53. Synthetic lethal interactions are dependencies in p53 mutant cancer but not in $p53^{WT}$ cells. p53 deficient cells have a compromised DDR, leaving then vulnerable to even further genomic instability by inhibiting DDR-related kinases. Metabolic rewiring introduces druggable dependencies on PIP4K2, cholesterol biosynthesis/HMGCR (statins), and IAPP (pramlinitide). Some p53 mutations can result in recognizable neoantigens, which has led to the development of mutant p53-targeted immunotherapy. p53 ablation can also modify antigen presentation efficiency justifying the investigation of immune checkpoint inhibition, especially when combined with other strategies.

Concluding Remarks

p53 has captured the fascination of cancer biologists, and its detailed characterization has produced fundamental insights into mechanisms of gene regulation and nature's safeguards against cancer. While the body of research on p53 is massive and sometimes even contradictory, it is now abundantly clear that cellular responses to p53 activation involve a complex interplay between activation triggers, cell lineage, and cell state. While such context-dependent effects on p53 have stymied attempts to generalize the mechanism of p53-mediated tumor suppression, they provide opportunities to exploit the network in cancer cells, while avoiding deleterious consequences of manipulating p53 in all tissues.

Despite decades of intensive research and countless discoveries, there remains much to learn about the roles and regulation of p53. A challenge in the coming era of p53 research will be to distill convergent truths assembled from comprehensive studies, and to translate knowledge of p53 into clinical application. Indeed, the enormous challenge associated with exploiting p53 therapeutically does not mitigate the astounding morbidity associated with *TP53* mutation. In the absence of new therapeutic innovations, *TP53* mutant cancer will lead to the deaths of over 500 million people alive today. New technologies, together with our ever-increasing understanding of the complexity of p53 action and the diverse consequences of p53 mutation will hopefully set the stage for more robust clinical advances.

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