The impact of chromosome 17p alterations on cancer

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Signature Page

Abstract

One of the most frequently altered regions in the genome is the short arm of chromosome 17 (17p). Mutational events target the tumor suppressor p53 whereas chromosome-arm deletions result in the loss of not only p53 but also other protein-coding genes located on 17p. Interestingly, p53 mutations together with loss of heterozygosity of 17p are selected for during tumorigenesis and are apparent in most cancer cells. This phenomenon raises many questions and my forthcoming thesis work sets out to address some of the issues regarding the functional consequences of p53 mutations, the tumor suppressive role of 17p genes, and the causal effects of cooperative tumor suppressor gene loss. Therefore, we first analyzed the mRNA profile of mutant p53 expressing cancer cells, and found that mutant p53 induces platelet-derived growth factor receptor b (PDGFRb) through a cell-autonomous mechanism to drive metastasis. Mutant p53 inhibits the p73/NF-Y complex that represses PDGFRb expression in p53-deficient, non-invasive cells. Blocking PDGFRb signaling prevented pancreatic cancer cell invasion and metastasis formation, implicating PDGFRb as a prognostic marker and possible target for attenuating metastasis in p53 mutant tumors. Second, we studied the miRNA profile of mutant p53 expressing cancer cells and used a systematic screening approach that identified miR-155 and miR-181b as mediators of mutant p53 to drive invasion. Both miRNAs function redundantly to modulate the MAPK pathway and to decrease active levels of MKK4, thereby inhibiting the anti-metastatic function of MKK4. Interestingly, the induction of this discrete miRNA signature by mutant p53 is Dicer-independent. Both studies together revealed at least two mechanisms that underlie the gain of function mutation of p53, suggesting that mutant p53 regulates several molecular mechanisms to execute its neomorphic activities. Lastly, we also investigated whether any of the other 274 protein-coding genes on 17p that often become heterozygously deleted in cancer cells has a tumor suppressive function. By conducting an RNAi screen we identified MKK4 as a potential tumor suppressor that modulates antiapoptotic and

survival genes to promote cancer development. Moreover, we observed that MKK4 functions in a haploinsufficient manner and drives tumorigenesis more potently with the simultaneous loss of p53. Therefore, the phenomenon of p53 mutations concurrent with 17p loss is selected for to potentiate tumorigenesis in a cooperative fashion. As 17p alterations are only one of many genetic changes that a cancer cell acquires during tumor development, a better understanding of the causal effects of combined genetic alterations is one of the most pressing needs in basic cancer research. Increased knowledge about the cancer genome will help to guide the development of more effective treatment options to reduce cancer mortality.

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List of Abbreviations

17p	short arm of chromosome 17
CHiP	**ch**romatin **i**mmuno**p**recipitation
CGH	**c**omparative **g**enomic **h**ybridization
Cre	Cre Recombinase
Ctrl	Control
DAPI	4',6-**d**i**a**midino-2-**p**heny**l**indole
DFS	**d**isease **f**ree **s**urvival
GIST	**g**astro**i**ntestinal **s**tromal **t**umors
GFP	**G**reen **F**luorescent **P**rotein
H&E	Hematoxylin and eosin
HCC	**h**epato**c**ellular **c**arcinoma
IF	**I**mmuno**f**luoresence
IHC	**I**mmuno**h**isto**c**hemistry
KPC	LSL-**K**ras$^{G12D/+}$, LSL-**p**53$^{R172H/+}$, Pdx1-**C**re
KP$_{fl}$C	LSL-**K**ras$^{G12D/+}$, LSL-**p**53$^{loxP/+}$, Pdx1-**C**re
LOH	**l**oss **o**f **h**eterozygosity
LPC	**l**iver **p**rogenitor **c**ells
LSL	**l**ox-**s**top-**l**ox cassette
MKK4	**m**itogen-activated protein **k**inase **k**inase 4
MOI	**M**ultiplicity **o**f **i**nfection
miRNA	microRNA
mRNA	messenger RNA
PanINs	**pan**creatic **i**ntraepithelial **n**eoplasias
PDAC	**p**ancreatic **d**uctal **a**deno**c**arcinoma (PDAC)
PDGFRb	**p**latelet-**d**erived **g**rowth **f**actor **r**eceptor **b**eta
RNAi	RNA interference
RNAseq	RNA sequencing
SCNAs	**s**omatic **c**opy **n**umber **a**lterations
shRNA	**s**hort **h**airpin RNA
TMA	**t**issue **m**icro**a**rray

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Chapter 1

Introduction

1. Chapter 1

Introduction

Comprehensive knowledge of genomic alterations in cancer is imperative for developing novel diagnostics, prognostics and targeted therapeutics. Increasingly sophisticated sequencing technologies have provided considerable insight into the landscape of genomic changes that occur in cancer, but functional studies are required to determine the mechanistic roles of mutated genes in tumorigenesis, and to discern whether their encoded proteins represent therapeutic targets. One of the most frequent genomic abnormalities in human cancers affects the short arm of chromosome 17 (17p). Such alterations include point mutations that target 17p13.1, the region that encodes the tumor suppressor p53, as well as heterozygous whole arm deletions that span most of the included 274 protein-coding genes. In many cancers, p53 mutations are missense, leading to expression of stable p53 proteins with gain of function activities that can cooperate with oncogenic mechanisms. p53 point mutations often accompany deletions of the remaining 17p allele and likely represent a major factor accounting for rapid disease progression, poor response to therapy, early relapse, and short patient survival. Mutations in p53 are primarily selected for during cancer development; however, the observation that loss of heterozygosity (LOH) of 17p occurs frequently raises the question whether altered gene function of other genes contributes to tumorigenesis. In this thesis, I try to shed light on the relevance of each of these genetic abnormalities to tumorigenesis and address several open questions regarding (i) the gain of new oncogenic functions of mutant p53, (ii) the identification of "driver" genes on 17p, and (iii) the consequences of attenuated gene function of multiple genes on 17p.

1.1. Genetic alterations in the cancer genome

Cancer development is driven by the step-wise acquisition of genetic alterations that result in either the enhanced or decreased activity of the corresponding proteins. Affected genes are called "oncogenes" or "tumor suppressor genes", referring to their respective abilities to either promote or inhibit cancer development. It was estimated that < 1% of genes in the human genome, 138 of the 20,000 coding genes, functionally affect tumorigenesis ("driver genes") (Vogelstein et al., 2013). Alterations in these genes are being identified recurrently in different tumor types, suggesting that almost all causal cancer genes mutated at high frequency have been identified. In addition to changes in driver genes, there are many additional alterations in "passenger genes" that do not confer a selective growth advantage but rather reflect residual evidences of cancer genome evolution. Since every cell division generates a few new mutations, the number of mutations that distinguish any two cells marks the time from their last common ancestor

Different types of somatic alterations can lead to the altered expression or activity of tumor suppressor genes and oncogenes. During the process of tumor evolution, the cancer genome is edited by single base substitutions, copy number alterations, and translocations (Figure 1.1.) (Futreal et al., 2004). Focal mutations mainly affect a single base or, in few cases, deletions or insertions of one or a few bases. On the other hand, chromosomal alterations, including amplifications or deletions, can affect small genomic segments, encompassing just a few genes, as well as large genomic areas that can affect megabases of DNA and large numbers of genes. Lastly, cancer cells also contain translocations that fuse two genes to create an oncogene and, in a small number of cases, that can inactivate a tumor suppressor

gene. However, regardless of the type of genomic alterations, the functional consequences of these alterations result in a suite of deleterious cellular outcomes, which include increased survival potential, altered cell fate, and/or disrupted genome maintenance processes (Vogelstein et al., 2013).

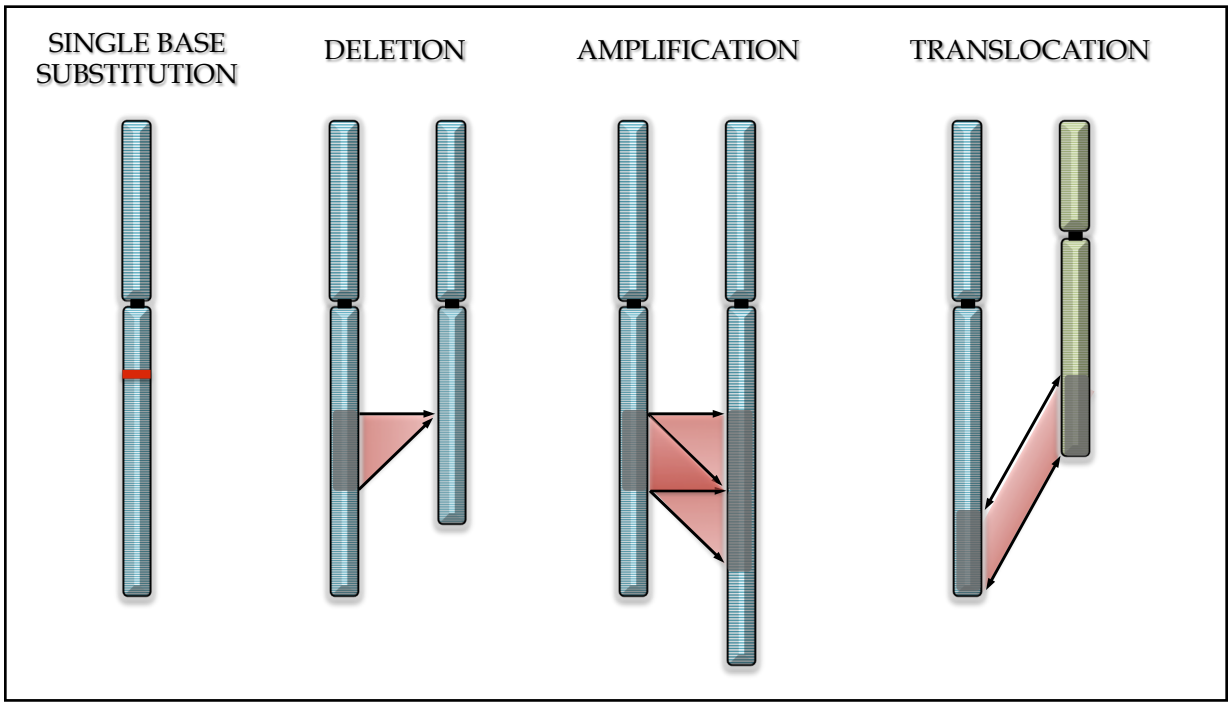


Figure 1.1. Genetic alterations as a hallmark of tumorigenesis
The DNA sequence of a gene can be altered in a number of ways: (i) small scale mutations, including a single base substitution or the insertion/deletion of small DNA fragments, (ii) larger scale somatic copy number alterations, including deletions/amplification ranging in size from focal regions to whole chromosome arms, and (iii) translocations, bringing together separate genes from the same or distinct chromosomes to form functionally novel fusion genes.

The distribution amongst the classes of genetic alterations in a tumor is uneven, with point mutations affecting 10 times more genes than any other aberration. However, ~80% of mutations are synonymous passengers with no causal effects on tumorigenesis (Stratton et al., 2009). It has been estimated that solid tumors display between 33 to 66 non-synonymous mutations in driver genes, with the exception of pediatric and leukemic malignancies (which have fewer mutations) and cancer types that are continuously exposed to mutagens,

such as melanomas or lung cancer (which have >200 mutations) (Vogelstein et al., 2013). The mutational signatures across different cancer types differ and develop further over time, after treatment, and through the inactivation of DNA repair processes that create greater genomic instability (Greenman et al., 2007; Alexandrov et al., 2013). Oncogenes are recurrently mutated at the same amino acid positions (such as RAS, mainly mutated at codon 12 (Sukumar et al., 1983)), whereas tumor suppressor genes can be inactivated by mutations that occur throughout their coding regions, resulting in protein-truncations (p16 (Kamb et al., 1994)). Despite the fact that sophisticated sequencing technologies have given us a glimpse at the diversity and complexity of mutational signatures buried within cancer genomes, the real challenges remains to identify causal consequences of somatic mutations by functional studies. Moreover, how different mutagens or the infidelity of the DNA maintenance machinery influence the mutational signature and the contribution of mutational signatures towards biological characteristics of each cancer remain elusive.

In contrast to point mutations that affect normal cells at similar rates as cancer cells, somatic copy number alterations (SCNAs) occur much more frequently in cancer than in normal tissue. SCNAs affect large regions of the genome, ranging from one kilobase to several megabases in size (Pelizzola et al., 1994; Vogelstein et al., 2013), and it might be that cancer cells tolerate these changes due to the inactivation of DNA damage response pathways. Deletion events eliminate tumor suppressor genes, whereas amplifications yield multiple copies of an oncogene in a cell. Focal deletion- and amplification peaks, which can be shared across cancer types, contain an average of 7 genes (Beroukhim et al., 2010). Extensive sequencing studies identified ~150 focal SCNAs across 26 different cancer types. However, only 11% of them contain yet identified tumor suppressor genes and 33% of them

known oncogenes, respectively. Larger arm-level SCNAs, which are shared across cancers of similar developmental lineages only, affect the cancer genome much more frequently than focal SCNAs (Beroukhim et al., 2010). Especially in the case of large chromosomal deletions, it is difficult to separate the specific target gene(s), whose loss confers a growth advantage to the cell, from the many passenger genes without tumorigenic effect (Vogelstein et al., 2013). In some cases, however, it is not the loss of one single tumor suppressor gene but the combined loss of several linked genes that cooperate to cause cellular defects (Solimini et al., 2012; Xue et al., 2012). Therefore, as with point mutations, evidence of genomic alterations is not enough to identify the genes with causal effects on tumorigenesis, and functional studies need to complement sequencing results.

Another frequently occurring class of genetic alterations encompasses chromosomal translocations, which juxtapose the regulatory region of one gene to another gene. These events have mostly dominant effects by combining two genes to create a "fusion" oncogene (e.g. *BCR-ABL*), but translocation can sometimes inactivate tumor suppressor genes by truncation or separation from their promoter. For example, the *TEL1-AML* gene fusion represses the expression of the tumor suppressor TEL1 (Kim et al., 1996). As most translocations target gene deserts devoid of known genes or fragile sites, the causal consequences mostly appear to be silent. These alterations occur predominantly in leukemias, lymphomas or mesenchymal tumors.

Identifying genetic modifications in the cancer genome has been of interest ever since the discovery that DNA sequences from transformed tumorigenic cells transform "normal" NIH-3T3 cells with oncogenic properties (Shih et al., 1979). One of the first genetic

alterations identified was an activating mutation in the HRAS oncogene (Der et al., 1982; Parada et al., 1982; Santos et al., 1982; Taparowsky et al., 1982). Since then, more than 100,000 additional aberrations in the cancer genome have been reported (Futreal et al., 2009). Results from multiple sequencing studies suggests that cancers are initiated by only 2-8 genetic alterations and continue to acquire ~150 additional genetic changes over a long period of time (Hanahan and Weinberg, 2011; Vogelstein et al., 2013). Consequently, strategies for eradicating cancer might be most fruitful during the early stages of cancer development, by means of early detection or preventative treatment, or by employing combination therapy that targets multiple pathways in advanced cancers.

The signaling pathways and cellular processes that are altered in cancer cells are versatile. Three core cellular processes become deregulated and include (i) cell fate, (ii) cell survival, and (iii) genome maintenance. Many altered genes interfere with the balance of differentiation and division, and push the cell towards the latter. Since differentiated cells eventually die or become quiescent, cancer cells gain a growth advantage by evading those fates. Conserved pathways that operate through this process include NOTCH, HH, and APC. Genes that encode chromatin-modifying enzymes can also determine over the fate of a cell and are frequently genetically altered in cancer cells. Additionally, cancer cells acquire a growth advantage due to an increased cell survival potential. Mutations in genes that stimulate proliferation under limiting nutrient concentrations occur frequently. Most mutations affect genes encoding growth factor receptors (such as EGFR, HER2, FGFR2, PDGFRA, and TGFBR2) or their downstream signaling mediators inside the cell (MET, KIT, RAS, RAF, PIK3CA, and PTEN) (Vogelstein et al., 2013). Cancer cells can also progress through the cell cycle in an uncontrolled way, thereby evading apoptosis. Genes encoding

for CDKN2A, MYC, and BCL2 become frequently altered and fall into this class. Increased angiogenesis also enhance cell survival and can be the result of mutations in VHL. Cancer cells with gross chromosomal changes or increased point mutation rates survive the damage and divide, instead of undergoing cell death. p53, ATM, MLH1, and MSH1 play a role in genome maintenance and are frequently mutated in cancers. Protein products from altered genes regulating cell fate, cell survival, and genome maintenance interact with each other and the pathways that govern such cellular processes overlap in cancer cells. It is not surprising that genetic modifications in different genes results in the same cellular outcome to drive growth advantage of tumor cells. But questions remain whether mutations in various components of a single pathway are mutually exclusive and as to how deregulated and uncontrolled pathways can be targeted for cancer therapy.

A greater knowledge of the functional consequences of genetic alterations and their downstream pathways will allow the development and improvement of therapy. Therapeutic success by directly targeting the driver gene of a cancer has been achieved in *PML-RXR*α promyelocytic leukemias by treatment with all-trans retinoic acic (ATRA), in *EGFR*^{mut}-driven lung cancer by treatment with gefitinib, or in *KIT*^{mut}-driven gastrointestinal stromal tumors by treatment with imatinib. Targeting the mediators of the driver gene represents another potentially effective therapeutic approach. Success has been observed when treating *KRAS*-driven tumors with MEK inhibitors. The dramatic, albeit transient, responses to agents that interfere with a single mutant gene product are difficult to reconcile given the genomic complexity of a cancer cell. Two points could explain this phenomenon. First, most of the genetic changes in a cancer genome are passenger alterations and are immaterial to tumorigenesis. Second, driver genes alter only a limited number of signaling

pathways through which the cancer develops. Therefore, a potential improvement to therapy involves the combined usage of multiple agents that target several genes of the same or parallel pathways to exploit synthetic lethal dependencies. Combination therapies targeting two affected molecular pathways or different components of the same pathway could potentially result in a more efficient inhibition. The detailed characterization of each molecular component of the affected pathways is imperative for designing such approaches. Novel strategies that exploit synthetic lethality in the context of loss-of-function alterations in tumor suppressor genes remain mainly unexplored (Stuart and Sellers, 2009). However, alterations in tumor suppressor genes, which are more frequently affected than oncogenes, can confer therapeutic susceptibility and, therefore, hold great promise for advancing the ability to cure cancer.

1.2. An example: Genetics of pancreatic cancer

The molecular analysis of pancreatic ductal adenocarcinomas (PDAC), the most common histological subtype of all pancreatic cancer cases, has provided insights into common genetic alterations, often implicating known cancer genes and classical cancer signaling cascades. PDAC arises from pancreatic intraepithelial neoplasms (PanINs), a preinvasive precursor state with cytological and genetic changes that become evident in invasive PDAC. These lesions stem from the small ducts of the exocrine pancreas, and are classified as PanIN-1 (low-grade dysplasia), PanIN-2 (moderate dysplasia) or PanIN-3 (high-grade dysplasia) lesions. PanINs are characterized, and most likely initiated, by oncogenic *KRAS* mutations, and are followed by *INK4A/ARF* loss, and finally, *p53* mutations and loss of *SMAD4*. Collectively, these observations support a genetic progression model of pancreatic

carcinogenesis, resulting in the formation of an infiltrating cancer (Figure 1.2). Extensive sequencing studies have revealed that PDAC is characterized by a few high-frequency mutations ("mountains") and many low-frequency mutations ("hills"), with the latter predominating in terms of the total number of alterations involved (Jones et al., 2008; Biankin et al., 2012). Less frequently mutated genes include *LKB1*, *TGFβ*, *MKK4*, *AKT2*, and *MYB* (Tuveson and Hingorani, 2005). The multitude of mutated genes affects the above-mentioned cellular processes in the great majority of PDAC patients; however, the pathway components that are altered in any individual tumor vary widely (Jones et al., 2008). Our knowledge about the relationships and causal consequences of these genetic changes to tumor progression and metastasis development remains rather limited.

Multiple genetic events, giving rise to e.g. epithelial-mesenchyman transition, upregulation of specific miRNA and/or oncogenic signaling pathways, are required for efficient metastasis to occur. The development of metastasis is a common feature of the natural history of PDAC, with up to 90% of patients having metastatic disease at death (Yachida and Iacobuzio-Donahue, 2013). The metastatic evolution requires at least 20 years from the first tumor-initiating mutation to the acquisition of metastatic abilities. The genetic changes discussed thus far represent initial events that occur during carcinogenesis upon which additional pro-metastatic events may be superimposed. When comparing mutation spectra from primary tumors and different metastatic lesions, two categories of mutations were identified: founder mutations, present in all samples analyzed from a given patient, and progressor mutations, present in a subset of samples from a patient (Yachida et al., 2010). Therefore, clonal populations that give rise to distant metastases are represented within the primary tumor; however, these clones further evolve genetically from the

original parental, non-metastatic clone. Loss of Smad4 and mutations in p53 might represent two of many mediators of metastasis development, but how they function remains unknown (Yachida and Iacobuzio-Donahue, 2013). Sequencing studies also illustrate the genetic heterogeneity present in the primary tumor and metastatic lesions, but fail to identify the mechanism by which genetic heterogeneity arises. As in most solid tumors, heterogeneity is observed among affected pathway components and different mutations affecting one gene, but whether those arise from preexistent small populations of cancer stem cells or due to waves of clonal evolution in association with the accumulation of genetic alterations, remains unclear. To date, the molecular features of the metastatic subclones in the primary tumor that promote metastasis formation remain elusive and no consistent genetic signature of metastatic subclones could be identified.

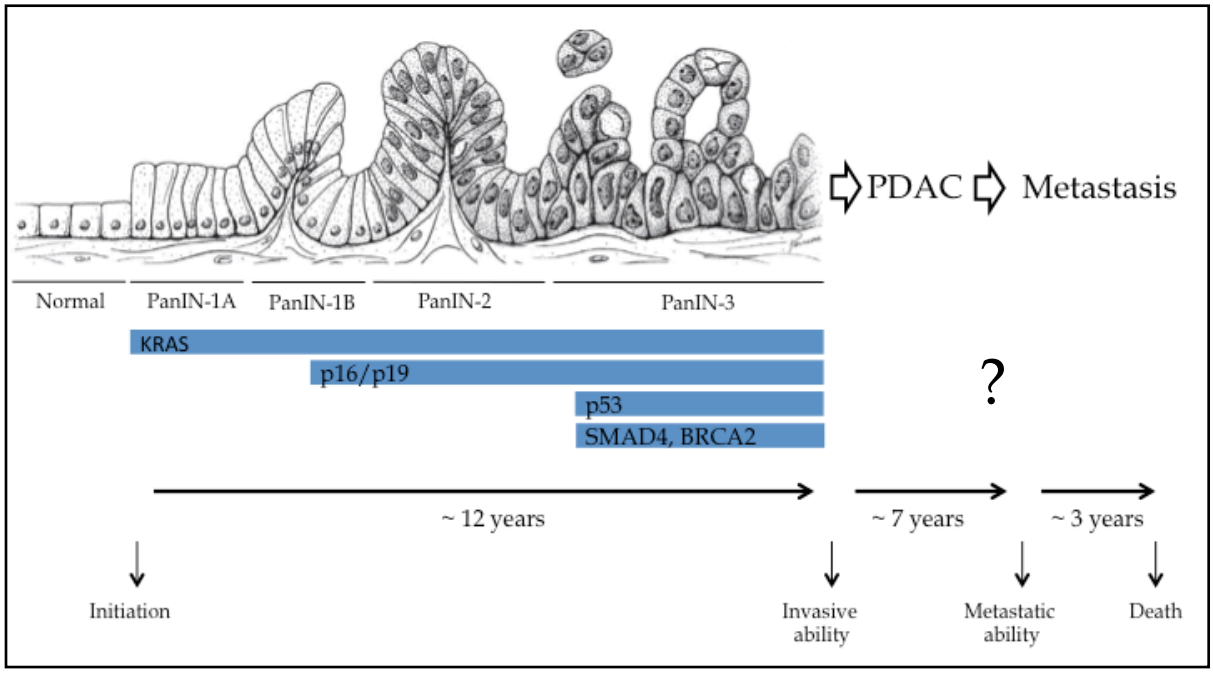


Figure 1.2. Pancreatic precursor lesions and genetic events involved in PDAC progression
The various genetic events are listed and divided into those that predominantly occur early or late in PDAC progression (adapted from Hruban et al., 2000).

Despite our knowledge of the genetic alterations in PDAC, the functional consequences on tumor initiation and progression as well as metastases formation remain unresolved. Engineered mouse models (GEMMs) of PDAC faithfully recapitulate the human disease and can therefore provide a more detailed understanding of the biological impact of the driver genes. *Pdx1-Cre* and *Ptf1-p48-cre* deleter strains have been used for Cre-mediated excision of *LoxP*-flanked elements (genes or STOP cassette) specifically in the pancreas. The *Pdx1* and *Ptf1-p48* promoters are active in the common progenitors of all pancreatic cell types with relatively restricted expression outside of the pancreas (Hezel at al., 2006). Therefore, this system can be used to mimic the acquisition of activating point mutations or the loss of tumor suppressor genes, frequently occurring in human cancers. Cre-induced activation of *LSL-Kras*^{G12D} rapidly initiates the development of PanIN lesions and the progression to frank PDAC after long latency (Hingorani et al., 2003). Thus, oncogenic *Kras* mutations need to be potentiated by other genetic alterations to induce neoplastic changes, as other rate-limiting events are likely to constrain progression of *Kras*^{G12D}-driven neoplasms towards invasive PDAC. When *Kras* activation is combined with mutations in the tumor suppressors *p53* or *Ink4a/Arf*, a rapid PDAC progression and lethal tumor burden is observed. Homozygous deletion of either *p53* or *Ink4a/Arf* in a *Kras*-mutant background gives a more rapidly developing PDAC phenotype and mice succumb within 7-11 weeks. These PDAC tumors resemble histologically and molecularly the human disease, including a proliferative stroma (Aguirre et al., 2003; Hingorani et al., 2005; Bardeesy et al., 2006). Since mice with a pancreas-specific deletion of only *p53* or *Ink4a/Arf* do not develop pancreatic neoplasia, the primary role of these two genes might not be in the onset of PanIN but, rather, to form a critical barrier in blocking progression of PanIN initiated by Kras^{G12D}. Interestingly, while tumors of these genotypes are locally invasive, gross metastases

formation seems to be restricted to mice with only heterozygous loss or mutations of tumor suppressor genes (Hingorani et al., 2005; Bardeesy et al., 2006). This may reflect the fact that the homozygous models develop multifocal tumors resulting in a rapidly lethal tumor, whereas the longer latency of heterozygous GEMMs allow enough time for clonal maturation, progression, and metastasis. Collectively, these mouse models recapitulate histologic variants from those seen in spontaneous human tumors and can be used to study the underlying mechanisms of cellular processes manipulated by genetic alterations.

Despite the data of many sequencing studies that have provided an outline of the genetic alterations associated with PDAC development and progression; the current picture remains static, with only correlative links to underlying tumor biology. Because PDAC is the fourth most common cause of cancer death, there is an urgent need for the identification of therapeutic targets and biological pathways of significance (Yachida and Iacobuzio-Donahue, 2013). The categorization of genetic events into driver and passenger events and the quest for specific genetic events that promote metastatic dissemination remains ongoing. Only a full comprehension of the intricate network of genetic alterations will allow for early detection, improved therapy, and increased patient survival.

1.3. The many faces of p53

1.3.1. Wild type p53 induces tumor suppression via multiple mechanisms

When p53 was first discovered in 1979 (Lane and Crawford 1979; Linzer and Levine 1979), it was believed to carry proto-oncogenic functions. For example, studies reported that p53 accumulates abundantly in cells (DeLeo et al., 1979), and others described p53's capacity to

transform primary cells in conjunction with oncogenic Ras (Eliyahu et al., 1984), and its ability to increase tumorigenesis when overexpressed in *p53*$^{-/-}$ cells (Wolf et al., 1984). Only upon analyzing the sequences of the over-expressed *p53* cDNA, which was isolated from tumor cells, it was found that the constructs carried mutations. Indeed, some of the tumors that were utilized to extract the *p53* sequences exhibited loss of heterozygosity (LOH) with one p53 allele mutated and the other lost, which represents a hallmark of tumor suppressor genes (Baker et al., 1989). Multiple lines of evidence subsequently reaffirmed the conclusion that p53 acts as a tumor suppressor. It was found that over expression of wild type *p53* suppressed oncogenic transformation (Hinds et al., 1987; Finlay et al., 1988; Eliyahu et al., 1989; Finlay et al., 1989), cancer-prone patients with the Li-Fraumenis syndrome (LFS) carry germline p53 mutations (Malkin et al, 1990), and *p53*$^{-/-}$ mice, although developmentally normal, have increased susceptibility to developing tumors (Donehower et al., 1992; Jacks et al., 1994). By now, thousands of studies have confirmed the role of wild type p53 in tumor suppression.

p53 is a tetrameric transcription factor, heavily regulated by posttranscriptional modifications, that binds to p53 response elements composed of two decamers separated by a spacer of 0–14 nucleotides (el-Deiry et al., 1992; Funk et al., 1992). The p53 protein is made up of the usual features of a transcription factor, with an amino terminal transactivation domain, a sequence-specific core DNA binding domain (DBD) and carboxy terminal tetramerization and regulatory domains (Bullock and Fersht, 2001). p53 regulates a myriad of different genes that are central to its critical role in mediating diverse aspects of cell and organismal biology, including metabolism (Gottlieb and Vousden, 2010), development (Sah et al., 1995), differentiation (Zheng et al., 2008), and aging (Maier et al., 2004). More

important to my work, however, is the ability of p53 to efficiently block cell proliferation in order to inhibit cancer development. Under physiological conditions, p53 levels are kept low by its negative regulator, the E3 ubiquitin ligase MDM2, which targets p53 for ubiquitin-dependent degradation through the proteasome (Kubbutat et al., 1997). However, stress signals, such as genotoxic damage, oncogene activation, and hypoxia, disrupt the MDM2-p53 interaction, triggering p53 stabilization and tumor suppressive responses. The p53 responses include: (i) cytoplasmic or mitochondrial-dependent apoptosis (Yonish et al., 1991); (ii) cell cycle checkpoint arrest (Kastan et al., 1992); (iii) DNA repair (Smith et al., 1994); (iv) altered differentiation (Schmid et al. 1991); (v) senescence; (Shay et al., 1991) (vi) autophagy (Feng et al., 2005); and (vii) induction of reactive oxygen species (Johnson et al., 1996) (Figure 1.2.).

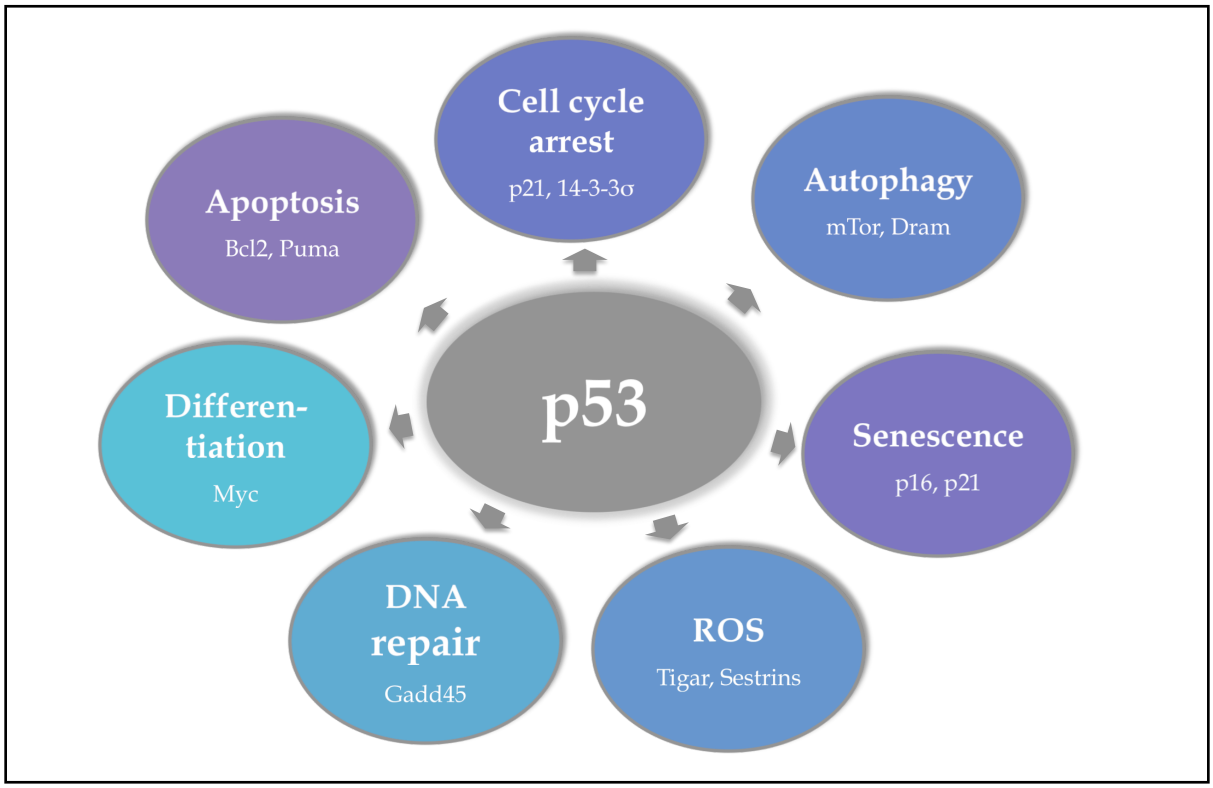


Figure 1.3. p53-dependent tumor suppressive pathways
Cancer development is stalled by the activation of p53 to control multiple pathways that influence proliferation and cell death. These mechanisms have the potential to lead to tumor suppression

p53's response in mediating tumor suppression strongly depends on the type of tissue, the nature of the stress signal, the extent of stress-induced damage, and the availability of survival signals (Oren, 2003). However, the mechanism of p53-mediated cell fate decisions remains to be scrutinized.

1.3.2. Missense mutations induce oncogenic properties

Remarkably, *p53* has been found to be the most frequently altered gene in cancer (Freed-Pastor and Prives, 2012). p53 expression and function is mainly perturbed by single-base pair substitutions but can also be altered through deletions, viral inactivation, and upregulation of negative regulators. Multiple lines of evidence have implicated that *p53* point mutations turn the p53 pathway into a tumor-promoting network that contribute to malignant progression. These mutations usually cancel the tumor suppressive effects of p53 by blocking its ability to bind DNA and transactivate p53 target genes. The presence of point mutations drastically alters p53 function and goes beyond simple loss of wild type function. On the one hand, the tetrameric nature of p53 allows many of the mutant forms to assemble with the wild-type protein, enabling the mutant forms to act in a "dominant- negative" manner in inactivating wild type p53 function. Thus, inactivating mutations that target only one of two wild type alleles in a cell can abrogate tumor suppression in a manner analogous to bi-allelic deletion of two wild type alleles. On the other hand, some missense mutations endow p53 with additional oncogenic properties, which actively promote the development of and aggressive and metastatic phenotype, collectively known as gain-of-function activities. Compelling evidence from animal and in vitro models, supported by human data, confirm that the acquisition of p53 point mutations is a crucial event during tumor

development and a driving force for overt malignant progression (Lozano, 2010). Even though the "gain-of-function hypothesis" has existed almost as long as p53 has been studied, the focus on understanding p53 mutants and their deleterious consequences on tumor progression has only been unveiled during the last decade (Girardini et al., 2014).

Mutations in *p53* occur in approximately half of all human cancers, but vary considerably between tumor types, ranging from 10% frequency in hematopoietic cancers to 70% in pancreatic malignancies (Brosh and Rotter, 2009). Mono-allelic single-base pair missense mutations comprise 77% of all mutant forms of *p53* and occur predominantly in the DNA binding domain. The resulting full-length proteins have no sequence specific-DNA-binding activity, and because *MDM2* is a p53-responsive gene, mutant p53 exhibits increased stability as compared to the wild type protein (Haupt et al., 1997; Kubbutat et al., 1997), leading to the accumulation of high levels of mutant p53 in tumor cells (Iggo et al., 1990; Strano et al., 2007). While 1,800 cancer-associated amino acid changes in the *p53* sequence have been reported (Soussi, 2011), mutations in ''hot spot'' residues occur with unusually high frequency. According to their effect on the thermodynamic stability of the p53 protein, mutations can be divided into two classes: "DNA contact" mutants refer to changes in residues that directly abrogate DNA binding ability (e.g. R248Q or R273H), and "conformational" mutants comprise those that cause local (e.g. R249S) or global (e.g. R175H) conformational distortions (Cho et al., 1994). Both categories not only result in a loss of function, but also in dominant-negative activity over the remaining wild type allele through the formation of hetero-oligomers (Chan et al., 2004; Xu et al., 2011). More importantly, a growing body of evidence supports the notion that p53 mutants acquire novel oncogenic functions. For instance, mutations in *p53* are accompanied with drug resistance through the

activation of the multi drug resistance 1 (*MDR1*) gene (Bush and Li, 2002) and are associated with poor clinical outcome of different cancer types (Goh et al., 2010). In fact, it seems that p53 mutants contribute to almost all of the so-called hallmarks of cancer: (i) invasion, (ii) metastasis, (iii) genomic instability, (iv) apoptosis resistance, (v) survival, (vi) drug resistance, and (vii) chronic inflammation (Figure 1.2.). However, a key question concerns how mutant p53 conveys a gain of function; knowing the answers may allow the identification of potential strategies to develop novel anti-cancer therapies.

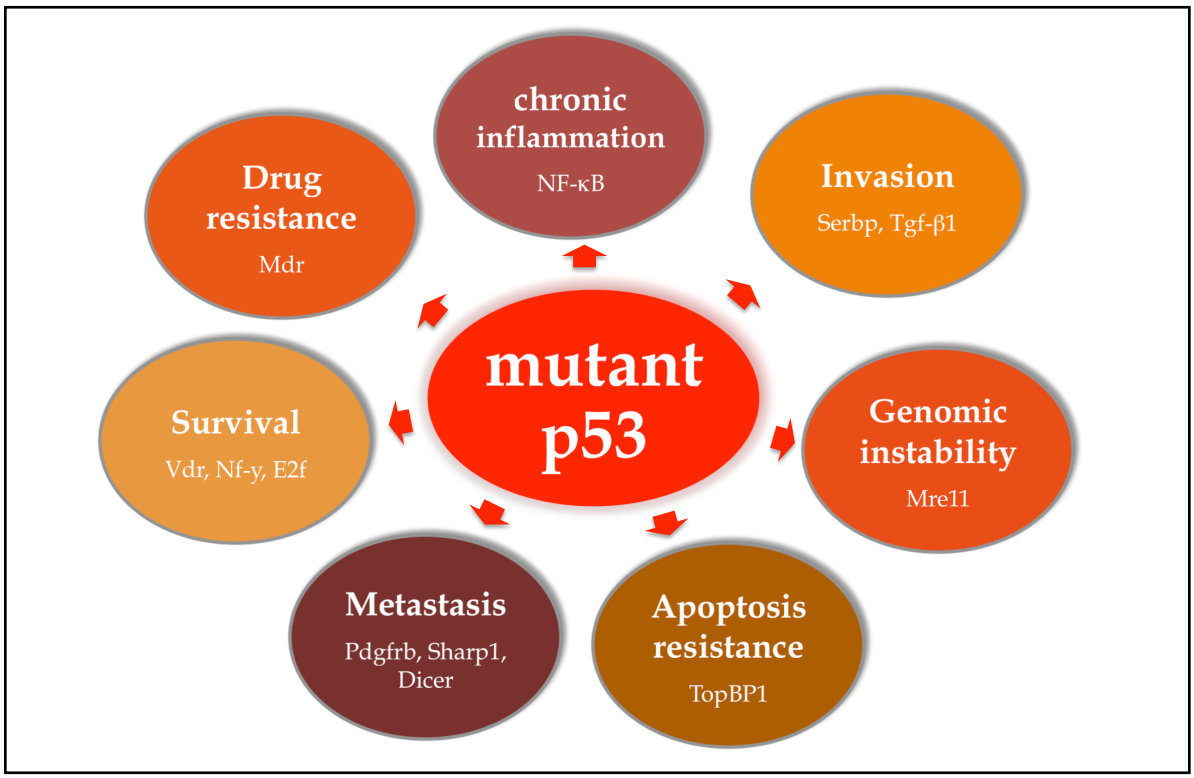


Figure 1.4. Gain of function activities of mutant p53
A summary of the biological effects of mutant p53 function and its mediators are indicated.

1.3.3. Gain of function mutation spectra

Mutant p53 carries out its gain of function properties through either one of two non-mutually exclusive mechanisms that involve the modulation of gene transcription or the

interaction of p53 with other cellular proteins to form aberrant protein complexes. Mutant p53 has been shown to form abnormal complexes with a wide variety of proteins to affect signaling through different pathways. For example, mutant p53 interacts with the prolyl isomerase PIN1 to potentiate formation of a complex with SMAD2 and p63 downstream of TGFβ signaling to increase invasiveness of breast cancers (Girardini et al., 2011). The nuclease MRE11 aggregates with mutant p53 to suppress the binding of the MRE11-RAD50-NBS1 (MRN) complex to DNA double-stranded breaks, thereby disrupting critical DNA damage-response pathways (Song et al., 2007). Moreover, mutant p53 can modulate gene transcription by interacting with other transcription factors, thereby impairing their ability to bind their target gene promoters to initiate transcription. The list of affected transcription factors is long and includes SP1, NF-Y, ETS1, ETS2, and the Vitamin D receptor, as well as transcriptional cofactors.

The most pivotal example of aberrant oligomerization with transcription factors represents the inhibitory effect of mutant p53 on its family members p63 and p73. Despite the homology of all three family members, neither p63 nor p73 form heterotetramers with wild-type p53, but do so, surprisingly, with different classes of mutant p53 (Di Como et al., 1999). These interactions, mediated through p53's DNA binding domain (Li and Prives, 2007), appear to explain a number of cellular properties conferred by p53 gain of function mutation, including chemoresistance (Sampath et al., 2001), cell migration (Adorno et al., 2009), and tumor metastasis (Muller et al., 2009).

As mentioned above, mutant p53 induces oncogenic functions also through DNA binding, a mechanism that is less well explored. Even though mutations in *p53* impair its

sequence-specific binding to DNA, mutant p53 retains its ability to bind to certain DNA structures with differential affinity (Gohler et al., 2005). These target structures are rich in repetitive elements and most likely adopt non-B conformations (Kogar et al., 2000). Hence, binding of mutant p53 to structural motifs rather than promoter sequences would explain the lack of consensus DNA sequences amongst genes regulated by mutant p53. Taken together, the study of p53 mutants has identified multiple routes to modify the gene expression landscape to impair or promote pivotal signaling pathways of a cell in order to exert oncogenic functions.

1.3.4. Mouse models of mutant p53

The most convincing evidence for neomorphic functions induced by a mutation in *p53* comes from genetically engineered mouse models (GEMMs) expressing mutant p53. The first proof of a gain of function *in vivo* came from germline transgenic mice that expressed a mutant *p53* genomic fragment under the control of its own promoter. These mice exhibited increased tumor susceptibility, including lung adenocarcinomas, osteosarcomas, and lymphomas (Lavigueur et al., 1989), which was later verified in mice that were manipulated at their endogenous *p53* locus to express mutant p53 under physiological control (Lang, et al., 2004; Olive, et al., 2004). Even though tumor-free survival was similar in $p53^{null}$ versus $p53^{mutant}$ mice, the mutant counterparts exhibited a higher incidence of metastasis (Lang et al., 2004) and were more prone to develop carcinomas instead of osteosarcomas (Olive et al., 2004). Taken together, mutant p53 acquires novel oncogenic potential beyond that conferred by loss of p53 function.

The role of mutant p53 has also been investigated in a tissue-specific manner. When mice expressing mutant p53 specifically in the liver or mammary tissues were challenged with carcinogens, tumorigenesis was accelerated and, in the latter context, increased genomic instability was observed (Ghebranious and Sell, 1998; Adams and Horton, 1998). Neomorphic functions of mutant p53 were also observed in collaboration with other oncogenes. For example, when $p53^{null}$, or $p53^{R172H}$, or $p53^{R270H}$ transgenic mice were crossed to animals harboring a lung-specific $Kras^{G12D}$ allele, mice expressing the R270H allele showed higher lung lesion progression than the $p53^{null}$ or the R172H mice, indicating that only some missense mutations exhibit observable gain-of-function in this cell type setting (Jackson et al., 2005). Many other mutant *p53* knock-in mice have exhibited not only increased tumor aggressiveness but also higher frequencies of metastasis. Examples include $Kras^{G12D}$, $p53^{R172H}$-driven non-melanoma skin tumors (Caulin et al., 2007); $Kras^{G12D}$, $p53^{R172H}$-driven pancreatic cancer (Morton et al., 2010); Apc, $p53^{R172H}$-driven intestinal cancer (Muller et al., 2009); and SV40 T-antigen, $p53^{R270H}$-driven breast cancer (Heinlein et al., 2008) models. Hence, tumors carrying a mutant form of p53 are more aggressive and invasive than those found in $p53^{null}$ mice. The *in vivo* evidence for mutant p53 gain of function is consistent with data from clinical studies of human cancer patients where a mutation in *p53* conveys with worse trends in prognosis and survival in patients with glioblastoma (Wang et al., 2013), colorectal (Russo et al., 2005), breast (Bonnefoi et al., 2011), ovarian (Levesque et al., 1995), and pancreatic cancer (Weissmueller et al, 2014).

The p53 family members p63 and p73 seem to be important downstream mediators of p53 tumor suppression. Interestingly, when p63 or p73 were deleted in $p53^{null}$ animals, compound mutant mice developed lymphomas, sarcomas and carcinomas that could more

readily metastasize (Flores et al., 2005; Tan et al., 2013). The tumor spectrum and enhanced metastatic burden were remarkably similar to the phenotypes observed in mice expressing $p53^{mutant}$ but exhibited reduced metastatic frequency, suggesting that mutant p53 signals through additional nodes beyond its family members.

In addition to insights about the neomorphic activities of mutant p53, GEMMs can also reveal intricate insights about the molecular characteristics of the mutant p53 protein. The question why p53 mutants accumulate to high levels in cancerous tissue but not in normal cells was tackled by manipulating its upstream regulators. When *$p53^{mutant}$* mice were studied in a *Cdkn2a*$^{-/-}$ or *Mdm2*$^{-/-}$ background, mutant p53 accumulated also in normal tissue and mice showed decreased overall survival (Terzian et al., 2008). Therefore, missense mutations in p53 alone are not sufficient to cause accumulation of high levels of mutant p53, but there are other events needed to impair MDM- or CDKN2A-mediated degradation.

The combined knowledge about mutant p53 raises the question whether targeting this core tumorigenic driver constitutes a promising therapeutic approach. Multiple approaches could potentially attenuate the oncogenic potential of mutant p53 by either (i) eliminating mutant p53, (ii) restoring mutant p53 to wild-type p53, (iii) targeting the p53 family members, or (iv) targeting downstream p53-mediators. Mouse models are ideal systems to study such therapeutic approaches and several of these approaches are currently being developed and tested. Most promising have been small molecules, such as PRIMA-1, which functions as a chaperon to stabilize the wild type p53 conformation, RETRA, which disrupts the interaction of mutant p53 with p63 and p73, and the HDAC inhibitor SAHA, which leads to mutant p53 destabilization. However, the validation of any beneficial

activities of these inhibitors in mice is only the first step in a long journey where the goal is to treat large numbers of cancer patients carrying mutant p53.

Mutant p53 GEMMs clearly support the gain of function hypothesis, but a number of critical questions continue to exist. First, it remains to be discovered whether different p53 mutants induce mutation-specific phenotypes. Second, questions remain regarding the selective pressure towards *p53* mutations rather than *p53* loss. In GEMMs, both modes of genetic alterations favor different tumor spectra and frequencies of metastasis but result in similar tumor latency, and, therefore, should be prevalent at equal rate in the cancer genome. Moreover, how mutant p53 drives cell dissemination and metastasis development remains unclear; however only a comprehensive understanding would allow the identification of novel targets and therapeutic strategies.

1.4. 17p deletions – simply LOH?

Cancer development is characterized by a stepwise progression. Pre-malignant lesions, such as dysplasia and hyperplasia, can be detected in diverse organs prior to the appearance of fully malignant invasive tumors. Expansion of pre-malignant lesions and further accumulation of genetic alterations results in malignant conversion and the development of a primary tumor. Fully malignant cells subsequently acquire the invasive and metastatic ability.

The chronology of genetic alterations during the tumorigenic process and their contribution to the distinct steps of malignant progression is an area of intense research.

Mutations in *p53* can occur in different stages of tumorigenesis and are extremely variable from one cancer to another. Moreover, they can contribute to the initiation of malignant transformation (Cagatay and Ozturk, 2002) or can arise as late events, leading to increased aggressiveness of advanced tumors (Hruban et al., 2000). However, the heterozygous state of mutated *p53* is transient and often followed by LOH for the short arm of chromosome 17 (17p), where the remaining wild type *p53* allele is located (Rivlin et al., 2011). Heterozygous 17p deletions occur frequently in human tumors and are often late events associated with the transition from benign to malignant states. For example, 17p loss is found in 7% and 15% of early chronic lymphocytic leukemia and colorectal cancer cases at diagnosis, respectively, but the frequency increases to 40% and 60% at the time of disease relapse or progression (Schnaiter and Stilgenbauer, 2013; Risio et al., 2003). Heterozygous 17p deletions might specifically arise from selective force to inactivate the remaining *p53* wild type allele to potentiate the gain of function activities of the mutated allele. However, losing the remaining *p53* allele could be achieved by focal deletions, a second mutation, or uniparental disomy (UPD) instead of losing an entire chromosome arm. Hence, the possibility remains that 17p loss attenuates the activity of multiple genes on the same chromosome arm that are separated by large distances. Therefore, the hypothesis arises that 17p events are selected during tumor progression not only to inactivate the *p53* allele but also because of the presence of linked tumor suppressor genes whose complete or partial loss individually may have only a modest effect on tumor growth.

The canonical view of the "two-hit hypothesis", where the inactivation of both copies of a tumor suppressor gene is considered a pre-requisite for tumor development, was first formulated in 1971. Tumor prone families with retinoblastoma were shown to transmit

one mutation in RB through the germ line and affected children developed disease early in life after acquiring a second mutation in the remaining wild type allele (Knudson, 1971). However, the cancer genome is rattled by the recurrent presence of large, heterozygous deletions and only 22% of such recurrent deletions can be explained by known or putative tumor suppressor genes (Solimini et al., 2012). Possible explanations for such frequent deleterious events go beyond Knudson's hypothesis and could include that (i) tumor suppressor genes on the remaining allele are silenced by mutations or epigenetic marks, (ii) unstable genomic regions induce large deletions randomly, or (iii) mono-allelic loss of one or multiple genes provides a selective growth advantage in a haploinsufficient manner. In depth analysis of chromosomal distribution of recurring deletions revealed that they preferentially overrepresent "STOP" genes and underrepresent "GO" genes, which negatively and positively regulate proliferation, respectively (Solimini et al., 2012). Therefore, the "cancer gene island hypothesis" proposes that heterozygous deletions do not occur randomly but are rather selected to target gene islands with high densities of STOP genes and low densities of GO genes. Moreover, recurrent cancer-associated deletions could reflect the selective advantage of simultaneously targeting multiple two-hit and/or haploinsufficient tumor suppressor genes. Those deletions might target more than just well-characterized tumor suppressor genes and could potentially encompass additional genes that can impact on tumorigenesis, supporting the idea that the biology mediated by these large deletions goes beyond the effects of individual genes. Functional studies support such hypothesis and elucidate that multiple genes within such regions contribute to the tumorigenic phenotype. For example, cosuppression of linked genes on chromosome arm 8p, a large deletion event occurring frequently in epelthelial cancers, promoted liver cancer formation more potently than loss of any individual gene (Xue et al., 2012). Collectively, the

prevalence of large, recurring heterozygous deletions results in the elimination of multiple haploinsufficient growth control genes to optimize fitness and drive tumorigenesis.

Chromosome	Frequency of LOH	Identified TSG
1p	32%	*p73*
4q	40 – 83%	-
6p	35 – 80%	*Igf2/M6p*
8p	50 – 60%	*Dlc1, Lpts*
10q	25%	*Pten*
13q	25 – 50%	*Rb, Brca2*
16p	40%	*Axin*
16q	36 – 70%	*E-cadherin*
17p	36 – 54%	*p53*

Table 1.1. Large somatic deletions occur frequently in human tumors (adapted from Nita et al., 2002)

Allelic loss of 17p and the presence of *p53* mutations are prognostic markers for resistance to treatment regimens. Given the gain of function caused by mutations in *p53*, it is attractive to hypothesize that LOH of the remaining allele is positively selected during tumorigenesis in order to lose the remaining *p53* wild type allele and potentiate mutant p53 neomorphic attributes. In some tumors, however, allelic losses of the entire 17p arm or encompassing the 17p13.3 band only have been detected in the absence of *p53* mutations (Risio et al., 2003), suggesting that the loss of gene function in this chromosome region is relevant for tumorigenesis. In fact, some tumor types favor a whole chromosome arm loss over focal peak deletions that encompass *p53* only, suggesting that the loss of other genes together with *p53* might have a cooperating effect to drive tumorigenesis (TCGA database).

The potential tumor suppressive function of other genes located on 17p has remained largely unexplored, despite evidence for the existence of a few, yet controversial, tumor suppressor genes in that region. Only few examples exist, including *MKK4* and *HIC1*.

The former is located on chromosome 17p11.2 and lies centromeric to p53. MKK4 is a mitogen-activated protein kinase (MAPKs) that translates external signals into activation of a series of downstream targets, including JNK and p38 (Whitmarsh and Davis, 2007). The MKK4 gene was first identified in 1995 during a search for MAP2Ks that could phosphorylate JNK, and its tumor suppressive role in cancer was first mentioned two years later (Teng at al., 1997). Multiple tumors exhibit loss-of-function mutations in, or LOH at, the *MKK4* locus (Su et al., 2002), and some studies suggest a link between the lack of MKK4 and metastasis formation (Yamada et al., 2002). However, the few existing studies about the role of MKK4 in tumorigenesis are contradictive, and some favor an oncogenic role of MKK4 in cancer development and progression (Wang et al., 2004; Finegan and Tournier, 2010).

HIC1 is another candidate tumor suppressor gene located on chromosome 17p, telomeric to *p53* (17p13.3). *HIC1,* a zinc-finger transcription factor, is a direct p53 target and represses the transcription of several genes, such as the SIRT1 deacytelase. Mono-allelic deletion of *HIC1* is frequently accompanied by hypermethylation of the second allele, indicating biallelic inactivation of the locus (Wales et al., 1995). The molecular mechanism underlying HIC1-mediated transcriptional and growth suppression remains elusive. One study suggested that HIC1 targets *E2F*-responsive genes responsible for transcriptional regulation and growth suppression whereas others have implicated HIC1 in the repression of *Ephrin-A1* transcription (Zhang and Yu, 2011)

How the loss of physically linked tumor suppressor genes synergizes to drive tumorigenesis remains elusive. The possibility remains, but is less likely, that none of these

frequently deleted genes is directly causally involved in tumorigenesis and that allelic losses are secondary effects of chromosomal instability. This might be especially true for 17p, as telomeres on 17p are shorter than the median telomere length (Martens et al., 1998), and the relatively short telomeres could possibly contribute to the frequent loss of 17p. Here, the loss of telomeres can result in sister chromatid fusion and prolonged breakage/fusion/bridge (B/F/B) cycles, leading to extensive DNA amplification and large terminal deletions (Bailey and Murnane, 2006). If indeed 17p deletions are initiated due to genomic instability, both alleles should be impacted at equal frequency. However, loss of 17p usually affects the chromosome arm carrying the wild type *p53* allele to allow the continued expression of mutant p53.

The idea of cooperating tumor suppressor genes that are simultaneously targeted by a chromosomal deletion challenges the view of a single "driver" gene in the region. The presence of large deletions on chromosomes, such as 3p, 5q, 8p, 9p, and 17p, suggest that deletion of linked cancer genes may play a broad role in cancer phenotypes. However, this idea greatly complicates the identification of genes with causal effects on tumorigenesis and strategies for cancer treatment if these genes affect different pathways.

In my graduate studies, I set out to understand causal effects of 17p alterations on tumorigenesis. As genetic changes are manifold and include mutations in the sequence of the tumor suppressor *p53* as well as subsequent LOH of the remaining allele, we studied both alterations individually to understand how they affect tumor growth and development. We first analyzed the gain of function of p53 mutants and how mutant p53 induces invasive and metastatic features of pancreatic cancer cells. By combining orthogonal

approaches, we found that mutant p53 regulates distinct mRNA and miRNA expression levels to carry out its enhanced oncogenic potential. On the other hand, we analyzed the causal effects of losing the remaining genes on 17p by LOH. We identified at least one additional tumor suppressor on 17p that can promote tumor growth and invasive potential in a haploinsufficient manner. Moreover, reduction of gene function of multiple genes on 17p has a cooperative effect on tumorigenesis. Together, these data suggest that 17p alterations result in different tumorigenic outcomes through contrasting molecular mechanisms. Therefore, *p53* mutations and LOH of the remaining 17p allele are under positive selective pressure, as both events allow for a more aggressive behavior of cancer cells than the individual alteration of *p53* alone.

Chapter 2

Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor beta signaling

2. Chapter 2

Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor beta signaling

2.1. Introduction

Mutations in the *p53* tumor suppressor gene represent the most common genetic lesions in cancer (Freed-Pastor and Prives, 2012). Functional studies indicate that wild-type p53 possesses a series of anti-proliferative activities that limit the proliferation and survival of pre-malignant cells. p53 exerts these activities, at least in part, through its ability to bind DNA in a sequence-specific manner to regulate gene expression, and the vast majority of mutations that occur in human tumors disable this property of p53 and, consequently, its anti-proliferative effects.

p53 mutations typically occur within the DNA-binding region and involve either DNA contact residues or residues important for conformational structure, both resulting in loss of DNA binding (Joerger and Fersht, 2007). Because p53 functions as a tetrameric transcription factor, mono-allelic *p53* mutations can exert dominant-negative effects on a coexpressed wild-type p53 protein. p53 activates E3 ubiquitin ligases that feed back to trigger p53 destruction and its rapid turn over; however, p53 missense mutants defective in regulating gene expression lead to the stable accumulation of the variant proteins (Oren et al., 2010). Interestingly, genetically engineered mice harboring common *p53* point mutations develop more aggressive and metastatic tumors compared to those arising in their *p53* heterozygous or null counterparts (Lang et al., 2004; Olive et al., 2004, Hanel et al., 2013),

suggesting that the mutant forms of p53 exert gain-of-function activities independent of their effects on wild-type p53. Accordingly, human tumors with mutant p53 are associated with poor patient prognosis (Soussi and Beroud, 2001) and drug resistance (Masciarelli et al., 2013).

Recently, targeting mutant p53 function has been proposed as an anti-metastatic measure. As p53 mutant proteins have to date proved undruggable (Levine and Oren, 2009; Lehmann and Pietenpol, 2012), efforts have focused on identifying the underlying mechanisms that mediate its effects. Such efforts have identified proteins involved in integrin recycling (Muller et al., 2009), the mevalonate pathway (Freed-Pastor et al., 2012) or miRNA biogenesis (Su et al., 2010) as potential mediators of mutant p53 action in invasion and metastasis. So far, most studies have been performed in breast cancer and the proposed mechanisms do not necessarily validate across cancer types. These observations underscore the importance of the cellular context in assessing mutant p53 action, and highlight the potential complexity of the effector network.

Pancreatic ductal adenocarcinoma (PDAC) is one cancer type in which mutant *p53* impacts disease progression. PDAC arises from indolent pancreatic intraepithelial neoplasias (PanINs) that frequently go undetected and persist for many years. However, the conversion of PanINs to highly aggressive, frankly invasive and metastatic PDACs, in which *p53* is mutated in 75% of cases, carries a dire prognosis due to late stage detection, the presence of metastases, and ineffective treatment options (Li et al., 2004). Even those patients with a surgically approachable pancreatic lesion develop recurrent and metastatic disease after local tumor resection (Hidalgo, 2010). Consistent with a role for mutant p53 in

this process, mice harboring pancreatic cancers driven by oncogenic *Kras* and a mutant *p53* allele show more metastases compared to identical mice harboring a *p53* null allele (Morton et al., 2010). However, it is not known whether mutant p53 is needed to sustain the metastatic phenotype and how it is regulated. Such information would produce insights into p53 action and validate mutant p53 as a therapeutic target.

In this study, we combined several orthogonal approaches and models to systematically explore the molecular basis whereby mutant p53 promotes invasion and metastasis in PDAC and the clinical implications of its effects. These studies identified the platelet-derived growth factor receptor beta (PDGFRb) as necessary and sufficient to mediate the effects of mutant p53 on invasion and metastasis in both a murine model and human PDAC cells. Further, we identified elevated PDGFRb expression as an indicator of poor metastasis-free survival in human PDAC patients. Taken together, our data identify a key mediator of mutant p53 activity and suggest that PDGFRb inhibitors may act as anti-metastatic agents in some patients with tumors expressing mutant p53.

2.2. Results

2.2.1. Transcriptional profiling and functional screening identifies PDGFRb as a downstream mediator of mutant p53

Genetically engineered mouse models of pancreatic cancer harboring a latent oncogenic *Kras* allele (*lox-stop-lox* $Kras^{G12D}$), a latent mutant $p53^{R172H}$, and a tissue specific *Cre* recombinase (*Pdx1-Cre*), also known as KPC mice, develop highly metastatic pancreatic cancer that faithfully mimics the human disease (Hingorani et al., 2005). To understand the impact of

p53 mutations on cell invasion and metastasis in this well-defined genetic system, we employed a murine KPC pancreatic cancer cell line that lost the remaining *p53* wild type allele during disease progression (Morton et al., 2010). The behavior of KPC cell lines stably expressing shRNAs targeting (mutant) *p53*, or control shRNAs targeting *Renilla* (KPC+sh.Ctrl), were compared to one another and to a *p53*-null $KP_{fl}C$ cell line (from a *Pdx-Cre, LSL-Kras*^{G12D}/+, *LSL-p53*$^{loxP/+}$) expressing the control shRNA ($KP_{fl}C$+sh.Ctrl).

We confirmed that KPC+sh.Ctrl cells expressing mutant p53 efficiently migrated in scratch-wound assays. This motility depended on mutant p53, as mutant p53 knockdown in KPC+sh.p53 cells reduced motility similarly to *p53*$^{-/-}$ $KP_{fl}C$ +sh.Ctrl cells (Figure 2.1.A). Next, we examined the invasive capacity of KPC cells into collagen gels in an inverted invasion assay. The presence of mutant p53 in KPC+sh.Ctrl cells enhanced invasiveness, which was significantly abrogated upon p53 knockdown (Figure 2.1.B). The ability of mutant p53 to drive cell invasion was also evident following enforced expression of $p53^{R175H}$ and $p53^{R273H}$, two mutants frequently found in human PDAC, in $KP_{fl}C$ cells. This result indicates that the differences in invasiveness of KPC and $KP_{fl}C$ cells depends on mutant p53 and were not acquired during generation and selection of cell populations expressing shRNAs or through RNAi off-target effects.

To test whether mutant p53 expression was required to sustain the metastatic potential of KPC cells, we orthotopically injected KPC+sh.Ctrl and KPC+sh.p53 cells into the pancreata of athymic mice and scored the number of metastases formed in both lung and liver, the most common sites of pancreatic cancer spread in patients. Although the primary tumor burden was independent of p53 status, tumors originating from

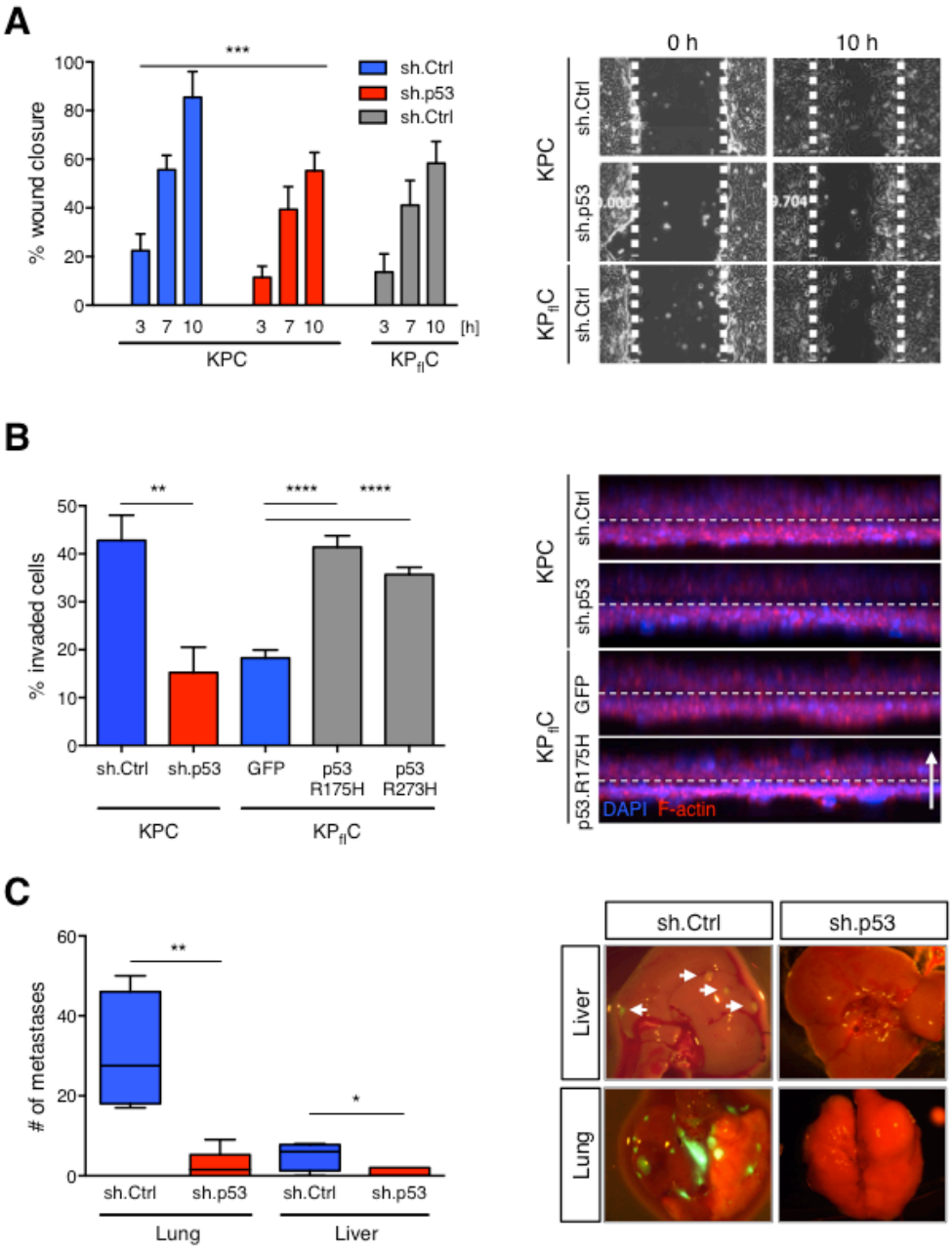


Figure 2.1. Depletion of Mutant p53 Abrogates Invasiveness of Pancreatic Cancer Cells

(A) Quantifications of wound distances in scratch-wound assays from 0, 3, 7, and 10 h after wounding of KPC cells stably expressing a nontargeting control shRNA (sh.Ctrl) or a shRNA targeting mutant p53 (sh.p53) or $KP_{fl}C$ cells stably expressing a sh.Ctrl (left panel). Data presented as mean ±SD. ***p < 0.001. Representative phase contrast images from live cell recordings of each condition are shown at 0 and 10 h (right panel). (B) KPC+sh.p53 and +sh.Ctrl as well as $KP_{fl}C$ cells expressing the GFP control and mutant p53 (175H and 273H) vector were allowed to invade into Collagen for 72 h before quantification as described in the Experimental Procedures (left panel). The average of invaded cells from 9 replicates ±SD is shown. A representative result of three repeated experiments is shown. **p < 0.01, ****p < 0.0001. Representative 3D reconstructions of each condition are shown (right panel). Cells were stained for F-actin (red) and DAPI (blue); dashed line indicates the approximate position of the Transwell membrane; the arrow indicates the direction of movement. (C) KPC+sh.p53 or +sh.Ctrl were orthotopically injected into the pancreata of athymic mice. When symptomatic, mice were euthanized and metastatic spread in lung and liver was quantified by counting GFP-positive macroscopic nodules (left panel). Data presented as mean ±SD. *p < 0.05, **p < 0.01. Panels show representative merged brightfield/GFP images from lung and liver (right panel).

KPC+sh.Ctrl cells expressing mutant p53 were significantly more metastatic than those in which mutant p53 was silenced, and metastases in the lung and liver were detected to a greater extent in mice that had been injected with cells expressing mutant p53 (Figure 2.1.C). Together, these results demonstrate that mutant p53 can contribute to PDAC invasion and metastasis and that inhibiting its activity can have an anti-metastatic effect.

To gain insight into how mutant p53 mediates the invasive phenotype of PDAC, we performed genome-wide transcriptome profiling of KPC cells by RNA sequencing (RNAseq). Four days following knockdown of mutant p53 in three independent clonal KPC populations, we observed a complex pattern of gene expression changes compared to three independent KPC+sh.Ctrl cell lines. We identified 441 genes either significantly up- or down regulated upon shRNA-mediated depletion of endogenous mutant p53 (Figures 2.2.A and S2.1.A). Ingenuity pathway analysis revealed that ~20% of the affected genes fall into the functional class of "cellular movement", supporting our experimental observations that mutant p53 can govern the invasive phenotype of pancreatic cancer cells (Figures 2.2.B and S2.1.C).

To facilitate the identification of mediators of mutant p53 activity, we focused on genes whose expression was positively regulated by mutant p53, as such molecules might both mediate effects of mutant p53 and be targets for pharmacological inhibition. Therefore, we generated pools of 3-6 shRNAs targeting individual upregulated genes, and screened them one-by-one to identify those that phenocopied the decreased invasion seen upon downregulation of mutant p53 (Table 2.1.). We identified three genes whose knockdown abrogated invasion driven by mutant p53 (Figure 2.2.C). SLC40A1 is a cell membrane

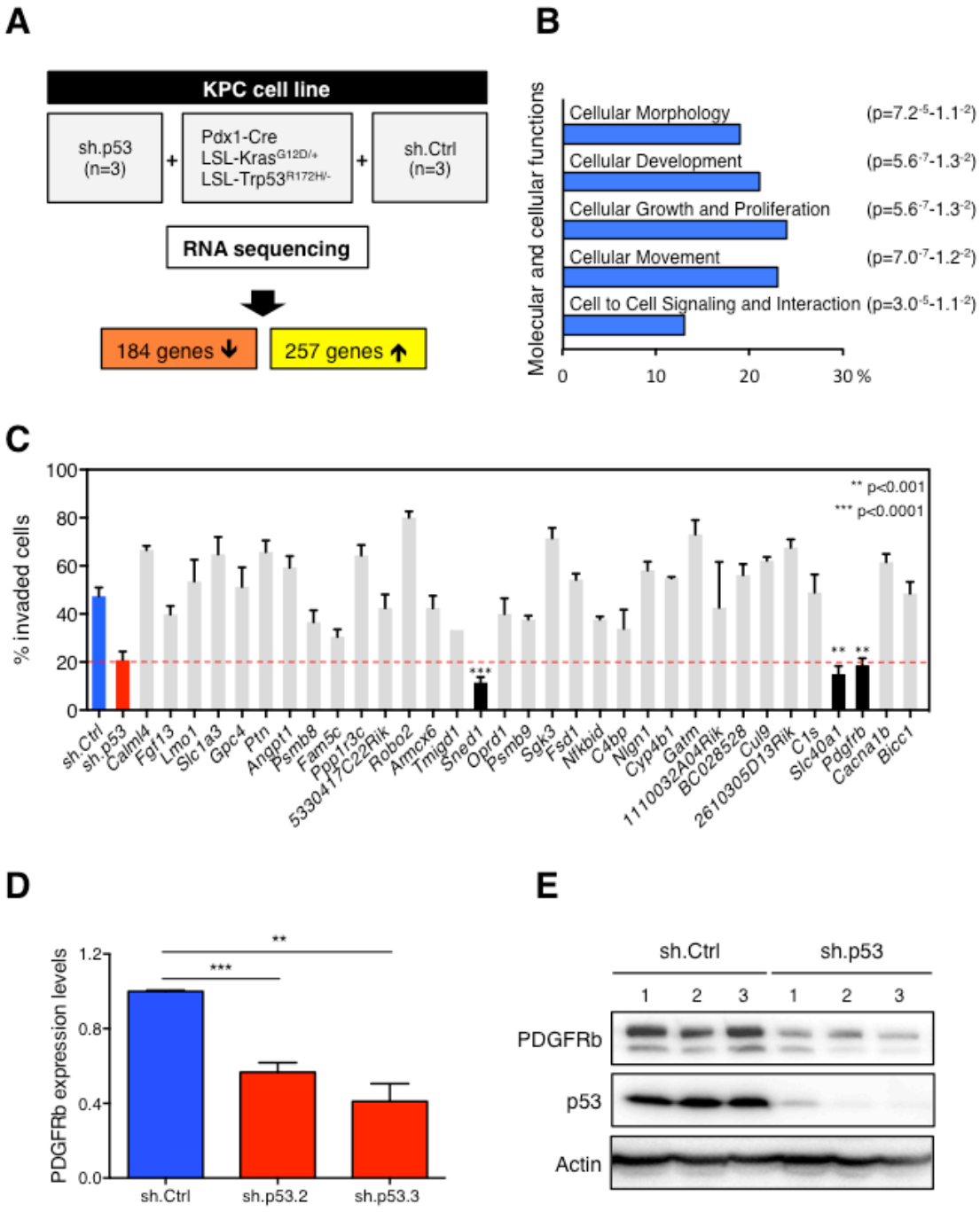


Figure 2.2. Identification of PDGFRb as a Downstream Mediator of Mutant p53 in Regulating Cell Invasion
(A) Schematic workflow of RNA sequencing. (B) Ingenuity pathway analysis (Ingenuity Systems, www.ingenuity.com). Bars ($p < 0.05$) represent molecular and cellular functions that are significantly changed following mutant p53 depletion. (C) One-by-one invasion assay screen. Quantification of invaded KPC cells infected with individual shRNA-pools (~3.6 shRNAs/gene) targeting the top 40 upregulated genes identified by RNAseq. Data presented as mean ±SD. (D) qRT-PCR for *PDGFRb* in KPC+sh.p53 (2 or 3) or +sh.Ctrl cells. Data presented as mean normalized *PDGFRb* expression ±SD of triplicate samples. A representative result of three repeated experiments is shown. (E) Western blotting analysis of PDGFRb, p53, and Actin in sh.p53- or sh.Ctrl-expressing KPC cells. The two bands of PDGFRb represent differentially glycosylated forms of the protein.

UP	DOWN
Slc1a3	Prl2c3
Calml4	Nckap1l
Fgf13	Vmn1r44
4933439K11Rik*	Gm14547
Lmo1	Armcx2
Gm13318*	1600029D21Rik
Gpc4	4930452B06Rik
Pde9a	Trim54
Ptn	Prl2c2
Angpt1	Selp
Pde8a	Serpinb9g
Psmb8	Prrg1
Fam5c	Slc25a48
Ppp1r3c	Prokr2
5330417C22Rik*	Rims2
Robo2	Fat2
Armcx6	Siglec15
Smarca1	Ctf1
Tmigd1	Spsb4
Sned1	Cd300lb
Oprd1	Gm15854
Psmb9	Klk8
Sgk3	Igfbp3
Fam198a*	Il18rap
Fsd1	Fam110c
Nfkbid	Acsbg1
C4bp	9030425E11Rik
Nlgn1	Nefl
Cyp4b1	Tnnt2
Gatm	Atg9b
1110032A04Rik	Stk39
BC028528*	Aqp8
Cul9	Tmem117
2610305D13Rik*	Klra4
C1s	Wnt10a
Unc5c	Hck
Slc40a1	Kcnf1
Pdgfrb	Ripply1
Cacna1b	Fam78b
Bicc1	Slc25a43

Table 2.1. Top 40 genes up- and downregulated in KPC cell expressing mutant p53 compared to p53-knockdown cells
** published transcriptional tragets of NF-Y*

protein that has been shown to mediate cellular iron efflux (Montalbetti et al., 2013); SNED1 is a stromal marker that induces cisplatin-resistance in head and neck squamous carcinoma (Longati et al., 2013); and PDGFRb is a receptor tyrosine kinase that mediates PDGF-regulated proliferation, survival and chemotaxis (Dai, 2010).

Oncogenic properties of mutated or amplified PDGFRa have been extensively studied in several tumor types, whereas PDGFRb has been exclusively linked to tumor angiogenesis via paracrine effects (Pietras et al., 2003; Cao et al., 2004). Based on our screening results, we hypothesized that PDGFRb may also have a cell-autonomous impact on cell invasion in pancreatic cancer. First, we verified by RT-qPCR and western blotting that PDGFRb mRNA and protein were reduced upon knockdown of mutant p53 (Figures 2.2D and 2.2.E). Expression of mutant p53 correlated with high PDGFRb expression levels and also with the expression of key downstream mediators of the PDGFRb signaling cascade (Figure S2.1.B).

We next examined the effects of depleting each PDGFR isoform on the invasive potential of KPC cells in vitro (Figure 2.3.A). Although knockdown of PDGFRa had no effect, depletion of PDGFRb decreased the ability of KPC cells to invade (Figure 2.3.B). Conversely, overexpression of PDGFRb in *p53*$^{-/-}$ $KP_{fl}C$ enhanced cell migration and invasion, similarly to cells over-expressing mutant forms of p53 (Figure S2.2.A). Reduced levels of PDGFRb in KPC cells neither altered the rate of cell proliferation (Figure S2.2.B) nor led to a competitive proliferative disadvantage over KPC cells expressing high PDGFRb levels (Figure S2.2.C). When GFP-positive KPC+sh.PDGFRb cells were mixed with dsRED-positive KPC+sh.Ctrl cells and injected subcutaneously into athymic mice, the GFP:dsRED ratio of

pre-injected cells was maintained in the established tumors, indicating that increased PDGFRb levels did not confer a selective advantage to tumor cell proliferation at the site of injection (Figure S2.2.D). Thus, cell-autonomous activity of PDGFRb is not required for the proliferation and tumorigenic potential of p53 mutant murine cancer cells but specifically impacts their invasive potential.

To determine if the mutant p53-PDGFRb signaling axis acts in human cancer cells, we analyzed PDGFRb expression levels in a panel of human pancreatic cancer cell lines. As in our model, PDGFRb mRNA levels were significantly higher in cells expressing mutant p53 compared to those in cell lines that maintained or lost the wild type *p53* allele (Figure 2.3.C). Furthermore, knockdown of mutant p53 in Miapaca2, BXPC3, CFPAC and A2.1 cell lines (carrying the 248W, 220C, 242R, and 155P alleles, respectively) decreased PDGFRb mRNA levels to varying degrees (Figure 2.3.D). Knockdown of mutant p53 also decreased PDGFRb expression in several human colon (SW620, $p53^{273H/P309S}$), lung (H1975, $p53^{273H}$), and breast (MDA-MB-231, $p53^{280K}$) cancer cell lines (Figure S2.3.A). Thus, the ability of mutant p53 to induce PDGFRb levels is not strictly confined to a particular *p53* allele or tumor type.

We further analyzed the functional connection between mutant p53 and PDGFRb in promoting the invasiveness of human PDAC lines. Consistent with our studies in mouse PDAC lines, knockdown of either mutant p53 or PDGFRb reduced invasiveness of the A2.1 pancreatic cancer cell line (Figure 2.3.E). Conversely, overexpression of PDGFRb in the human $p53^{-/-}$ ASPC pancreatic cancer cell line enhanced invasion compared to cells infected with a GFP control vector (Figure S2.3.B). Collectively, these results confirm that

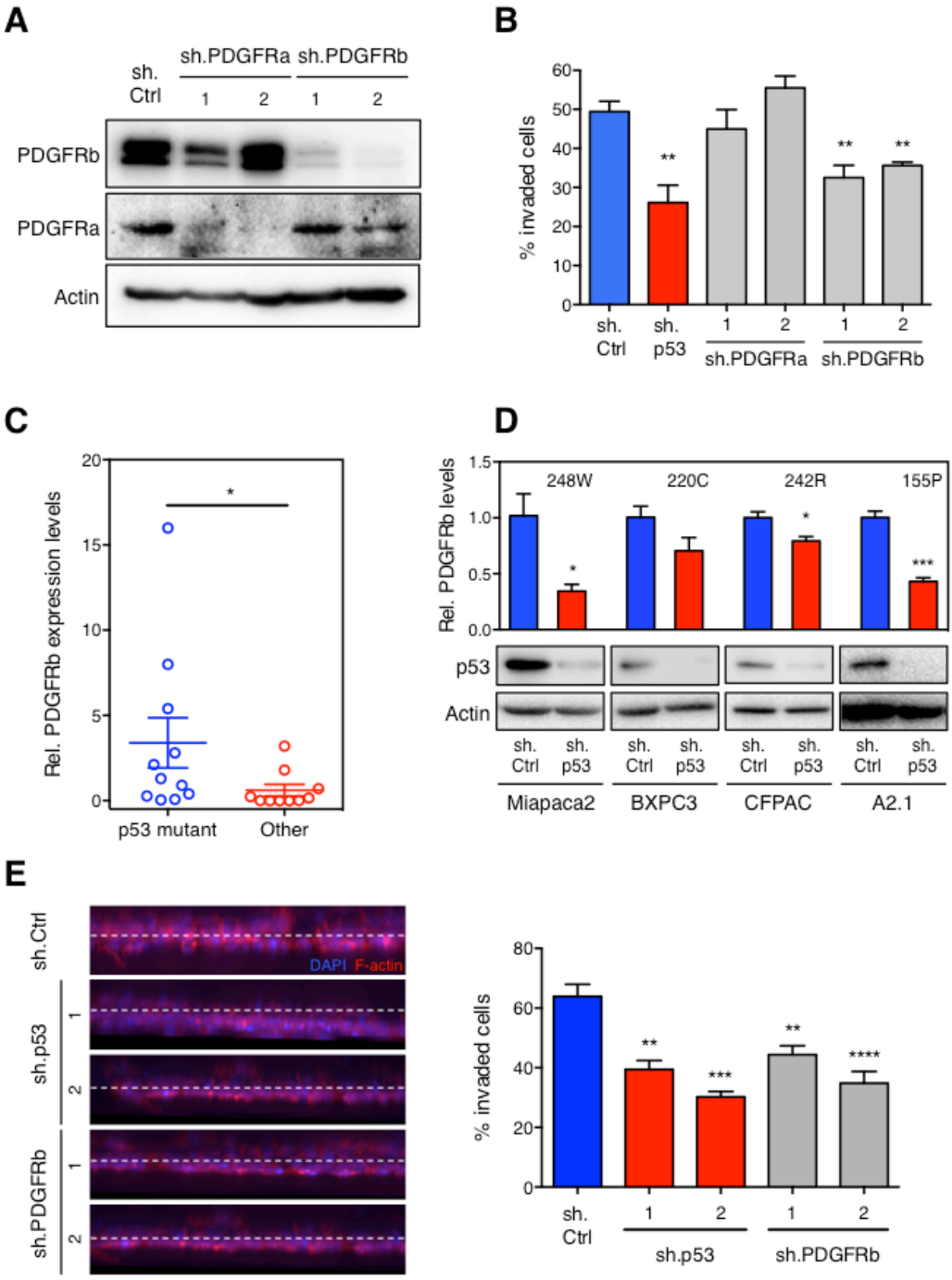


Figure 2.3. Depletion of Mutant p53 in Murine and Human Pancreatic Cancer Cells Decreases PDGFRb Expression Levels to Enhance Cell Invasion

(A) PDGFRa, PDGFRb and Actin levels of KPC cells infected with shRNAs targeting PDGFRa, PDGFRb or a nontargeting control (Ctrl) as determined by western blotting. (B) Quantification of the invasion into collagen of cell lines from (A), compared to KPC+sh.p53. Data presented as mean ±SD. **p < 0.005. (C) qRT-PCR for PDGFRb in 21 human pancreatic cancer cell lines of different *p53* status. Data presented as mean normalized PDGFRb expression ±SD. (D) qRT-PCR for *PDGFRb* in the human pancreatic cancer cell lines Miapaca2, BXPC3, CFPAC, and A2.1 expressing sh.p53 or sh.Ctrl. Data presented as mean normalized *PDGFRb* expression ±SD. *p < 0.05, ***p < 0.001. p53 mutation of each cell line as indicated. p53 and actin levels were determined by western blotting (lower panel). (E) Quantification of invasion of human A2.1 cells infected with sh.PDGFRb, sh.p53 or sh.Ctrl (right panel). Data presented as mean ±SD. **p < 0.005, ***p < 0.001, ****p < 0.0001. Representative 3D reconstructions of invaded cells are shown (Left panel).

upregulation of PDGFRb receptor is important for the action of mutant p53 in PDAC and possibly other tumor types.Mutant p53 disrupts the p73/NF-Y complex to mediate PDGFRb expression and tumor cell invasion

2.2.2. Mutant p53 disrupts the p73/NF-Y complex to mediate PDGFRb expression and tumor cell invasion

Several pro-oncogenic properties of mutant p53 depend on its ability to physically interact with and inhibit the p53 family members, p63 and p73 (Li and Prives, 2007). Since a previous report indicated that p73 can repress the transcription of PDGFRB (Hackzell et al., 2002), and because our KPC cells expressed p73 but not p63 as determined by RNA-seq (data not shown), we aimed to understand whether the physical interaction of mutant p53 with p73 might impair the ability of p73 to negatively regulate the expression of PDGFRb.

First, we verified the interaction between p73 and mutant p53 proteins by reciprocal co-immunoprecipitation (Figure 2.4.A) and, consistent with previous reports, p73 binding to a "conformation" p53 mutant (R175H) appeared stronger than to a "DNA-binding" p53 mutant (R273H) (Gaiddon et al., 2001; Muller et al., 2009). Next, using a luciferase reporter driven from the *PDGFRB* promoter, we confirmed that overexpression of p73 in $KP_{fl}C$ cells decreased transcriptional activity of *PDGFRB* and, conversely, that knockdown of endogenous p73 increased luciferase expression (Figures 2.4.C and S2.4.A). We also observed a similar increase in luciferase signal upon overexpression of two distinct forms of mutant p53 in $KP_{fl}C$ cells (Figure 2.4.C) as well as a significant decrease upon depletion of mutant p53 or overexpression of p73 in KPC cells (Figure S2.4.C). Importantly, depletion of endogenous p73 in KPC cells expressing mutant p53 did not enhance the transcription of

PDGFRB (Figure S2.4.C), indicating that the repressing activity of p73 is regulated by its interaction with mutant p53. Hence, mutant p53 cancels the ability of p73 to repress PDGFRb transcription, leading to an increase in its expression.

To better understand how p73 represses *PDGFRB* transcription, we performed chromatin immunoprecipitation (ChIP) analysis in $KP_{fl}C$ cells but failed to detect direct binding of p73 to the *PDGFRB* promoter (data not shown), a result consistent with previous reports (Matys et al., 2006). Nevertheless, promoter analysis of *PDGFRB* (Transfec®, Biobase) identified a conserved CCAAT binding motif for NF-Y, a well-characterized heterotrimeric transcriptional activator (NF-YA, NF-YB, and NF-YC) of *PDGFRB*, where the NF-YB subunit interacts with p73 but is devoid of transcriptional activity (Ballagi et al., 1995; Ishisaki et al., 1997; Serra et al., 1998). We verified NF-Y binding to the *PDGFRB* promoter by use of ChIP analysis (Figure 2.4.B). Remarkably, this binding was prevented by p73 overexpression, suggesting that the p73/NF-Y interaction hampers its ability to bind and activate the *PDGFRB* promoter (Figure 2.4.B). Indeed, when we immunoprecipitated p73 in $KP_{fl}C$ cells, we detected direct binding of NF-YB to p73 (Figure 2.4.A). This interaction was abrogated upon expression of mutant p53, indicating that it disrupts or interferes with the formation of the inhibitory p73/NF-Y complex (Figure 2.4.A).

Next, we tested whether the repressive action of p73 on *PDGFRB* transcription was mediated by NF-Y and modulated by mutant p53, and the implications of this regulatory circuit for invasion. Interestingly, the ability of p73 overexpression to inhibit the *PDGFRB*-luc reporter was abolished by depletion of NF-YB (Figures 2.4.D and S2.4.B), and NF-YB knockdown suppressed the ability of mutant p53 to enhance PDGFRb expression in $KP_{fl}C$

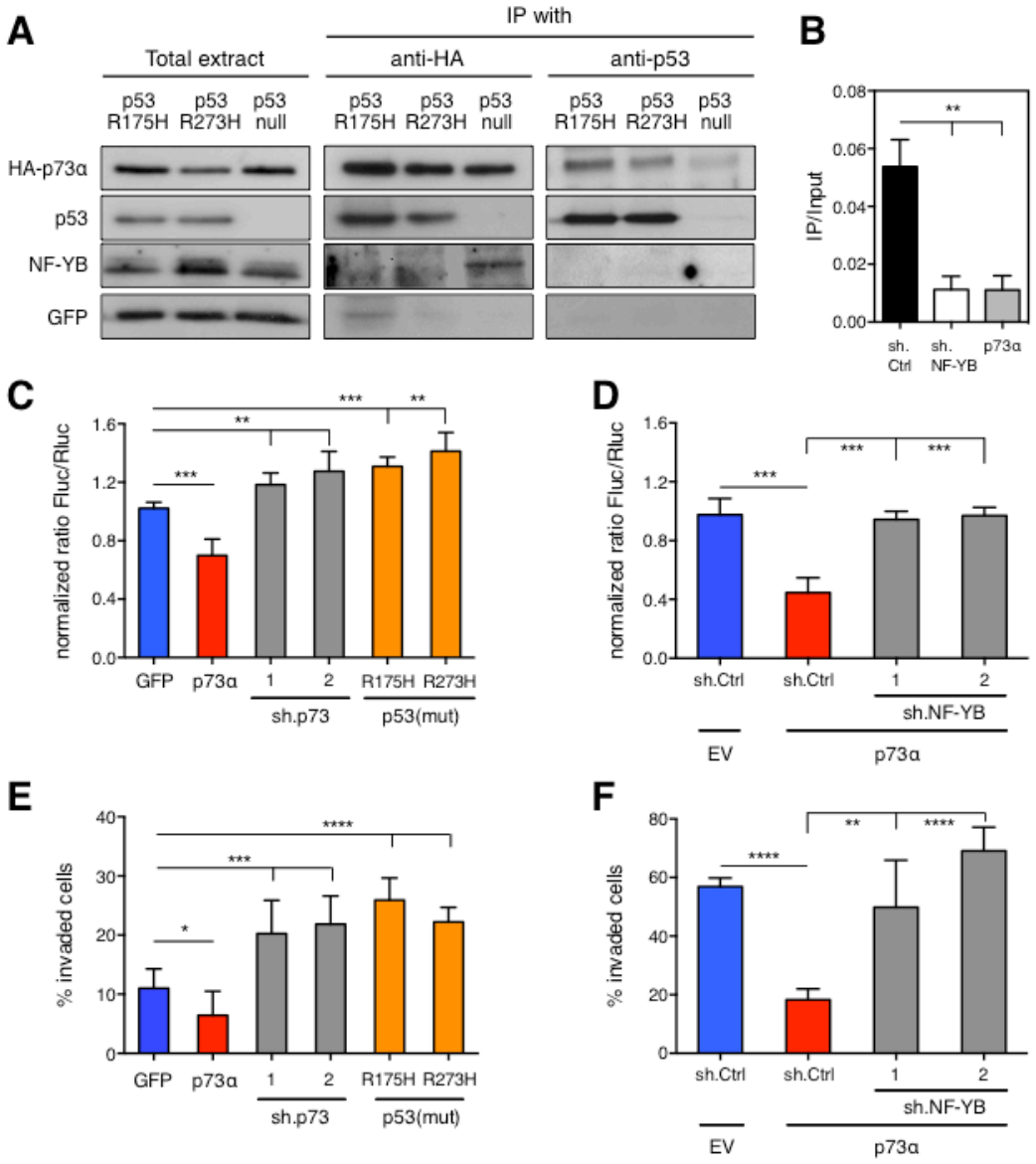


Figure 2.4. Mutant p53 Sequesters p73 to Impede the Repressive Function of the p73/NF-Y Complex on the *PDGFRB* Promoter

(A) $KP_{fl}C$ cells stably expressing a GFP-, $p53^{R175H}$-, or $p53^{R273H}$ vector were transfected with HA.TAp73□. Either p53 or HA were immunoprecipitated and the expression of HAp73α, p53, or NF-YB was determined in both the input (10% of lysates) and immunoprecipitation. (B) Chromatin immunoprecipitation (ChIP) using NF-YB antibodies in $KP_{fl}C$ cells stably expressing sh.Ctrl, sh.NF-YB or HAp73α. Values are means ±SD. **p < 0.01. (C) $KP_{fl}C$ cells stably expressing a GFP-, HAp73α-, $p53^{R175H}$-, or $p53^{R273H}$ vector or sh.p73 (1 or 2) were co-transfected with the *PDGFRB*-promoter-luciferase construct and renilla-luciferase vector. Firefly-luciferase activity of GFP-vector cells was set to 1. Values are relative Firefly-luciferase (Fluc) units normalized by renilla expression (Rluc) ±SD of quadruplicate samples. **p < 0.01, ***p < 0.001. A representative result of three repeated experiments is shown. (D) $KP_{fl}C$+GFP cells as well as $KP_{fl}C$+HAp73α superinfected with sh.Ctrl, or sh.NF-YB (1 or 2) were co-transfected with the *PDGFRB*-promoter-luciferase construct and *Renilla*-luciferase vector. Luciferase activity was measured as described above. ***p < 0.001. (E) Quantification of invasion of the same cells as in (C). Data presented as mean ±SD. *p < 0.05, ***p < 0.001, ****p < 0.0001. (F) Quantification of invasion of same cells as in (D). Data presented as mean ±SD. **p < 0.01, ****p < 0.0001.

cells (Figure S3D). As expected, p73 overexpression reduced the invasive potential of $KP_{fl}C$ cells, whereas knockdown of p73 or overexpression of mutant p53 significantly increased invasiveness (Figure 2.4.E). Conversely, as occurred in mutant p53 cells following p73 overexpression, depletion of p53 also reduced the invasive behavior in KPC cells (Figure S2.4.E). Finally, NF-YB knockdown restored the invasive potential of $KP_{fl}C$ cells that overexpressed p73 (Figure 2.4.F) and suppressed the ability of mutant p53 to enhance cell invasion (Figure S2.4.F). Together these results support a model in which mutant p53 promotes invasion in pancreatic cancer cells, in part, via an indirect mechanism that depends on its ability to enhance PDGFRb expression through the disruption of the inhibitory p73/NF-Y complex (Figure 2.5.).

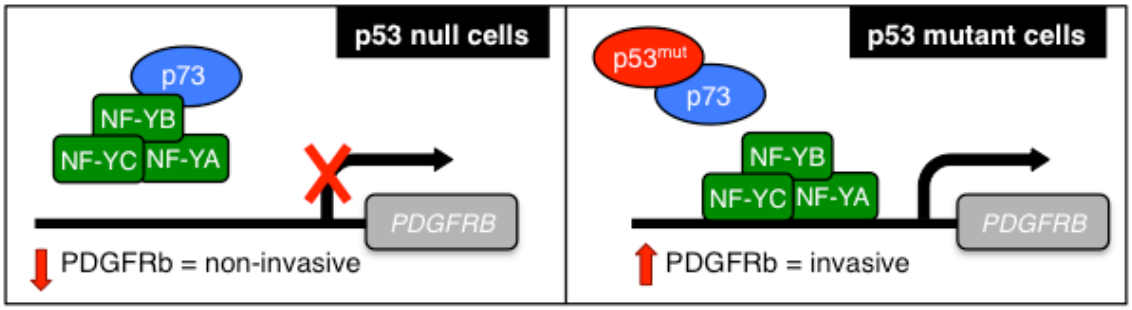


Figure 2.5. Scheme summarizing the mechanism of action of mutant p53 in promoting invasiveness

2.2.3. Modulation of PDGFRb expression levels mediates the phenotypic effects of mutant p53 depletion in vivo

Following the observation that p53 mutants induce the expression of PDGFRb to promote cell invasion in PDAC cultures, we investigated whether PDGFRb levels regulate metastatic behavior of PDAC cells in mice. To this end, we performed a lung colonization assay by injecting KPC+sh.p53, KPC+sh.PDGFRb, KPC+sh.PDGFRa or KPC+sh.Ctrl-cells intravenously via the tail vein into athymic mice and scored the number of colonies formed

in the lungs. We found that, whereas KPC+sh.Ctrl and KPC+sh.PDGFRa cells expressing mutant p53 formed tumor nodules in the lungs at high frequency, PDGFRb depletion significantly reduced the number of lung colonies, phenocopying the anti-metastatic effect observed upon knocking down mutant p53 (Figures 2.6.A and S2.5.A). However, depletion of PDGFRb did not affect the size of the metastatic foci, suggesting that PDGFRb does not alter their capacity to grow and proliferate in a new environment (Figure S2.5.B). In whole lung sections, GFP-positive signals coincided with metastatic nodules, indicating that metastases formed from cells expressing shRNAs, and likely not from the proliferation of tumor cells that lost shRNA expression (Figure S2.5.C).

We next sought to examine whether pharmacologic inhibition of the PDGFRb pathway recapitulates the effects of PDGFRb or mutant p53 depletion (Figure 2.6.A and S2.5.A). We used the compound crenolanib, a small molecule inhibitor of type III tyrosine kinases, potent against PDGFRa, PDGFRb, and FLT3 but not other known receptor tyrosine kinases (VEGFR, FGFR), serine/threonine (RAF) or tyrosine kinases (ABL1) (Lewis et al., 2009). We assessed the potency and efficacy of crenolanib on inhibiting the viability of murine KPC and human A2.1 pancreatic cancer cells and found that the dose-response patterns were comparable between the two cell lines, with IC50 values of 13.1 μM and 8.5 μM, respectively (Figure 2.6.B). Strong inhibition of PDGFRb activity, as measured by phospho-PDGFRb, was achieved in both cell lines at 0.3 μM, a dose at which no toxicity was observed (Figures 2.6.C and S2.5.D). Time course experiments revealed that strong target inhibition was achieved within 10 min of drug treatment (Figure S4E). Accordingly, crenolanib treatment of KPC and A2.1 cells substantially reduced invasion relative to that seen with cells treated with DMSO (Figure 2.6.D).

To test whether crenolanib can suppress metastasis, KPC cells were pretreated with the drug overnight and injected intravenously into recipient mice that were subsequently assessed for colony formation in the lung. Although drug treatment had no effect on the viability of the injected cell population, mice injected with drug-treated KPC cells showed significantly fewer lung nodules compared to controls pre-treated with DMSO (Figures 2.6.E and S2.6.A). Conversely, the same concentration of crenolanib did not reduce the metastatic potential of $KP_{fl}C$ cells in a lung colonization assay, suggesting that PDGFRb acts autonomously in KPC cells to potentiate cell invasion and metastasis (Figure S2.6.B). Further supporting this notion, conditioned media from KPC cells and most human pancreatic cell lines tested triggered PDGFRb phosphorylation in serum-starved 3T3 cells, indicating pancreatic cancer cells can provide a source of PDGF ligand that could trigger autocrine activation of PDGFRb (Figure S4H and data not shown). Therefore, abrogation of this signaling by RNAi or small molecule inhibitors leads to a significant reduction of invasion and metastasis driven by mutant p53 in vitro and in vivo.

The results described above imply that pharmacologic inhibition of PDGFRb could have anti-metastatic effects. We therefore sought to determine whether PDGFRb inhibition prevents metastasis in KPC mice that develop metastatic disease with a variable latency of 3 to 10 months in 50 - 80% of animals (Hingorani et al., 2005). For long-term treatment of KPC mice, we decided to use FDA-approved imatinib, a potent inhibitor of PDGFRb, c-KIT and BCR-ABL activity. Notably, the c-KIT and BCR-ABL kinases have not been linked to PDAC development (Jones et al., 2008).

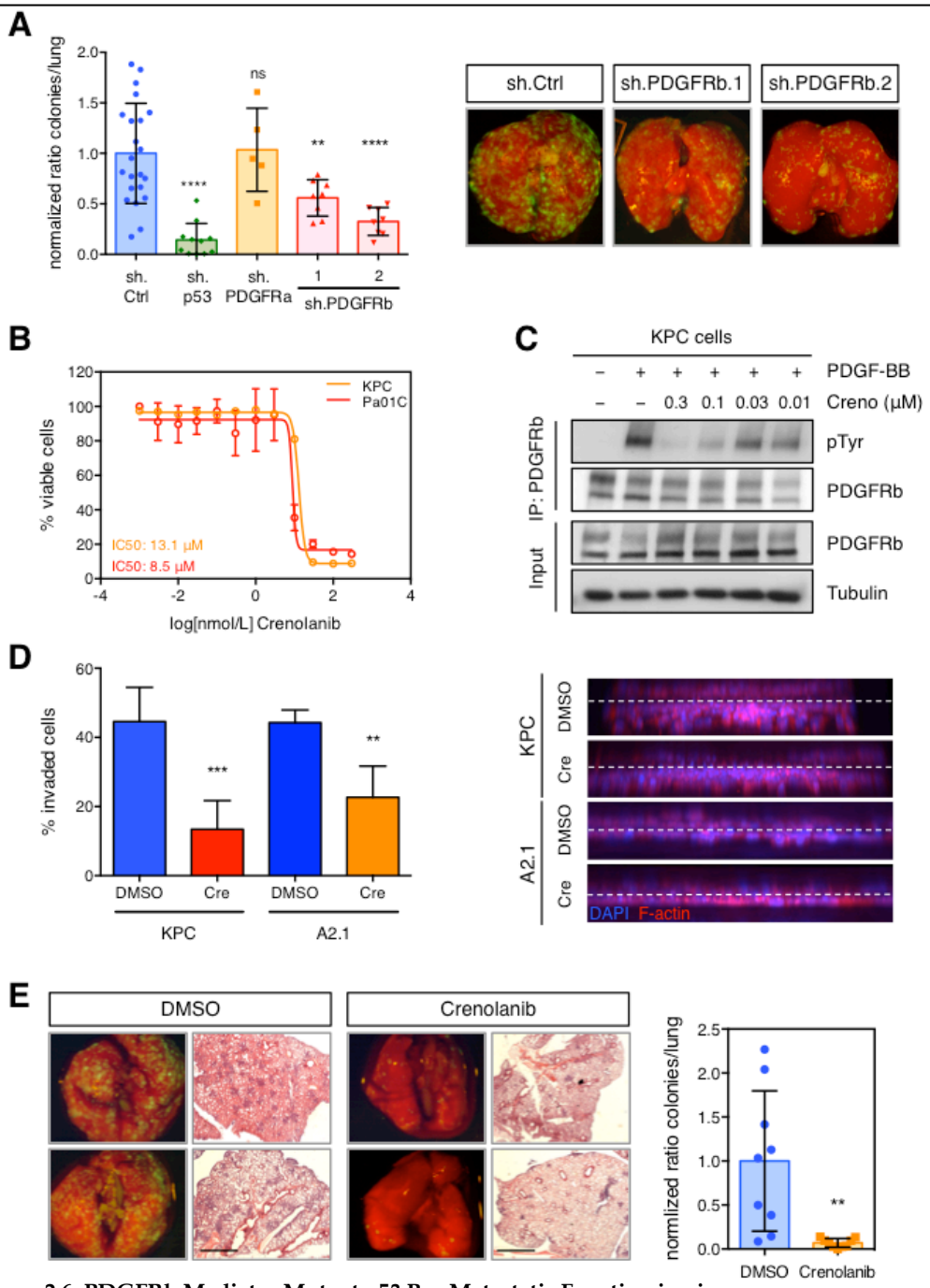


Figure 2.6. PDGFRb Mediates Mutant p53 Pro-Metastatic Function in vivo

(A) Lung colonization assays after tail vein injection of KPC cells +sh.PDGFRa, +sh.PDGFRb (1 or 2), +sh.p53, or +sh.Ctrl. Total number of lung metastatic nodules in individual mice (n>6) was counted on serial histological sections (left panel). Data presented as mean ±SD. **p < 0.01, ****p < 0.0001. Representative merged brightfield/GFP images of whole lung from indicated mice (right panel). (B) MTS assay (E_{490}) of murine KPC and human A2.1 cells treated with crenolanib with various doses for 72 h. Normalized values presented as mean ±SD form quadruple replicates. (C) Immunoprecipitation of PDGFRb from KPC cells stimulated with 50 ng/ml PDGF-BB, after crenolanib or DMSO treatment for 4 h. Protein levels of PDGFRb, phospho-Tyrosine and Tubulin were determined by western blotting. (D) Quantification of invasion of murine KPC and human A2.1 treated with either DMSO or crenolanib at 300 nM (left panel). Data presented as mean ±SD. **p < 0.01, ***p < 0.001. Representative 3D reconstructions of invaded cells are shown (right panel). (E) Lung colonization assays after tail vein injection of crenolanib (300 nM)- or DMSO-treated KPC cells. Representative merged brightfield/GFP imaged of whole lung as well as H&E stains of pulmonary lobes are shown (left panel). Quantification of total number of lung metastatic nodules in individual mice (n > 6) (right panel).

Inhibition of PDGFRb by imatinib in KPC cells strongly reduced PDGFRb tyrosine phosphorylation at 3 uM (Figure 2.7.A), a dose of the drug that is significantly lower than that required to inhibit cell proliferation (IC50 of 29.7 μM) (Figure S5A). Nonetheless, imatinib treatment significantly reduced the invasive potential of KPC cells in vitro (Figure S5B). More importantly, pre-treatment of KPC cells with imatinib decreased their potential to colonize the lungs of recipient athymic mice to a similar extent as that seen upon crenolanib treatment (Figure 2.7.B).

We next treated KPC mice with imatinib to assess its effects on metastasis. To this end, mice were treated with a dose of 50 mg/kg imatinib by oral gavage twice daily, a regimen previously shown to produce therapeutic concentrations of imatinib in mice (Wolff et al., 2003). Treatment was initiated in mice of 8 weeks of age, a time at which KPC mice have developed preneoplastic lesions (Hingorani et al., 2005), and mice were monitored until they became symptomatic. Imatinib had no impact on tumor volume in the pancreas or overall survival, suggesting that the high disease burden in the pancreas was the primary cause of death (Figures 2.7.C and S5C).

However, Imatinib induced a striking reduction in the occurrence of metastasis. The incidence of metastasis was 92% in vehicle-treated animals compared to 15% in mice treated with imatinib, as assessed by macroscopic examination and confirmed by histopathological analyses ($\chi 2$ test, $p<0.0001$) (Figures 2.7.D and S5D). The anti-metastatic effect was observed across several organs such as liver, peritoneum and lung (Figures 2.7.E and 2.7.F). As expected, imatinib was able to effectively inhibit PDGFRb activity in primary tumors based on reduced levels of phospho-PDGFRb in the tumor cells (Figures 2.7.G and S5E). Together,

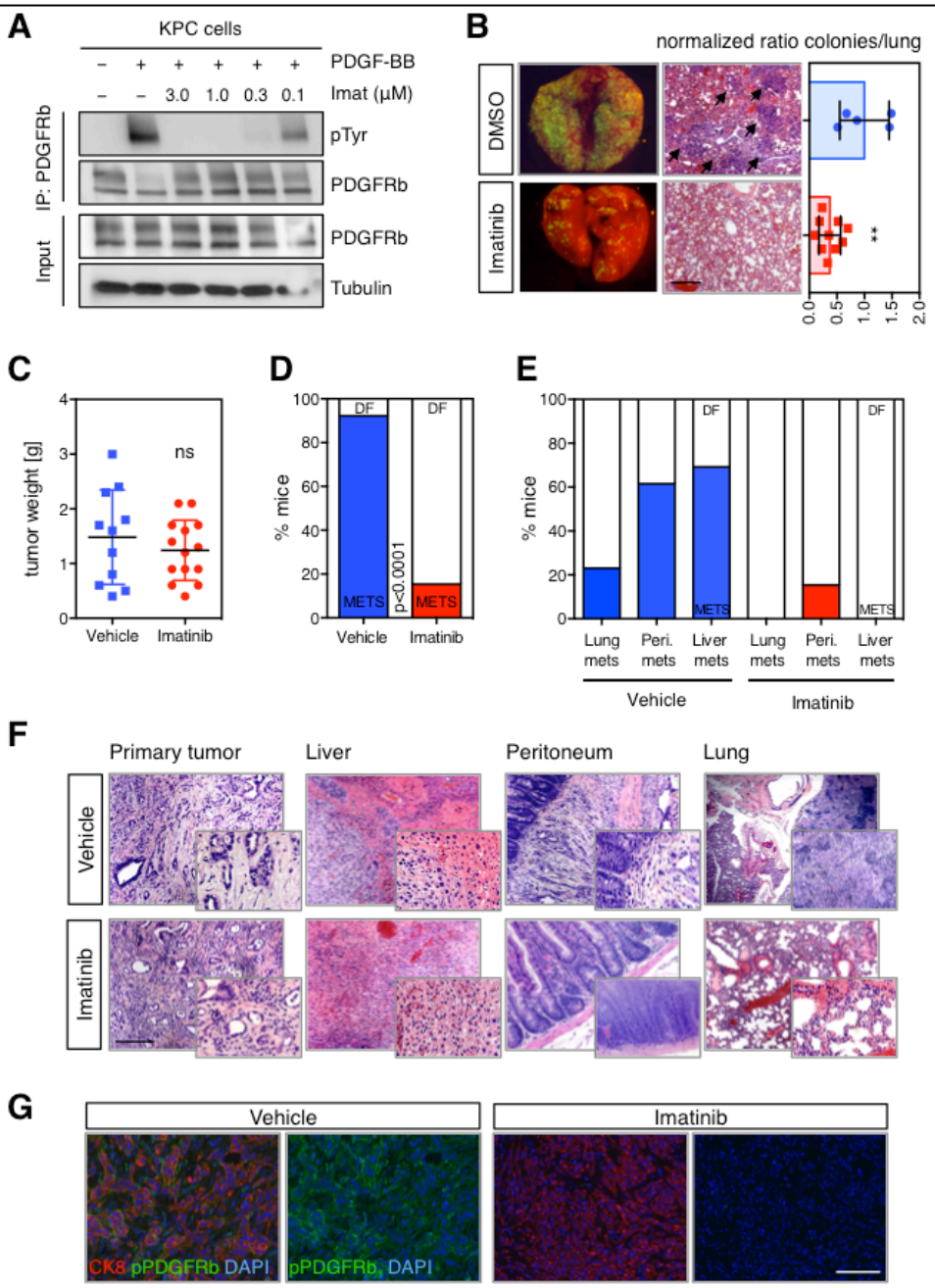


Figure 2.7. Imatinib Reduces the Incidence of Metastasis in KPC Mice Through PDGFRb Inhibition
(A) Immunoprecipitation of PDGFRb from KPC cells stimulated with 50 ng/ml PDGF-BB, after imatinib or DMSO treatment for 4 h. Protein levels of PDGFRb, phospho-Tyrosine and Tubulin were determined by western blotting. (B) Lung colonization assays after tail vein injection of imatinib (3 μM)- or DMSO-treated KPC cells. Representative merged brightfield/GFP imaged of whole lung as well as H&E stains of pulmonary lobes are shown. Arrows indicate metastases (left panel). Quantification of total number of lung metastatic nodules in individual mice (n > 5) (right panel). Data presented as mean ±SD. **p < 0.01. Scale bars represent 100 μm. (C) Weight of pancreatic tumors of KPC mice treated with vehicle or imatinib at time of death. (D) Quantification of the number of mice with metastatic disease at the time of death. Values are percentages of the total number of mice in each cohort. Colored columns represent mice with metastases (METS) and white columns represent disease-free (DF) animals. (E) Quantification of the number of mice with lung, peritoneal (Peri.) or liver metastatic disease at the time of death. Values are percentages of the total number of mice in each cohort. (F) Representative H&E stains of harvested organs (primary tumor, lung, liver, peritoneal tissue) from vehicle and imatinib-treated animals. (G) Representative immunofluorescence images of pancreatic tumors of vehicle- or imatinib-treated KPC mice. DAPI, blue; CK8, red; and pPDGFRb, green. Scale bars represent 100 μm.

these data suggest that by inhibiting the kinase activity of PDGFRb, imatinib significantly diminishes the metastatic potential of pancreatic cancer cells.

2.2.4. PDGFRb Expression Correlates with Disease Free-Survival in Human Pancreatic, Colorectal, and Ovarian Cancer Patients

To investigate the clinical significance of PDGFRb expression, we examined whether upregulation of this gene is correlated with prognosis or with the clinicopathological characteristics of PDACs in patients. To avoid confounding signals from the tumor stroma, PDGFRb mRNA levels were assessed in tumor samples with high purity score. Strikingly, we observed that pancreatic cancer patients with tumors expressing high levels of PDGFRb showed a poor disease-free survival and, hence, shorter time to relapse including metastases in distant organs (p=0.019) (Figure 7A). Additionally, PDGFRb expression levels were significantly elevated in late-stage PDAC as compared to the earlier stages (Figure 7B). Patients with high PDGFRb levels also displayed an increase in tumor cells invading the vascular space, another clinicopathological characteristic of tumor dissemination (Figure 7C).

Next, we tested whether PDGFRb levels correlate with the status of *p53* by analyzing a panel of PDAC tissue microarrays (TMAs). Of importance, we observed significantly higher levels of activated PDGFRb in those tumors that showed an accumulation of p53 (p=0.009), which generally represents tumors with *p53* mutation (Cooks et al., 2013) (Figures 7D and S6A). These data confirmed results obtained with mice and underscore a role of mutant p53 in regulating the PDGFRb signaling in human pancreatic cancer.

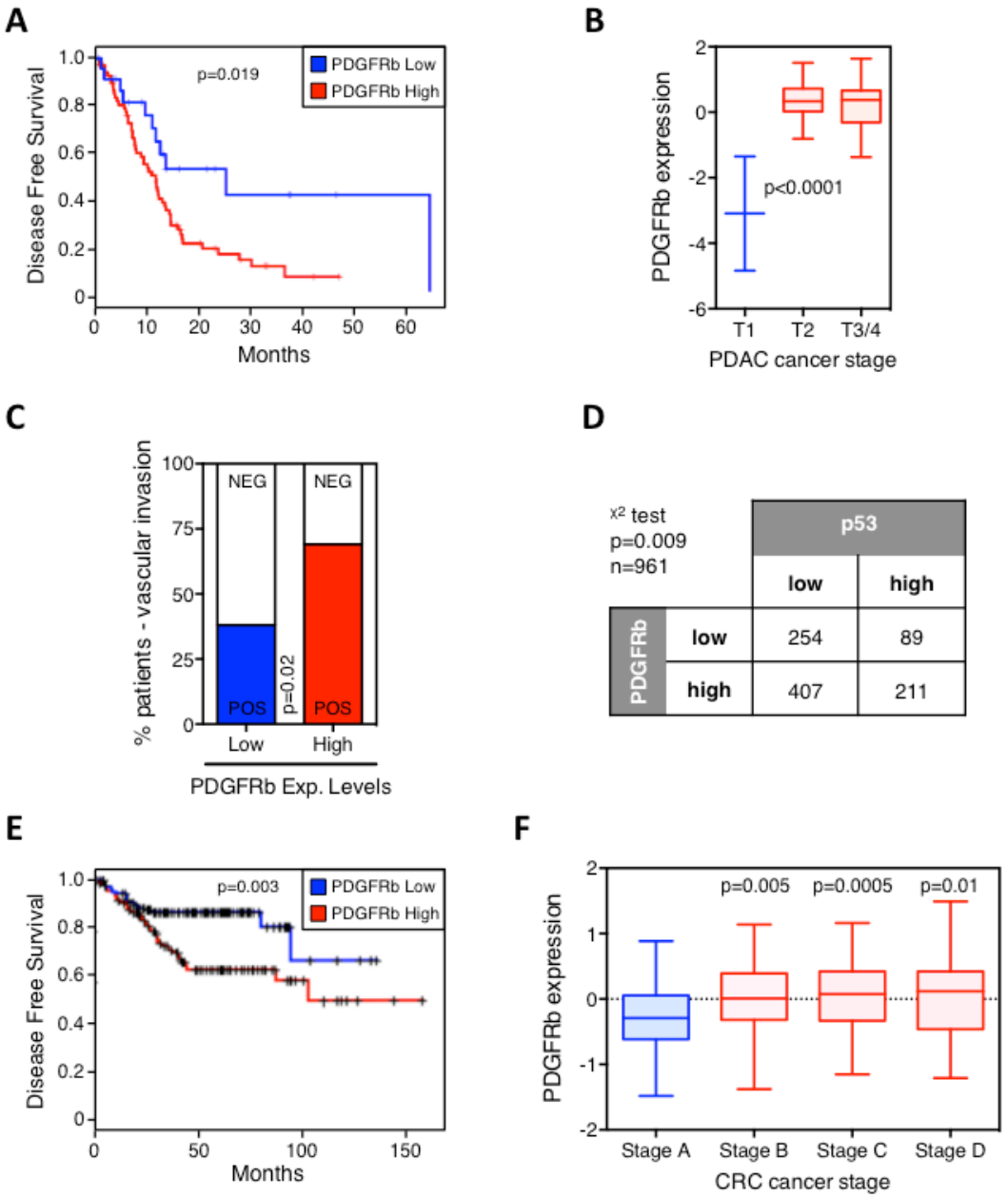


Figure 2.8. High PDGFRb Expression in Human Pancreatic and Colorectal Cancer Correlates with Reduced Metastases Free Survival

(A) Kaplan-Meier survival curves of 103 pancreatic cancer patients (clinical variable = DFS) as a function of PDGFRb-high versus PDGFRb-low expressing tumors. (B) Box plot of PDGFRb expression versus tumor grade of pancreatic tumors. (C) Clinicopathologic analysis of vascular space invasion of pancreatic cancer patients stratified by the expression levels of PDGFRb in the primary tumor. (D) Stratification of human PDAC samples (n = 961) based on high and low pPDGFRb and p53 expression levels. Chi-Square test was performed (p = 0.009). p53 and pPDGFRb levels were assessed by IHC and scored using a relative scale from 0 to 3. (E) Kaplan-Meier survival curves of colorectal cancer patients (clinical variable = DFS) as a function of PDGFRb-high versus PDGFRb-low expressing tumors. (F) Box plot of PDGFRb expression versus tumor grade of colon tumors.

Other tumor types for which *p53* mutations are predictive of metastatic disease are colorectal and ovarian cancer (Russo et al., 2005; Levesque et al., 1995). Thus, we analyzed the clinical significance of PDGFRb in these cancer types and found that levels of PDGFRb significantly stratified colorectal and ovarian tumor patients into two distinct cohorts. Patients with tumors expressing low PDGFRb levels exhibit a lower probability to form metastases compared to patients with PDGFRb high-expressing tumors ($p=0.003$ and $p<0.0001$) (Figures 7E and S6C). As in PDAC, a significant increase in PDGFRb expression was observed in higher stage colorectal cancers (Figure 7F). In addition to PDGFRb, our mutant p53 gene signature significantly scored as a prognostic marker in colorectal and ovarian tumor patients (top 40 genes upregulated in KPC cells; Figure 2C and Table S1). When we analyzed the three genes that scored in our invasion assay screen, we found that the *PDGFRB* gene was the strongest predictor for the probability to develop metastasis in colorectal and ovarian cancer patients (Figures S6B and S6C). In summary, consistent with our functional studies, elevated PDGFRb expression correlated significantly with the status of p53, higher tumor stage, and a poorer disease-free survival rate in pancreatic, colorectal and ovarian cancer patients.

2.3. Discussion

Mutations that occur in the *p53* tumor suppressor inactivate wild-type p53 functions but can also produce "gain-of-function" oncogenic properties that can contribute to cell proliferation, survival and metastasis. Here, we explored the phenotypic effects of mutant p53 in pancreatic cancer and showed that the sustained expression of the mutant *p53* allele is necessary to maintain the invasive phenotype of PDAC cells by increasing the expression

of PDGFRb. These results have several ramifications for our understanding of mutant p53 action as well as the behavior and potential treatment of pancreatic cancer.

Signaling through PDGFRb contributes to multiple tumor‐associated processes including cell invasion and metastasis. Given the generally restricted expression of PDGFRb to mesenchymal cell types, most of its oncogenic properties are thought to reflect paracrine effects of tumor cell-secreted PDGF. Indeed, previous work on the role of PDGFRb in carcinoma progression and metastasis suggest that it mainly elicits responses in the tumor stroma by promoting tumor angiogenesis (Pietras et al., 2003; Cao et al., 2004). In contrast, our study provides new evidence for tumor cell-specific expression of PDGFRb in promoting metastasis. We show that genetic or pharmacological inhibition of PDGFRb in the pancreatic cancer cells themselves dramatically reduces their invasive and metastatic potential, and that treatment of mice harboring genetic and histologically relevant tumors prevents metastatic spread in vivo. Together, our data indicate that pancreatic tumor cells expressing mutant p53 not only synthesize PDGF but also up-regulate PDGFRb, leading to a tumor autocrine, cell-autonomous effect.

The ability of the PDGF signaling axis to induce migration occurs also in wound repair, where fibroblasts respond to PDGF released from platelets (Seppä et al., 1982). When platelets aggregate early in clot formation they release PDGF, inducing the recruitment of fibroblasts into the wound. Here, PDGF-A has a more prominent role during early stages of healing whereas the expression of PDGFRb appears later in the wounded epithelium, indicating that PDGF-B regulates later wound healing events (Green et el., 1997). In addition to the induced migratory phenotype, mitogenic activity of PDGF also causes a rapid

proliferation of fibroblasts and the synthesis of constituents of the extracellular matrix of connective tissue to repair the wound. Inappropriate responses lead to fibrosis.

Though increases in PDGFRb expression were necessary and sufficient to mediate mutant p53 effects in our model, we identified at least two additional genes (*SNED1* and *SLC40A1*) that also contribute to the invasive phenotype through as yet unknown mechanisms. Studies in other systems, primarily breast cancer, have suggested that CXCR4, cyclin-G2, and the mevalonate pathway are important mediators of the pro-metastatic activities of mutant p53 (Mehta et al., 2007; Adorno et al., 2009; Freed-Pastor et al., 2012). In addition, mutant p53 has also been reported to drive invasion by regulating several miRNAs such as miR155 and miR130b (Neilsen et al., 2013; Dong et al., 2013). In agreement, we found that miR155 is positively regulated by mutant p53 in KPC cells and can promote metastasis in our model (data not shown); however, in contrast to a previous report (Su et al., 2010), no effects on Dicer expression were observed, arguing that in pancreatic cancer the mutant p53-associated changes in microRNA expression and metastasis are Dicer independent. Additionally, miR34a expression levels were not dependent on p53 status in our system (data not shown), even though this miRNA acts as negative regulator of PDGFRb in lung cancer (Garofalo at al., 2013). Regardless, as for wild type p53, mutant p53 exerts effects through the regulation of multiple genes rather than by modulating a single signaling pathway.

Most of our understanding of how mutant p53 mediates its oncogenic activity has been derived from exploring the consequences of the physical interaction between the mutant protein and the p53 family members, p63 and p73. Whereas the mutant p53-p63

interaction modulates the expression of p63 target genes to enhance invasion and metastasis (Adorno et al., 2009; Muller et al., 2009), how p53-p73 interactions produce similar outcomes is poorly understood. Here, we show that mutant p53 enhances pancreatic cancer cell metastasis by modulating p73 and its interaction with the transcriptional activator NF-Y. This model is consistent with previous studies showing that: (1) loss of p73 in a *p53*-null background might be functionally equivalent to the expression of mutant p53 (Lang et al., 2004); (2) aberrant transcriptional regulation by mutant p53 is mediated through the transcriptional activator NF-Y (Di Agostino et al., 2006); and (3) mutant p53 promotes recycling of receptor tyrosine kinases to initiate invasion (Muller et al., 2009). Whether structurally distinct p53 mutants enhance metastasis to the same extent and through the same mechanism remains unclear, and certainly most truncating mutants arising from the nonsense mutations occurring in a fraction of pancreas cancers are predicted to behave as if *p53* null. Still, in our study both conformational (e.g. R175H) and structural mutants (e.g. R273H) were capable of inducing *PDGFRB* through a similar mechanism.

Questions remain as to how the mutant p53/p73/NF-Y regulatory axis acts mechanistically. For instance, it remains unclear whether p73 acts to suppress the transactivation capacity of NF-Y or whether it sequesters the activator and prevents its binding to the *PDGFRB* promoter. Even though our results indicate that p73 overexpression hampers the ability of NF-Y to bind to the *PDGFRB* promoter, further studies will be required to distinguish between mechanisms. In addition, we noted slightly stronger induction of *PDGFRB* transcription as well as higher levels of invasion upon overexpression of mutant p53 compared to depletion of p73, indicating that mutant p53 may exert additional regulatory effects on PDGFRb expression. Although studies suggest that mutant

p53 can directly bind to NF-Y to regulate its transcriptional activity (Di Agostino et al., 2006), we failed to observe any physical interaction between mutant p53 and NF-Y in our cells. The discrepancy could reflect different extraction conditions or biological settings, since the mutant p53/NF-Y interaction has been shown to occur upon DNA damage (Liu et al., 2011).

Mutations in *KRAS, p53, CDKN2A, BRCA2* and *SMAD4* define the genetic landscape of PDAC; however, it remains unclear how each mutation contributes to the malignant evolution of this aggressive disease. We present evidence for a crucial role of mutant p53 in metastasis formation, which supports the attractive concept of targeting its gain-of-function activities to limit cancer cell dissemination and metastasis. However, mutant p53 is neither a targetable cell surface protein nor a druggable enzyme (Levine and Oren, 2009), and novel therapeutic modalities such as RNAi or restoring wild type p53 conformations have yet to show efficacy in clinical studies (Lehmann and Pietenpol, 2012). Hence, targeting downstream pathways or genes that mediate the activity of mutant p53, such as PDGFRb, pose an alternative treatment strategy.

Owing to the early metastatic spread of pancreatic cancer, widespread use of PDGFRb inhibitors might require advances in early detection or combination with other therapies. Nonetheless, our study suggests that PDGFRb inhibition might prove immediately useful in pancreatic patients harboring *p53* missense mutations either before (neoadjuvant) or after surgical resection (adjuvant) of localized disease (10-15% of PDAC cases), in patients with locally advanced inoperable non-metastatic disease (~30% of PDAC cases), or as a preventative approach in patients with familial predisposition to cancer

development. Moreover, PDGFRb inhibition as a therapeutic approach could be extended to other metastatic cancer types, e.g. colorectal, where the disease is often diagnosed before tumor cell dissemination.

High levels of PDGFRb expression have recently been associated with tumor recurrence in primary colorectal cancer, another gastrointestinal tumor in which p53 is frequently mutated (Stellar et al., 2013). Accordingly, we noted correlations between elevated PDGFRb levels, more advanced tumor stage, and poorer disease-free survival in pancreatic as well as colorectal and ovarian cancer patients. These results indicate that PDGFRb levels might be used as a prognostic biomarker for cancer progression, and eventually used in conjunction with p53 to identify patient cohorts most likely to respond to therapies targeting this axis. Although further studies will be required to explore this notion, pharmacological inhibition of PDGFRb with the tyrosine kinase inhibitor imatinib, in combination with standard chemotherapy, has shown promise in treating metastatic colorectal cancer patients (Hoehler et al., 2013).

In summary, we describe a gain-of-function activity of mutant p53 that promotes invasion and metastasis through increasing *PDGFRB* transcription and reverting the repressive function of the p73/NF-Y complex. While other activities of mutant p53 on cell behavior and survival exist (Freed-Pastor and Prives, 2012), our study provides a detailed molecular understanding of at least one aspect of the invasive behavior of cells expressing mutant p53, and offers a potential new target for therapy that might interfere with this activity.

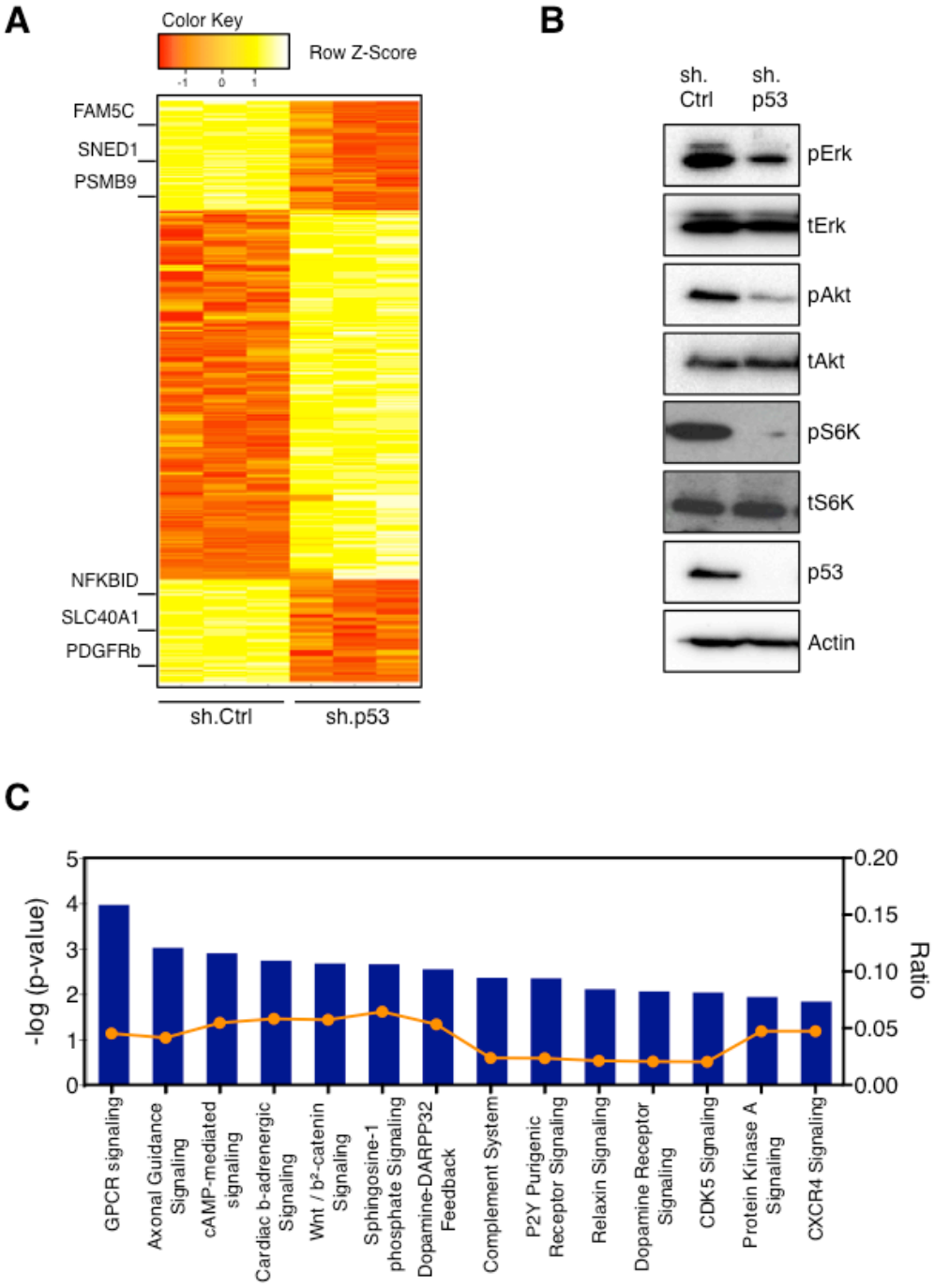
2.4. Chapter Contributions

S.W. and E.M. designed and performed the majority of the experiments and contributed equally to this work. M.S., J.P.M., D.F.T., and T.K. contributed intellectually to the design of the project and helped performing experiments (treatment of KPC mice, IHC/IF, TMAs and invasion assay, respectively.). E.W. and C.A.D. conducted and analyzed RNA sequencing. S.M., N.T.P., and C.Pr. conducted and analyzed ChIP experiments. E.K.M., J.W., S.M.G., and A.V.B. collected, analyzed, and interpreted human data. C.Pi. provided TMAs and their p53 status. S.W., E.M., and S.W.L. wrote the manuscript with the assistance from all authors. S.W.L. conceived and supervised the project.

2.5. Citation

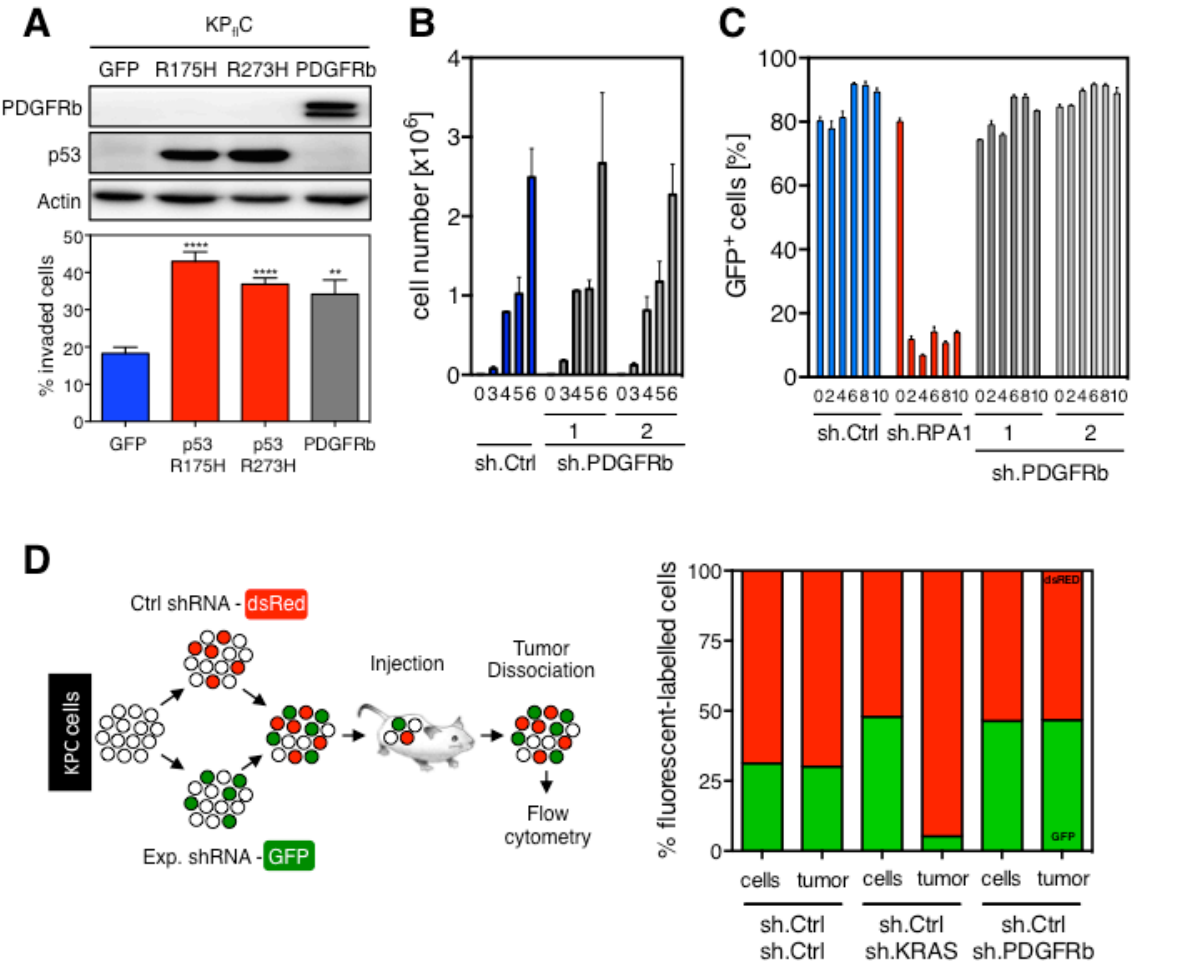
Weissmueller S*, Manchado E*, Saborowski M, Morris JP IV, Wagenblast E, Davis CA, Moon S, Pfister NT, Tschaharganeh DF, Kitzing T, Aust D, Markert EK, Wu J, Grimmond SM, Pilarsky C, Prives C, Biankin AV, Lowe SW. 2014. Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor beta signaling. *Cell*. **157(2):** 382–94.

2.6. Supplementary Figures



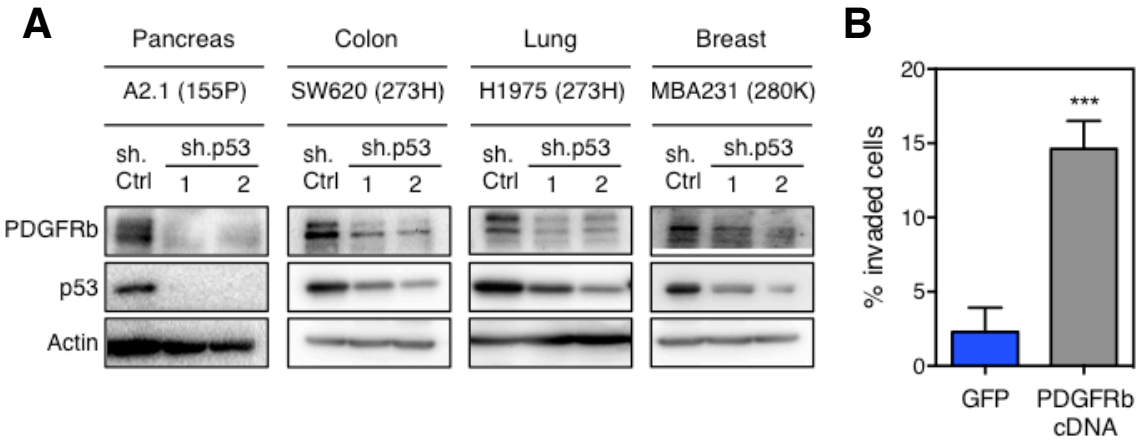
Suppl. Figure 2.1. Knockdown of Mutant p53 in Pancreatic Cancer Cells Alters a Myriad of Genes and Pathways

(A) Heatmap of significantly changed genes ($p_{adj} < 0.05$) following mutant p53 depletion, as identified by RNA sequencing. Three individual clonal cell lines of KPC+sh.p53 and +sh.Ctrl were analyzed and representative top scoring genes are labeled. (B) Western blotting analysis of activated downstream PDGF receptor b pathways following knockdown of mutant p53 in KPC cells. Actin expression was used as loading control. (C) Blue bars that cross the threshold line ($p < 0.05$) represent top scoring pathways that are significantly changed in mutant p53-depleted KPC cells. Data was analyzed through the use of ingenuity pathway analysis.

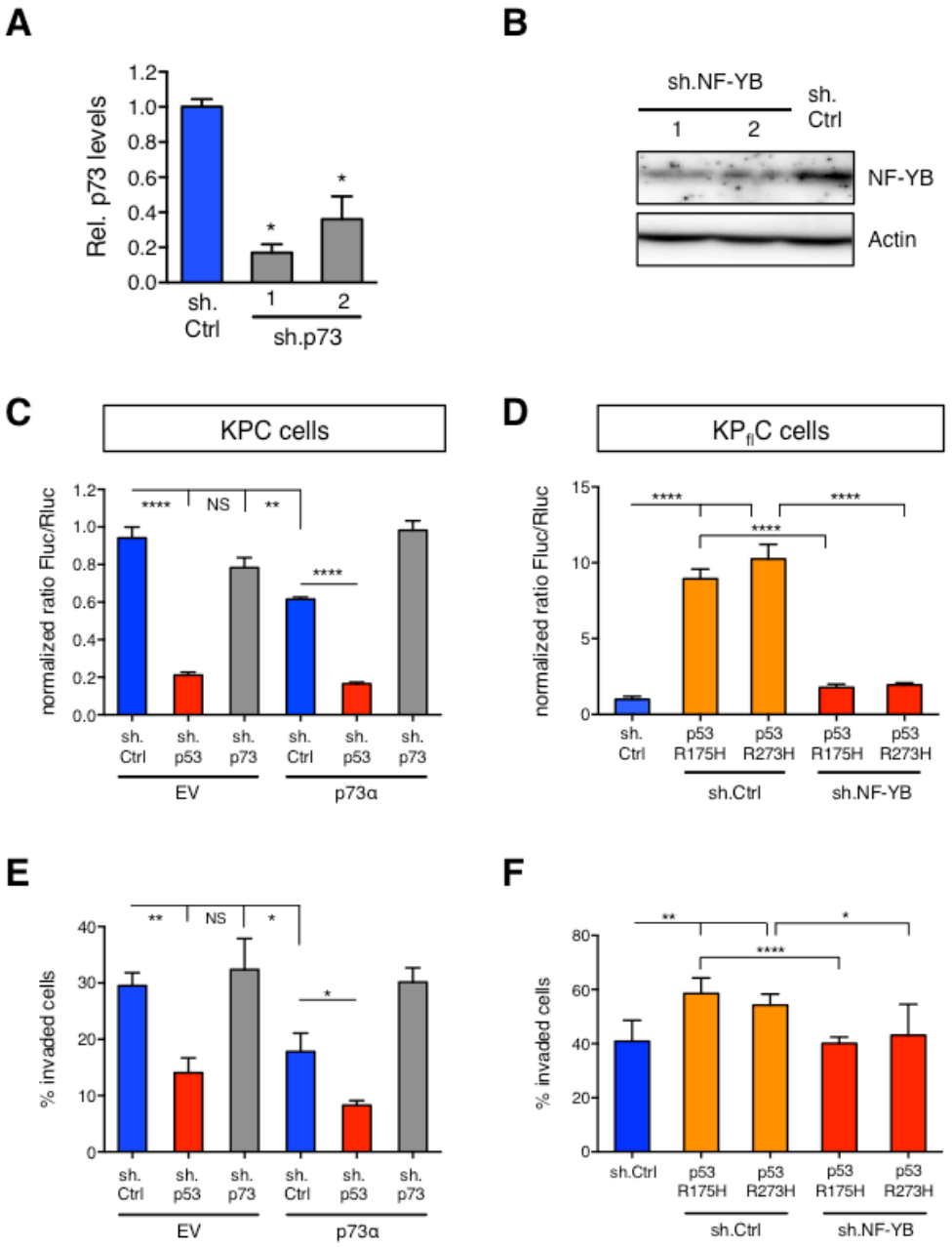


Suppl. Figure 2.2. PDGFRb Expression Levels Determine the Invasive Ability of Murine and Human Cancer Cells without affecting cell proliferation

(A) Western blotting analysis of PDGFRb, p53, and actin from $KP_{fl}C$ cells stably expressing a GFP-, $p53^{R175H}$-, $p53^{R273H}$-, or PDGFRb-cDNA vector (upper panel). Quantification of invasion into collagen from the same cells (lower panel). The average of invaded cells from 9 replicates ±SD is shown. **p < 0.01, ****p < 0.0001. (B) Cell number over time (days) of KPC cells stably expressing sh.Ctrl or sh.PDGFRb (1 or 2). (C) Negative selection RNAi studies in KPC cells stably expressing dox-inducible sh.Ctrl, sh.RPA3 or sh.PDGFRb (1 or 2) using the tet-on TRMPV system. Graphs represent the percentage of shRNA-expressing (Venus+ dsRed+) cells over time (days), normalized to initial measurement 1 d after dox treatment. (D) Schematic of dual-color competitive proliferation assay in vivo for evaluating effects of RNAi-mediated PDGFRb suppression in tumor growth. KPC cells were transduced with indicated experimental shRNAs (GFP+) and a neutral control shRNA (dsRED+) (left panel). Percentage of cells expressing indicated experimental shRNA (GFP+) or the neutral control shRNA (dsRED+) in pre-injected cells and tumors 2 weeks after injection. Values represent the mean of multiple pre-injected or tumor-derived cell lines (right panel).

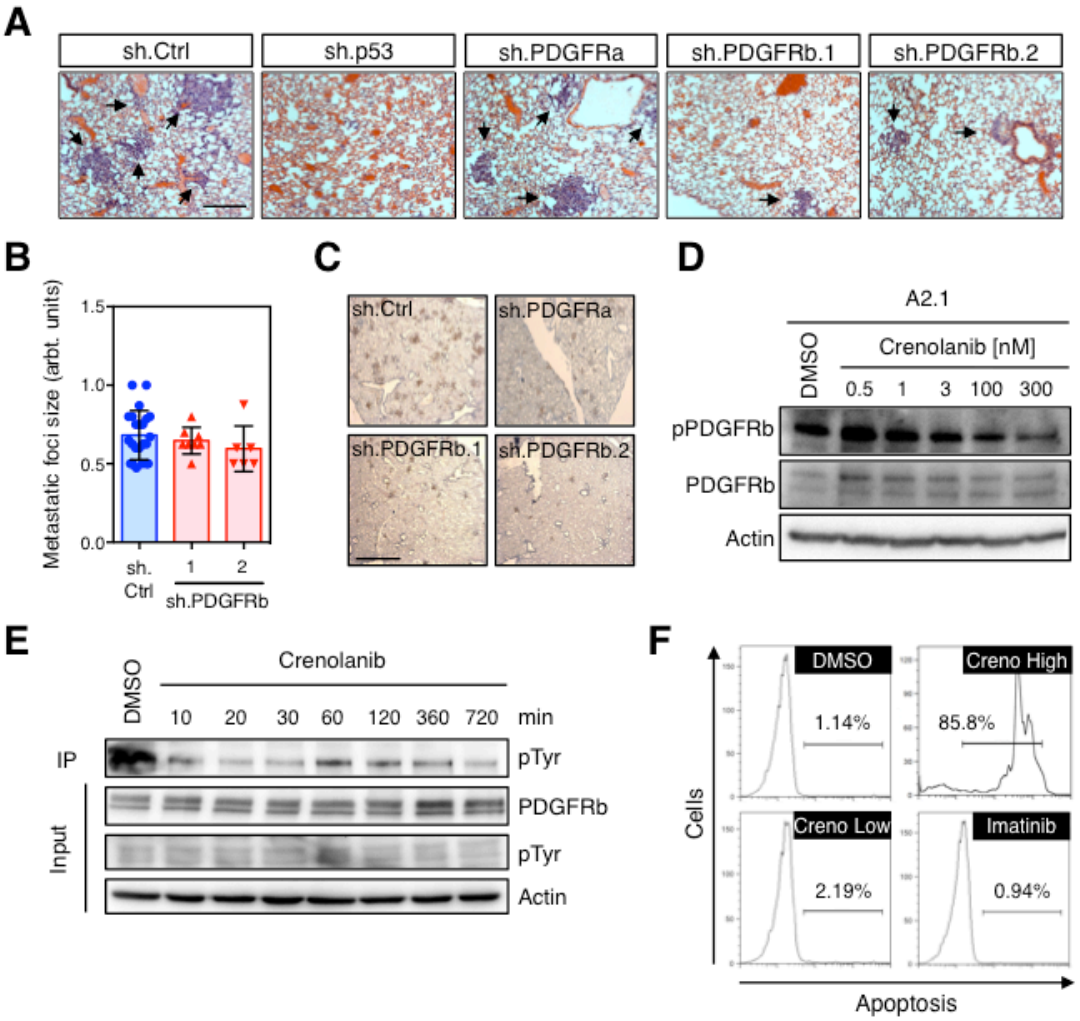


Suppl. Figure 2.3. PDGFRb Expression Levels of Different Human Cancer Cells Depend on the p53 Status
(A) Western blotting analysis of PDGFRb, p53, and actin levels in human pancreatic, colon, lung, and breast cancer cells stably expressing sh.Ctrl or sh.p53 (1 or 2). Mutation of p53 as indicated. (B) Quantification of the invasion into collagen of human $p53^{-/-}$ ASPC pancreatic cancer cells stably expressing a GFP- or PDGFRb-cDNA vector. The average of invaded cells from 9 replicates ±SD is shown. ***$p < 0.001$.



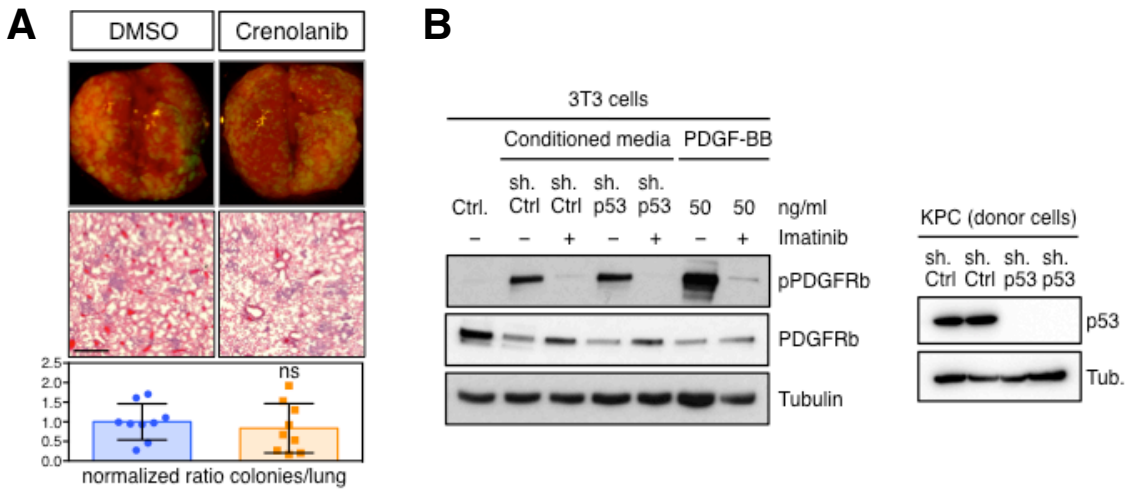
Suppl. Figure 2.4. Mutant p53 Drives PDGFRB Transcription by Opposing the Repressive Function of the p73/NF-Y Complex

(A) qRT-PCR for *p73* in KPC+sh.p73 (1 or 2) or +sh.Ctrl cells. Data present mean normalized *p73* expression ±SD of triplicate samples. A representative result of three repeated experiments is shown. *p < 0.05. (B) NF-YB and actin levels of KP$_{fl}$C cells infected with sh.NF-YB or sh.Ctrl as determined by western blotting. (C) After double infection using an empty control or HAp73 α vector together with sh.Ctrl, sh.p53, or sh.p73, KPC cells were co-transfected with the PDGFRB-promoter-luciferase construct and renilla-luciferase vector. Firefly-luciferase activity of GFP-vector cells was set to 1. Values are relative Firefly-luciferase (Fluc) units normalized by renilla expression (Rluc) ±SD of quadruplicate samples. **p < 0.01, ****p < 0.0001. A representative result of three repeated experiments is shown. (D) KP$_{fl}$C cells stably expressing sh.Ctrl or sh.NF-YB together with mutant p53 (175H or 273H) were co-transfected with the *PDGFRB*-promoter-luciferase construct and *Renilla*-luciferase vector. Luciferase activity was measured as described above. ****p < 0.0001. (E) Quantification of invasion of the same cells as in (C). The average of invaded cells from 9 replicates ±SD is shown. A representative result of three repeated experiments is shown. *p < 0.05, **p < 0.01. (F). Quantification of invasion of the same cells as in (D). The average of invaded cells from 9 replicates ±SD is shown. A representative result of three repeated experiments is shown. *p < 0.05, **p < 0.01, ****p < 0.0001.

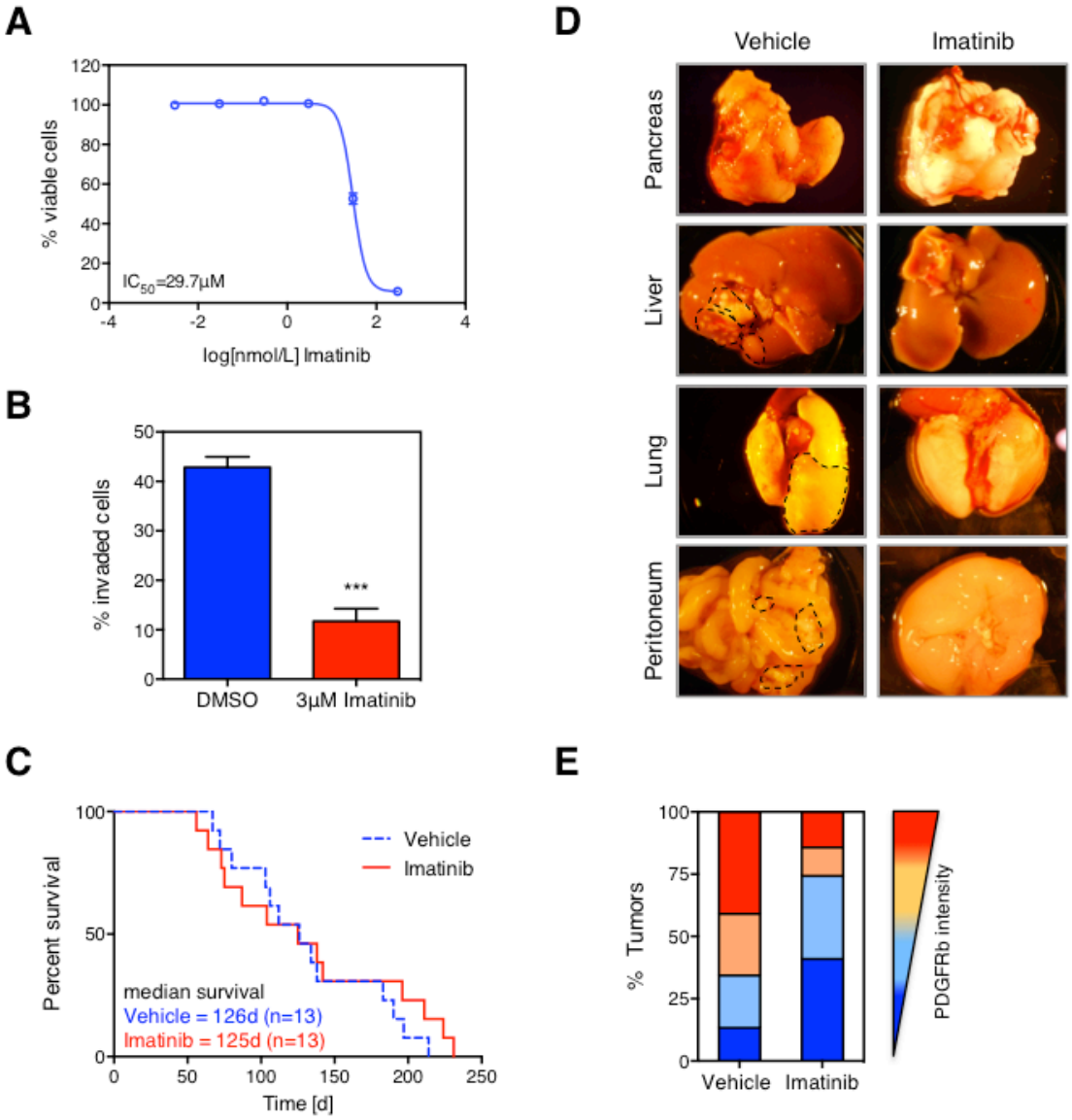


Suppl. Figure 2.5. Inhibition of PDGFRb Activity by RNAi or Small Molecules Decreases Metastatic Potential of Pancreatic Cancer Cells

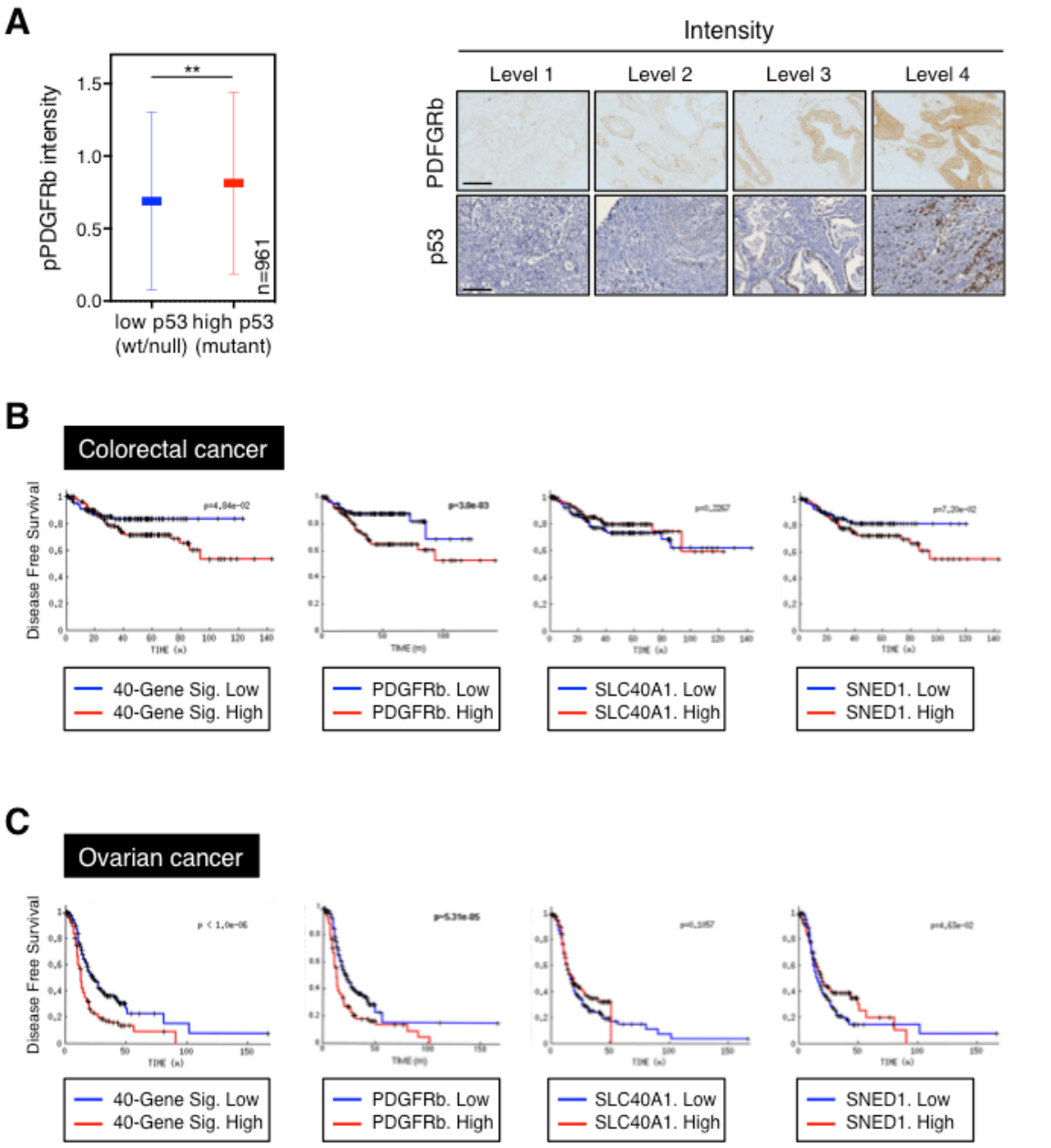
(A) Lung colonization assays after tail vein injection of PDGFRa-, PDGFRb (1 or 2)-, p53-, and control-depleted KPC cells. Hematoxylin and eosin (H&E) stains of representative sections of pulmonary lobes from indicated mice are shown, arrows indicate metastases. Scale bars represent 50 μm. (B) Relative nodule size of lung metastases from mice intravenously injected with PDGFRb (1 or 2)-, and control-depleted KPC cells. Data represent mean ±SD. (C) GPF immunohistochemistry on histological lung sections from lung colonization assay in (B). shRNA expression correlates with GFP signal, as the fluorescent marker is linked to the hairpin. Scale bars represent 1000 μm. (D) Western blotting for pPDGFRb, PDGFRb, and actin in human A2.1 cells after treatment with DMSO or crenolanib at varying doses. (E) Immunoprecipitation of PDGFRb from KPC cells treated with DMSO or crenolanib (300 nM) for different time periods. The input protein levels for PDGFRb, phospho-Tyrosine, and actin and those present in immunoprecipitates for phospho-Tyrosine were determined by western blotting. (F) Propidium Iodide staining of KPC cells treated overnight with either DMSO, Crenolanib (0.3 (Creno Low) or 25 μM (Creno High)) or imatinib (3 μM)..



Suppl. Figure 2.6. PDGFRb acts autonomously in KPC cells to potentiate cell invasion and metastasis
(A) Lung colonization assays after tail vein injection of crenolanib- or DMSO-treated $KP_{fl}C$ cells. Representative merged brightfield/GFP images of whole lung as well as H&E stains of representative sections of pulmonary lobes are shown. Quantification of total number of lung metastatic nodules in individual mice (n > 6) (lower panel). Data presented as mean ±SD. Scale bars represent 100 µm. (B) Western blotting for pPDGFRb, PDGFRb, and tubulin of starved 3T3 cells, which were pre-treated with either DMSO or Imatinib (3 µM) for 4 h and subsequently stimulated for 15 min with conditioned media from KPC (sh.Ctrl or sh.p53) or 50 ng/µl PDGF-BB (left panel). Western blotting for p53 and tubulin of KPC+sh.Ctrl and +sh.p53 (right panel)



Suppl. Figure 2.7. Imatinib Reduces Invasiveness of KPC Cells and Metastases Formation in KPC Mice
(A) MTS assay (E490) of KPC cells treated with imatinib with various doses for 72 h. Normalized values are expressed as means ±SD form quadruple replicates. (B) Quantification of invasion of KPC cells treated with either DMSO or imatinib at 3 μM. The average of invaded cells from 9 replicates ±SD is shown. A representative result of three repeated experiments is shown. ***$p < 0.001$. (C) Kaplan–Meier survival curves of mice treated with vehicle or imatinib (50 mg/kg) from 8 weeks of age. (D) Representative bright field images of pancreatic tumor, liver, lung and peritoneum from KPC mice treated with vehicle or imatinib. Black arrows denote metastases. (E) Quantification of pPDGFRb intensity in pancreatic tumors of KPC mice treated with vehicle or imatinib. pPDGFRb was assessed by immunofluorescence and its intensity scored from 0 (pPDGFRb low levels) to 3 (pPDGFRb high levels). 15 images per tumor from 7 mice per group were analyzed.



Suppl. Figure 2.8. Metastasis free survival of Colorectal and Ovarian Cancer Patients is Dictated by Mutant p53-regulated Genes

(A) pPDGFRb levels in human PDAC samples (n = 961) stratified by p53 (left panel). Levels of pPDGFRb and p53 were determined by IHC and representative images are shown (right panel). **p < 0.01. Scale bars represent 100 μ m. (B) Kaplan-Meier survival curves of colorectal cancer patients (clinical variable = DFS) as a function of the expression levels of the 40 genes from the mutant p53 gene signature, PDGFRb, SLC40A1, and SNED1. (C) Kaplan-Meier survival curves of ovarian cancer patients (clinical variable = DFS). Patients were stratified as in (B).

Chapter 3

Distinct miRNA signature of invasive pancreatic cancer

3. Chapter 3

Distinct miRNA signature of invasive pancreatic cancer

3.1. Introduction

Pancreatic cancer remains one of the leading causes of cancer deaths and has a 5-year survival rate of less than 5%. Given its resistance to both radiation and chemotherapy, surgical resection is the only curative treatment option to date. However, the number of patients that are eligible for curative surgery is limited due to the late stage detection of this disease. Moreover, front-line treatment options for advanced pancreatic cancer are not effective and prolong survival only by a few weeks. Thus, further studies of the molecular mechanisms underlying the aggressive nature of this disease are needed. These investigations might lead to the identification of biomarkers for early detection of pancreatic cancer as well as new therapeutic targets for the treatment of late stages of this disease.

microRNAs (miRNAs or miRs) are small, non-coding RNA molecules of 22 nucleotides in length, which regulate gene expression in a post-transcriptional manner. miRNAs coding sequences can appear as single genes or as polycistronic primary transcripts that are processed into multiple individual mature miRNAs (Mendell, 2008). The genomic organization of the miRNA clusters is often highly conserved; however, the role of the coordinated regulation and function remains unclear. The biogenesis of miRNA is a well-characterized biological process that has been studied in detail in the past few years. During the miRNA maturation process, the long pri-miRNA transcript that contains stem-loop hairpin structures is processed by Drosha and Dicer to yield the mature miRNA

duplex. One strand of the mature duplex is then incorporated into the RISC complex, the effector machinery that mediates post-transcriptional silencing of target genes. The RISC complex containing a guide miRNA strand bind together to the 3'-untranslated region of messenger RNAs (mRNAs) and initiate degradation or inhibition of translation of their target mRNA, resulting in attenuated protein levels. miRNAs recognize their targets through imperfect sequence complementarity, which allows each of them to potentially regulate a large number of protein coding genes. More than 1,400 miRNAs, identified so far, have been defined to exhibit functional roles in widespread cellular processes, including proliferation, differentiation and development.

Deregulated miRNA expression is a characteristic of human diseases, including cancer. miRNAs can serve as cancer biomarkers (Wang et al., 2014), and are implicated in the tumor development and maintenance (Szafranska et al., 2007). miRNA activity can be perturbed at different levels, including genetic alteration, transcriptional regulation, posttranscriptional modification and processing steps during miRNA biogenesis. Altered control of any of these steps provokes abnormal miRNA profiles that can lead to the aberrant expression of cancer genes (Esquela-Kerscher and Slack, 2006) or interfere with the molecular crosstalk within well-characterized cancer pathways (Olive et al., 2010). Examples for the former include the "oncomiR"-21 whose expression is enhanced in cancers, resulting in the downregulation of tumor suppressor genes, such as Pten and Bcl2, to induce proliferation and attenuate apoptosis. On the other hand, the tumor suppressor let-7 and its repressive function on the oncogenes RAS and HMGA2 are diminished in many cancers (Kong et al, 2012). Finally, a candidate miRNA that has emerged as a key component of the p53 tumor suppressor network is mir-34, the first identified miRNA to be transcriptionally

regulated by p53. miR-34 mediates the tumor suppressor effects of p53 and, when deregulated, confers resistance to apoptosis in cancer cells (He et al., 2007).

Pancreatic ductal adenocarcinoma is a highly aggressive disease characterized by extensive invasion into the surrounding tissues and metastasis to distant organs during disease progression. Because miRNAs regulate important cancer genes and tumor networks, it is very likely that they are involved in tumor invasion, functioning as either metastasis suppressors or –mediators. Indeed, several miRNAs have been directly or indirectly linked to invasion and metastasis in different tumor types. While the tumor suppressor miR-124 represses the invasive capabilities of pancreatic cancer cells through the inactivation of the Rho GTPase RAC1 (Wang et al., 2014), the oncogenic miR-155 acts as a metastasis mediator in breast cancer through the regulation of the TGFβ response in the epithelial-mesenchymal transition (Johansson et al., 2013). Together, miRNAs can modulate the metastatic phenotype of human cancers through the regulation of oncogenic and tumor suppressive networks.

Many metastatic cancers acquire mutations in the tumor suppressor *p53*, which can gain neomorphic functions to promote tumorigenesis. As discussed in Chapter 2, mutant p53 drives the invasive phenotype of pancreatic cancer cells via upregulation of PDGFRb. However, there are additional mechanisms by which mutant p53 exerts its gain of function such as the regulation of miRNA expression in cancer cells. On the one hand, it was reported that Dicer and global miRNA levels become attenuated by mutant p53 to increase invasion through enhanced recycling and activation of the growth factor receptors MET and EGFR (Muller et al., 2014). On the other hand, it was shown that mutant p53 drives the

expression of only miR-155 through a p63-dependent mechanism in breast cancer cells (Neilsen et al., 2013). Therefore, although the role of mutant p53 in modulating miRNAs to mediate oncogenesis has be revealed, whether mutant p53 alters the entire miRNA biogenesis pathway and/or impinges on distinct miRNAs are open questions.

One of the most relevant gene networks in human cancer is the mitogen-activated protein kinase (MAPK) signal transduction pathway. The MAPK pathway is evolutionarily conserved and plays a key role in a myriad of biological processes, such as cell growth, differentiation, and apoptosis. MKK4, a modulator of the MAPK pathway, is a dual-specific tyrosine and serine/threonine protein kinase that activates both p38 and JNK in response to stress signals. MKK4 plays a role in suppressing prostate, ovarian, and liver cancer metastasis, and its decreased activation correlates with increased cell motility and invasiveness (Kim et al., 2001; Yamada et al., 2002; Tsai et al., 2014). How MKK4 mediates invasiveness seems to be cell type-specific and might involve JNK or NF-κB (Kim et al., 2001; Tsai et al., 2014). However, how activated MKK4 levels become downregulated and whether miRNAs are involved in fine-tuning the complex MAPK cascade to induce an invasive phenotype remains elusive.

In Chapter 3, we aim to explore whether the mutant p53-dependent miRNA signature contributes to cell invasion and metastasis in pancreatic cancer. RNA sequencing of miRNAs in pancreatic cancer cells expressing mutant p53 and a subsequent RNAi screen, identified miR-155 and miR-181b as mediators of mutant p53 that drive invasion. Moreover, we found that both miRNAs execute their oncogenic potential via the MAPK pathway, by decreasing the levels of activated MKK4. Our data suggest that mutant p53 drives invasion

through several mechanisms, including the regulation of miRNAs and their targets. This observation opens the question whether inhibition of one downstream effectors of mutant-p53 are sufficient to effectively inhibit its gain of function.

3.2. Results

3.2.1. Mutant p53 regulates a distinct miRNA signature in pancreatic cancer

To study whether mutant p53 regulates the miRNA profile in pancreatic cancer, we utilized pancreatic cancer cell lines isolated from tumors of genetically engineered KPC or $KP_{fl}C$ mice. Both strains harbor a *Cre*-responsive, latent oncogenic *Kras* allele (*lox-stop-lox* ***K****ras*^{G12D}), together with either a latent mutant ***p****53*$^{R172H/wt}$ knock in allele or a ***fl****oxed* ***p****53*$^{fl/wt}$ allele, and a tissue-specific *Cre* recombinase (*Pdx1-****C****re*) (Morton et al., 2010). Pancreas-specific activation of *Kras* occurs in both strains, whereas the KPC strain expresses mutant p53, while $KP_{fl}C$ cells lose p53 function. Importantly, the *p53* status of these cells could be manipulated by retroviral transduction to either mediate knockdown of mutant p53 in KPC cells or to restore different mutant forms of p53 in $KP_{fl}C$ cells. As described in the previous chapter, mutant p53 contributes to PDAC invasion and metastasis by modulating the expression of several genes. Accordingly, inhibition of the activity of mutant p53 or its downstream mediator PDGFRb had an anti-metastatic effect. Does mutant p53 also modulate the expression of miRNAs to influence the invasive phenotype of pancreatic cancer cells?

To study whether miRNA networks are perturbed in pancreatic cancer cells, we performed small-RNA profiling of KPC cells to identify those miRNAs that are specifically regulated by mutant p53. Therefore, we infected KPC cells with retroviral vectors encoding

validated shRNAs directed to *p53* (sh.p53) or control gene (sh.Ctrl). Four days following knockdown of mutant p53 in three independent clonal KPC populations, we conducted deep sequencing of RNAs of < 200 nt in size. Interestingly, we observed distinct miRNA expression changes in experimental versus control cells, rather than a global alteration of the miRNA signature. More specifically, we identified 9 and 7 miRNAs as significantly up- and down regulated, respectively, upon shRNA-mediated depletion of endogenous mutant p53, respectively (Table 3.1. and Figure 3.1.A).

UP		DOWN	
miRNA	**Fold change**	**miRNA**	**Fold change**
miR-195	9.59	miR-1965	0.23
miR-148a	6.35	miR-147	0.38
miR-10b	5.40	miR-221	0.38
miR-138	3.07	miR-3096	0.46
miR-139	2.82	miR-222	0.46
miR-187	2.41	miR-188	0.51
miR-1306	2.21	miR-22	0.55
miR-155	1.79		
miR-181b	1.68		

Table 3.1. miRNAs expression changes in mutant-p53 expressing KPC cells compared to p53 knock down

Mutant p53 can promote invasion and metastasis through a global reduction of miRNA levels mediated by downregulation of Dicer expression (Muller et al., 2014). However, our study revealed that the regulation of miRNAs by mutant p53 is Dicer-independent in pancreatic cancer. First, as measured by western blotting and long RNA seq profiling (Figure 3.1.B), we did not observe downregulation of Dicer protein or mRNA transcript levels in response to mutant p53. Second, if Dicer levels were downregulated by mutant p53, we would have expected an accumulation of pri-miRNAs due to a failure in its maturation. However, we did not observe global alterations of pri-miRNAs in pancreatic

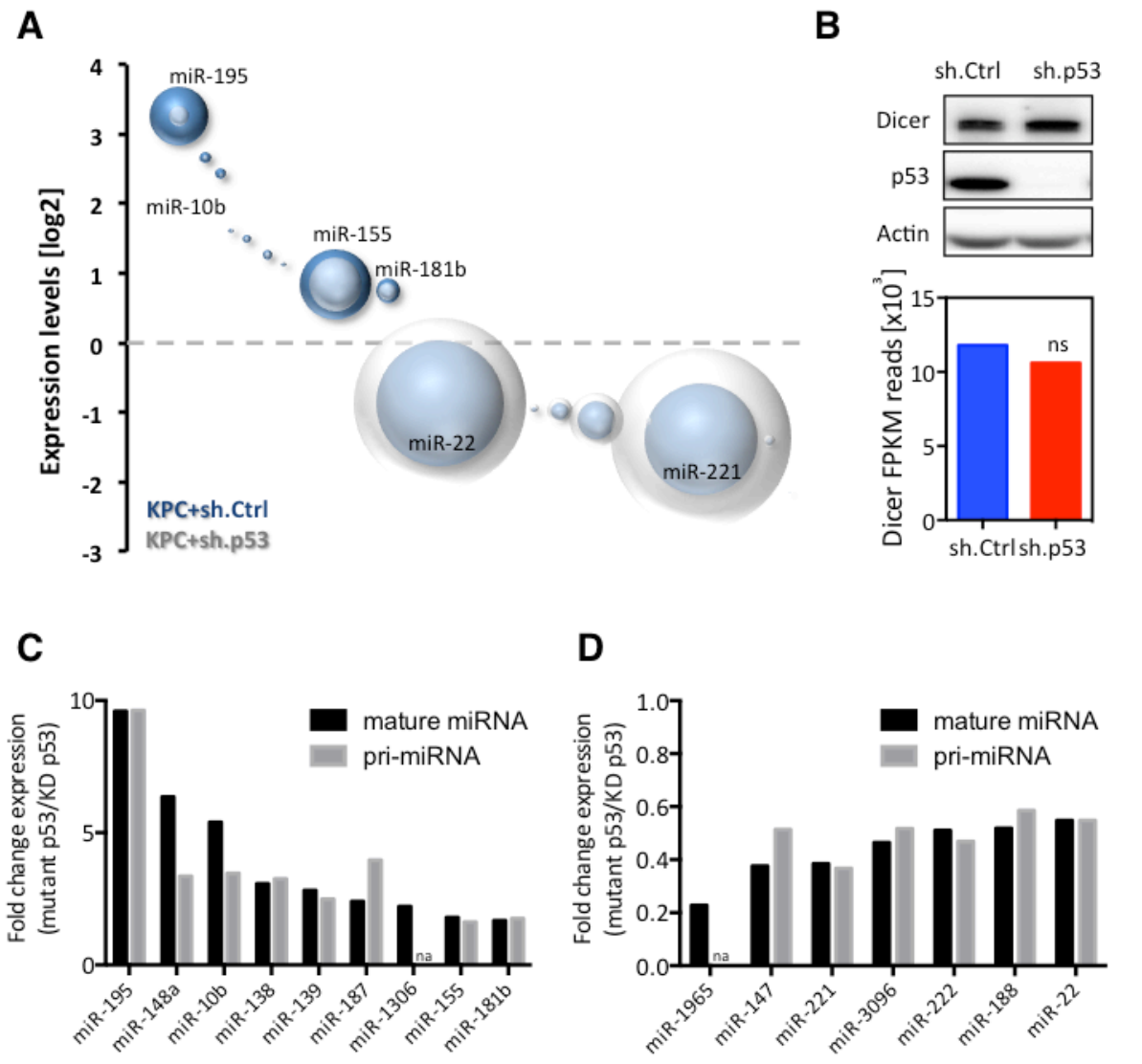


Figure 3.1. Mutant p53 regulates a distinct miRNA signature in a Dicer-independent way
(A) Bubble plot depicting the relative abundance and log2 ratio of miRNAs in KPC+sh.Ctrl relative to KPC+sh.p53 cells. (B) Protein and mRNA levels for Dicer in KPC+sh.Ctrl or +sh.p53. (C and D) Fold change expression levels of pri- and mature miRNA of KPC cells expressing mutant p53 compared to knock down. miRNA that are upregulated shown in (C) and downregulated shown in (D).

cancer cells expressing a mutant form of p53. Moreover, the same changes in pri-miRNAs were detected for mature miRNAs upon knockdown of mutant p53 (Figures 3.1C and 3.1.D). Third, previous studies in human non-small cell lung carcinoma cell lines reported that, by interacting with p63, mutant p53 to activates transcription of Dicer (Muller et al., 2014). Unlike these studies, we could not detect p63 expression in our pancreatic cancer system (described in Chapter 2) as analyzed by long RNAseq or western blotting. Hence, the underlying molecular mechanism whereby mutant p53 controls the expression of miRNAs seems to vary in different cell types and need not be linked to mechanisms that involve the regulation of Dicer expression levels.

3.2.2. miR-155 and miR-181b are downstream mediators of pancreatic cancer invasion driven by mutant p53

To elucidate whether miRNAs regulated by mutant p53 mediate the invasive and metastatic properties of pancreatic cancer, we screened each miRNA that was significantly altered upon knock down of mutant p53 (Figures 3.1.C and 3.1.D), as identified by small RNAseq, one-by-one in an in vitro invasion assay. miRNAs positively regulated by mutant p53 were overexpressed in $KP_{fl}C$ cells that lack p53 function, in order to identify those that might mimick the enhanced invasive phenotype driven by mutant p53. In contrast, we transduced KPC cells with miRNAs that were negatively regulated by mutant p53 and looked for miRNAs that induced a decreased invasive phenotype, similar to the effect seen upon downregulation of mutant p53 (Figure 3.2.B). Although we failed to find miRNAs whose expression abrogated invasion driven by mutant p53 in KPC cells, we identified three miRNAs whose overexpression in $KP_{fl}C$ increased invasiveness. miR-155 is highly

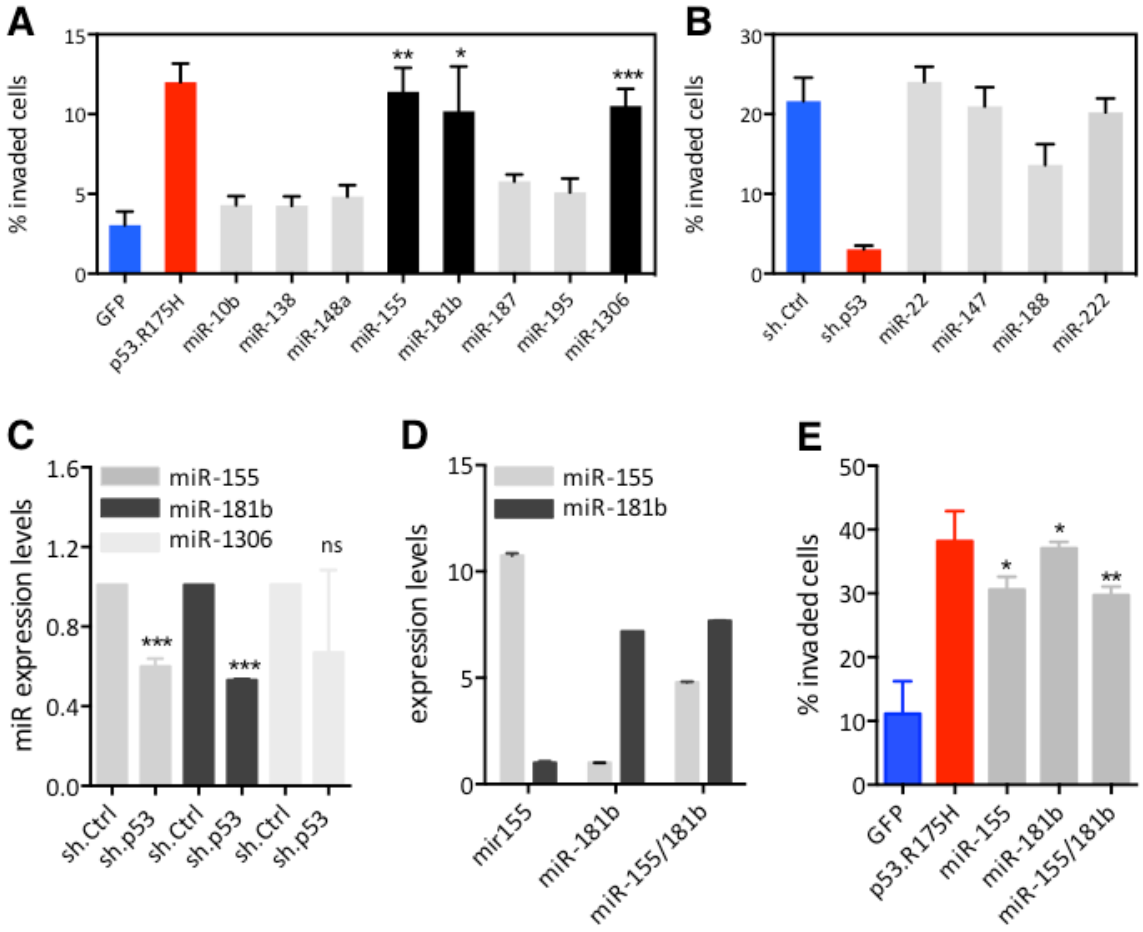


Figure 3.2. Identification of miR-155 and miR-181b as Downstream Mediators of Mutant p53 in Regulating Cell Invasion

(A) One-by-one invasion assay screen. Quantification of invaded $KP_{fl}C$ cells infected with miRNAs upregulated by mutant p53 (left panel) and KPC cells infected with miRNAs downregulated by mutant p53 (right panel). (B) qRT-PCR for miR-155, -181b, and -1306 in KPC+sh.Ctrl or +sh.p53 cells. (C) qRT-PCR for miR-155 and -181b in $KP_{fl}C$ cells infected with miR-151, -181b, or -151/-181b. (D) Quantification of the invasion into collagen of cell lines from (C) compared to $KP_{fl}C$+GFP or +p53.R175H. Data presented as mean ±SD. *p < 0.05, **p < 0.01, ***p < 0.001.

expressed in hematopoietic cells and was one of the first miRNAs implicated in cancer (Spoerl et al., 2013); miR-181b promotes the activation of the NF-κB signaling pathways and is involved in the resistance of pancreatic cancer cells to gemcitabine (Ma et al., 2011; Takiuchi D et al. 2013); and miR-1306 is a less well characterized miRNA for which direct targets and pathways are still unknown.

We next verified by RT-qPCR that miR-155 and -181b expression levels were reduced upon knockdown of mutant p53, indicating that their expression depends on p53 status (Figure 3.2.C). Conversely, miR-1306 expression levels did not change upon manipulation of mutant p53 levels, suggesting that its oncogenic properties are not regulated by mutant p53 (Figure 3.2.C). Overexpression of either miR-155 or miR-181b increased the invasive properties of $KP_{fl}C$ cells to a level similar to that observed upon overexpression of a mutant form of p53. Simultaneous overexpression of miR-155 and -181b did not have a cooperative effect on invasiveness of $KP_{fl}C$ cells, suggesting that both miRNAs might function redundantly and target the same mRNA transcripts (Figure 3.2.E). In short, mutant p53 orchestrates a cell invasion program in pancreatic cancer cells through the upregulation of miR-155 and miR-181b.

3.2.3. miR-155 and miR-181b target *Map3k10* to modulate invasiveness

To gain an understanding of the downstream modulators regulated by miR-155 and -181b, we first generated a list of putative targets of both miRNAs (mirbase.org) and compared the overlap of predicted targets with the mutant p53-responsive genes identified by long RNAseq from our previous experiment (described in Chapter 2). We found 12 genes to be

putative targets of both miR-155 and -181b, and to be dependent on the *p53* status (Figure S3.1, and 3.3.A). Amongst those genes, *Map3k10* was the most promising candidate because it (i) carries a central role in the MAPK pathway; (ii) is a validatedmiR155 target (Figure 3.3.B) (Zhu et al., 2012); and (iii) is overexpressed in PDAC tissue to promote the viability, proliferation, drug resistance (An et al., 2013). MAP3K10 phosphorylates the downstream kinase Mkk4 and is an upstream component of the JNK/p38 signaling pathways that result in the activation of a diverse range of proteins that play roles in cellular proliferation and differentiation, inflammation and immune responses. Although a previous study had suggested a repressive function of miR-155 on the MAPK pathway member MAP3K10 (Zhu et al., 2012), the role of miR-181b remains unknown.

In order to test the role of MAP3K10 in mutant p53-driven invasion, we first verified the dependency of MAP3K10 levels on mutant p53 by RT-qPCR and western blotting. *Map3k10* mRNA and protein levels were increased upon knockdown of mutant p53, indicating that mutant p53 negatively regulates its expression, consistent with our RNAseq data (Figures 3.3.C and 3.3.D). Next, we tested the functional connection between mutant p53, miR-155 and -181b, and MAP3K10 in promoting tumor cell invasion of pancreatic cancer cells. According to our hypothesis, mutant p53 should enhance miR-155 and -181b expression to promote the degradation of the *Map3k10* transcript and to potentiate the invasive phenotype of pancreatic cancer cells. Indeed, knockdown of MAP3K10 with two different shRNAs enhanced the invasive potential of $KP_{fl}C$ cells to a similar extent as seen upon overexpression of mutant p53. Together, our data points to a mutant p53/miR-155 and -181b/MAP3K10 signaling axis that specifically impacts upon the invasive potential of pancreatic cancer cells (Figure 3.3.E)

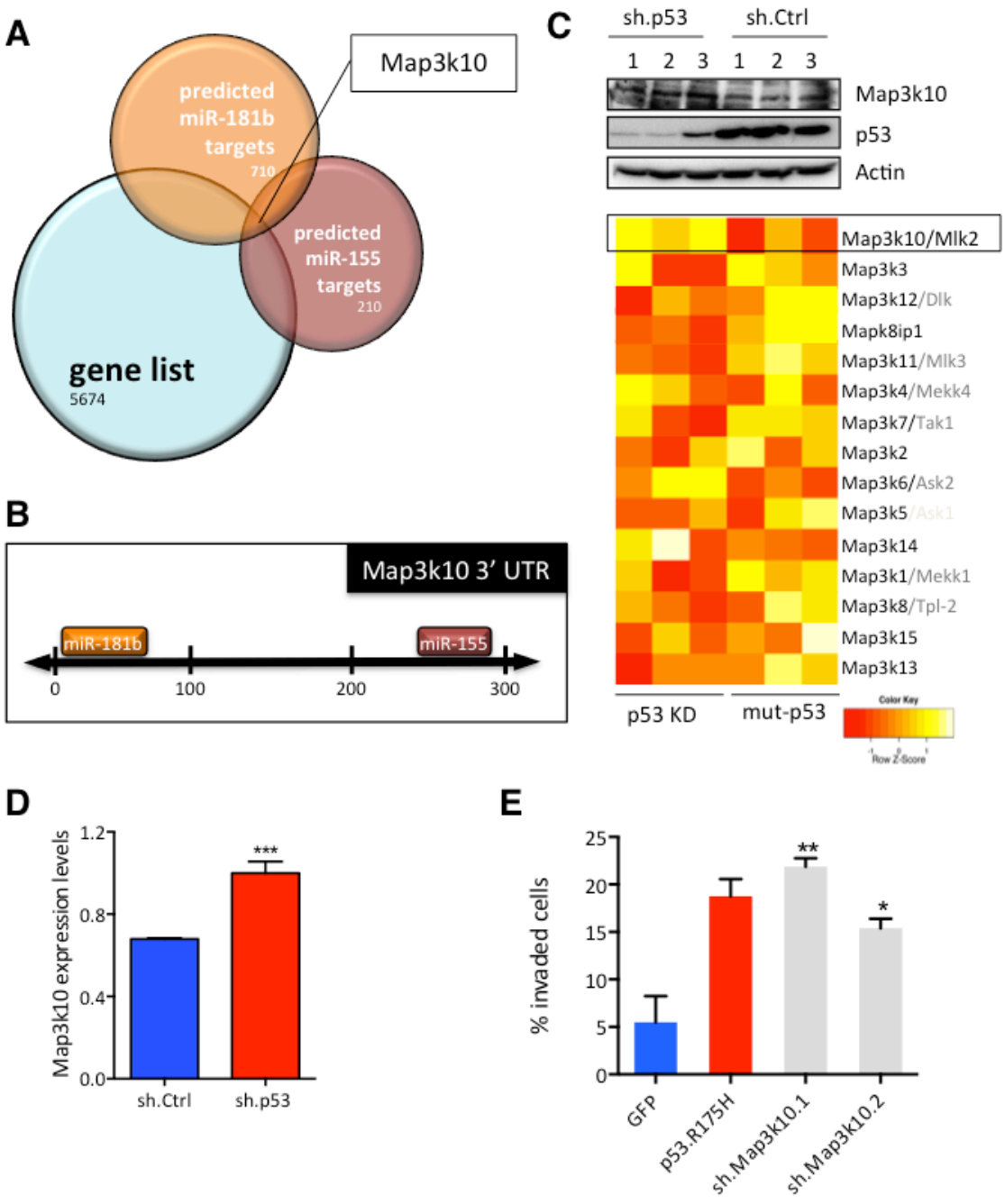


Figure 3.3. mutant p53 induces an invasive phenotype through the miR-mediated downregulation of MAP3K10

(A) Venn diagram of predicted targets of miR-155 and -181b, and mutant-p53-response genes as identified by long RNAseq. *Map3k10* is the only overlapping gene. (B) *Map3k10* 3' UTR and the putative binding sites for miR-155 and 181b. (C) Western blotting analysis of MAP3K10, p53, and Actin in sh.p53- or sh.Ctrl-expressing KPC cells (upper panel). Heatmap of *Map3k's* and their expression changes upon p53 depletion as identified by RNAseq. (D) qRT-PCR for *Map3k10* in KPC+sh.p53 or +sh.Ctrl cells. Data presented as mean normalized *Map3k10* expression ±SD of triplicate samples. A representative result of three repeated experiments is shown. (E) Quantification of the invasion into collagen of $KP_{fl}C$+GFP, +p53.R175H, and sh.Map3k10 (1 or 2). Data presented as mean ±SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.2.4. Mutant p53 inhibits MKK4 phosphorylation through the miR-155/-181b-Map3k10 axis

MKK4 is one of the downstream effectors of MAP3K10 that acts as a suppressor of metastasis in different tumor types, such as lung and ovarian cancer (Ahn et al., 2011; Yamada et al., 2002). Following the observation that mutant p53 represses MAP3K10 levels to promote cell invasion in pancreatic cancer cells, we investigated whether mutant p53 regulates the activation of MKK4. To this end, we measured the levels of Ser-257 phosphorylated Mkk4 levels upon manipulation of mutant p53. Levels of activated Ser-257 pMKK4 were increased when mutant p53 was knocked down in KPC cells, whereas inhibitory phosphorylation of MKK4 at residue Ser-80 was not affected (Figure 3.4.A).

To further explore whether mutant p53 exerts its oncogenic function through the regulation of MKK4 activity, we genetically manipulated MAP3K10 and mutant p53 in pancreatic cancer cells and analyzed MKK4 status by western blotting. In non-invasive, *p53*-null $KP_{fl}C$ cells, MAP3K10 and activated Ser-257 pMKK4 were expressed at high levels (Figure 3.4.B, lane 2). Accordingly, knock down of MAP3K10 resulted in reduced levels of Ser-257 pMKK4, whereas total levels of MKK4 remained unchanged. Importantly, overexpression of a mutant form of p53 in $KP_{fl}C$ cells resulted in attenuated expression of MAP3K10 (presumably through elevated miR-155 and miR-181b expression levels) and consequently, decreased levels of activated pMKK4, rendering these cells more invasive as previously described (Figure 3.4.B lane 1). Despite the expression of a mutant form of p53 leading to reduced levels of activated MKK4, consistent changes in the activated levels of the canonical substrates of MKK4, JNK and Jun, were not observed. Similarly, levels of phosphorylated JNK and c-JUN were not reduced upon depletion of p53 in KPC cells,

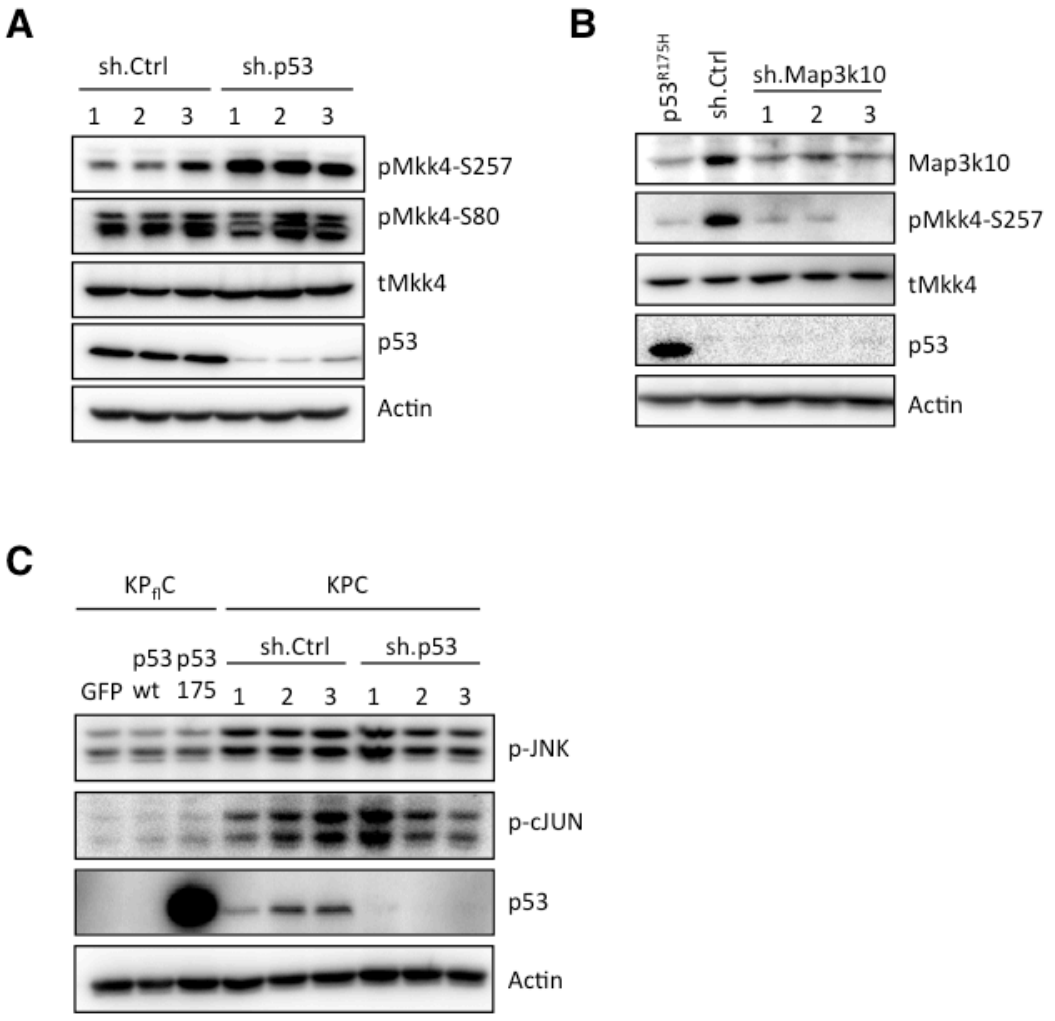


Figure 3.4. Levels of activated Mkk4 are dependent on the p53 status
(A) Western blotting analysis of activated (S247), suppressed (S80), and total levels of MKK4, p53, and Actin in sh.p53- or sh.Ctrl-expressing KPC cells. (B) Western blotting analysis of MAP3K10, MKK4 (S257 and total), p53, and Actin in p53.R175H, sh.Ctrl, sh.Map3k10 (1, 2, or 3)-expressing cells. (C) Canonical downstream targets of MKK4. Protein levels of phospho-JNK, phospho-c-Jun, p53 and Actin were measured in $KP_{fl}C$ cells expressing a GFP, p53.wt, or p53.R172H vector, and in KPC cells expressing sh.Ctrl (1,2, or 3) and sh.p53 (1,2, or 3).

suggesting the role for other compensatory regulators in these cells (i.e., MKK7) (Figure 3.4.B). Together these results support the idea that mutant p53 governs the MAPK pathway through the regulation of the miR-155/miR-181b/MAP3K10 axis, resulting in the suppression of the metastasis suppressor MKK4.

According to previous reports (Ahn et al., 2011), we did not see a proliferative advantage of $KP_{fl}C$ cells infected with two different shRNAs targeting *Mkk4* (Figure 3.5.A). However, we confirmed that $KP_{fl}C$ +sh.Mkk4 cells efficiently migrated in scratch-wound assays, despite the absence of mutant p53, which usually renders cells less migratory as seen with p53-null $KP_{fl}C$+sh.Ctrl cells (Figure 3.5.B). Next, we examined whether the invasive capacity of $KP_{fl}C$ cells depends on MKK4 activity. As observed before, p53-null KPflC+sh.Ctrl cells invaded very poorly but their invasive potential was enhanced when E-Cadherin, a crucial factor in epithelial cell-cell adhesion, was knocked down. Similarly, knockdown of MKK4 in $KP_{fl}C$ initiated invasiveness (Figure 3.5.C), suggesting that MKK4 functions as a suppressor of invasion.

Others have proposed tumor- and metastasis suppressive functions of MKK4. Our studies in murine pancreatic cancer cells further support this idea. Limited data from pancreatic cancer patients show frequent heterozygous deletions of *MKK4*, a common characteristic of genes with tumor suppressive functions (Figure 3.4.F). Moreover, the tumor suppressor p53 is mutated in 75% of PDAC patients and mutations in this gene have been correlated with a worse disease-free outcome (see Chapter 2). The fact that tumors harboring mutations in p53 are also characterized by heterozygous loss of *MKK4* could reinforce our findings that mutant p53 attenuates activating levels of MKK4 to increase

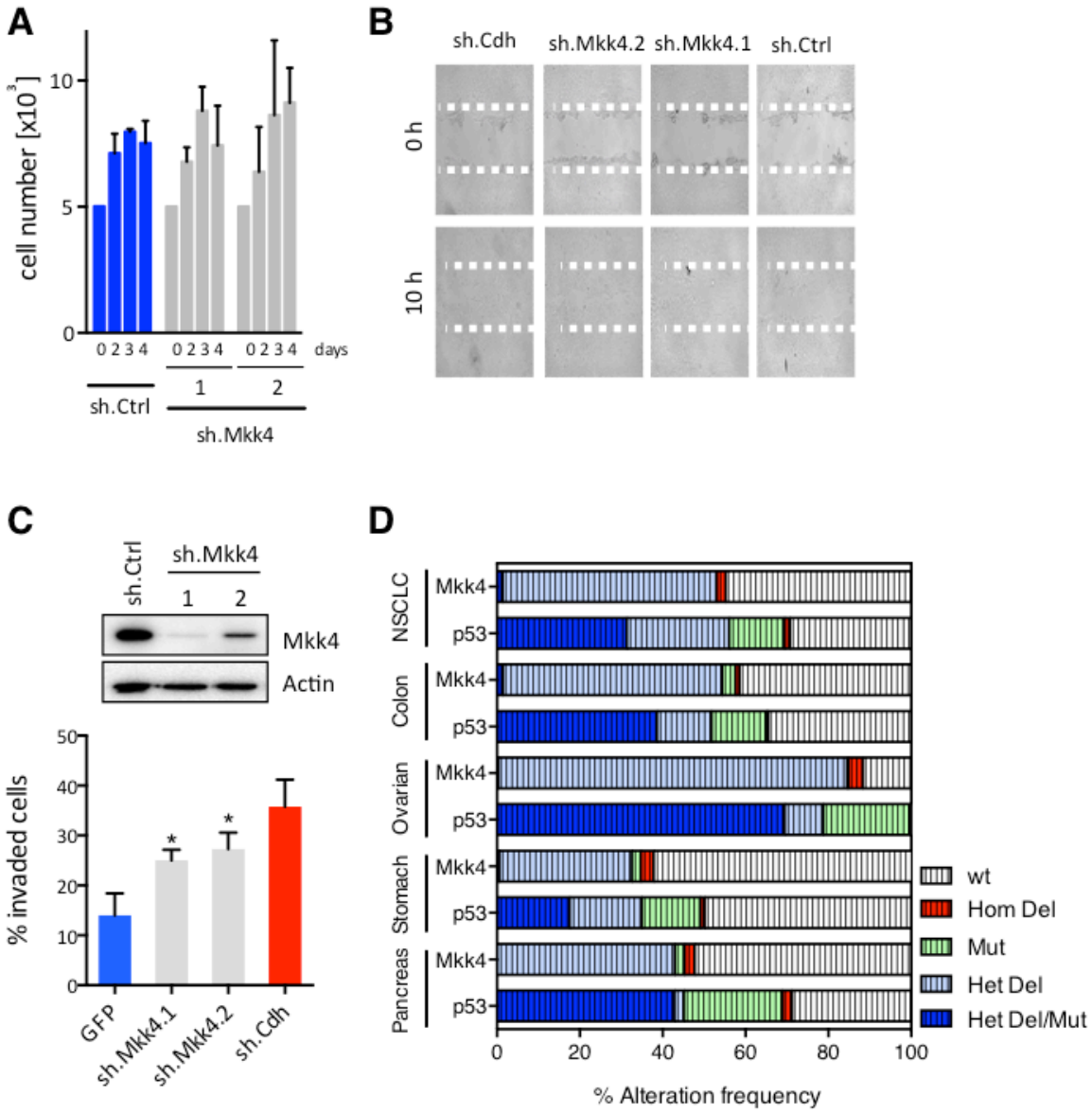


Figure 3.5. Low levels of activated MKK4 induce invasiveness
(A) Cell number over time (days) of $KP_{fl}C$ cells stably expressing sh.Ctrl or sh.Mkk4 (1 or 2). (B) Representative phase contrast images from live cell recordings of scratch-wound assays of $KP_{fl}C$ cells stably expressing sh.Ctrl, sh.Mkk4 (1 or 2), and sh.Cdh at 0 and 10 h. (C) MKK4 and Actin levels of $KP_{fl}C$ cells infected with shRNAs targeting a non-targeting control (Ctrl) or Mkk4 (1 or 2) as determined by western blotting. (D) Genetic configuration of *p53* and *MKK4* in human pancreatic, stomach, ovarian, colon and lung cancer. Depicted is the percentage of heterozygous or homozygous deletions in the presence or absence of mutations.

invasion. Together these results support a model in which mutant p53 promotes invasion in pancreatic cancer cells, in part, via an indirect mechanism that depends on its ability to repress the metastasis suppressor MKK4 through the regulation of the miR-155/miR-181b/MAP3K10 axis (Figure 3.5).

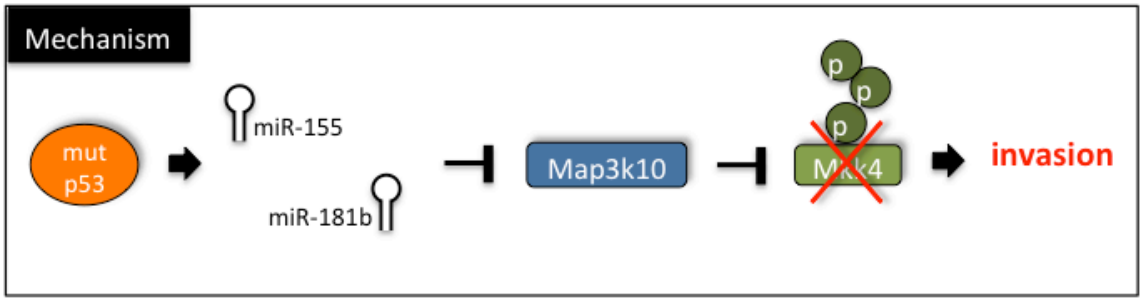


Figure 3.6. miRNA-mediated mechanism of action of mutant p53 in promoting invasion

3.3. Discussion

miRNAs govern many cellular processes and deregulation of their biogenesis has been associated with several aspects of malignant progression. Here, we have shown that mutant p53 regulates a distinct miRNA profile to potentiate the invasive capacity of pancreatic cancer cells. By enhancing the expression of miR-155 and -181b, mutant p53 reduces the protein level of Map3k10 and, thereby, attenuates the ability of MAP3K10 to activate MKK4, a well-known metastasis suppressor gene in several cancer types. Therefore, mutant p53 not only potentiates the invasive capacity of pancreatic cancer cells through the direct regulation of genes such as PDGFRb (see Chapter 2), but also through the modulation of miRNA levels.

A global decrease of mature miRNA levels is a characteristic of human and murine cancers, and is involved in several aspects of tumorigenesis such as cell invasion and

metastasis. General downregulation of miRNA is often associated with a decreased expression of miRNA biogenesis enzymes, such as Dicer (Lu et al., 2005). Dicer is a transcriptional target of p63, and decreased levels of p63 coincide with attenuated levels of Dicer and mature miRNAs (Muller at al., 2014). However, as described here, pancreatic cancer cells expressing a mutant form of p53 exhibit an enhanced invasive potential, which is independent of a global decrease of mature miRNAs or mutant p53-dependent regulation of Dicer. Instead, mutant p53 promotes the aberrant expression of several distinct miRNAs in pancreatic cancer cells. The discrepancies may be explained by the fact that p63, a p53 family member negatively regulated by mutant p53, is not expressed in KPC cells. Supporting a Dicer-independent activity of mutant p53 in enhancing pancreatic cancer cell invasion and metastasis, we observed an equal number of up- and downregulated miRNAs in response to mutant p53 knockdown. Moreover, only upregulated miRNAs seem to contribute to the invasive and metastatic phenotype conferred by mutant p53. Collectively, our data indicate that mutant p53 exerts its effects in pancreatic cancer cells through the regulation of particular miRNAs rather than by modulating the global biogenesis of their genetic regulators.

Recently, miRNAs have been proposed to function as biomarkers of pancreatic cancer development and progression (Khan et al., 2013). Because the current 5-year survival rate for patients with pancreatic cancer is less than 5%, and surgical resection remains the most effective therapy, identifying markers affecting overall survival may improve our ability to define subsets of pancreatic cancer patients and will help to develop effective targeted therapies. Combination of clinicopathological data, follow-up analyses and miRNA expression studies have led to the identification of miR-21, miR-155 and miR-200 as bona-

fide predictors of survival and clinical outcome in pancreatic cancer patients. Importantly, these miRNAs regulate, and are regulated by, genes that are typically altered in pancreatic cancer including *p53, KRAS, p16,* and *BRCA1/2*, thereby influencing DNA repair and cell cycle progression (Tang et al., 2013). miR-34 is involved in pancreatic cancer stem cell self-renewal and/or cell fate determination, potentially via the direct modulation of downstream targets Bcl-2 and Notch, and restoration of miR-34 in mutant p53 or p53-null pancreatic tumors may hold significant promise as a novel molecular therapy (Ji et a;., 2009). Moreover, miRNAs have been linked to tumor progression and metastasis by mediating epithelial-mesenchymal transition (EMT). In breast cancer cells, miR-200 prevents EMT by inhibiting ZEB1 and ZEB2, repressors of E-Cadherin, whereas overexpression of miR-429 reverses the transition in ovarian cancer cells (Kong et al., 2012). Thus, understanding the reciprocal relationships between miRNAs and these genes will be of significant value for determining the driving forces underlying this aggressive disease and for the development of new therapeutic avenues.

Questions remain as to how mutant p53 regulates miR-155 and -181b expression. Elevated expression of both miRNAs in solid tumors, including pancreatic cancer, is well established (Liu et al., 2014; Papaconstantinou et al., 2014); however, the molecular mechanism underlying the Dicer-independent upregulation of these two miRNAs remains unclear. Expression of both miRNAs might be the consequence of the interaction of mutant p53 with specific transcription factors to induce miRNA transcription and expression. For example, the proto-oncogene c-MYC transcriptionally activates the oncogenic miR-17-92 cluster, resulting in the tight control of E2F1 levels and cell cycle progression (O'Donnell et al., 2005). Alternatively, mutant p53 could modulate levels of miR-155 and -181b by

regulating the activity of epigenetic regulators. For example, BRCA1 epigenetically represses miR-155 expression via its association with HDAC2, which deacetylates histones H2A and H3 on the miR-155 promoter (Chang et al., 2011).

Human pancreatic tumors are frequently characterized by a mono-allelic mutation in the tumor suppressor *p53* and LOH of the remaining short arm of chromosome 17 (17p). An open debate has been why a cancer cell is under selective pressure to sustain both events. As described in Chapter 2 and 3, p53 mutants carry neomorphic functions and render a tumor more aggressive. LOH of 17p, and thereby the remaining wild type allele of *p53*, is thought to potentiate the gain of function ability of the mutant form. However, does the loss of chromosome arm 17p equal solely the loss of the remaining *p53* allele? Here we propose another idea by which LOH of 17p reduces metastasis suppressive function of MKK4, located 10 cM centromeric to *p53* on chromosome 17 (Figure 4.4). The data at hand suggest a metastasis-suppressive phenotype of activated MKK4, which is attenuated by heterozygous loss of 17p and further disabled in the presence of mutant p53. Mutant p53 is able to suppress activation of MKK4, resulting in increased invasion through yet unknown downstream mediators. This idea is supported by the fact that all heterozygous deletions of *MKK4* occur in tumors with *p53* mutations (Figure 3.4.F), but Mkk4 inactivating mutations are mutually exclusive with *p53* mutations.

Understanding the function of metastasis suppressor proteins can potentially help to identify patients at risk for metastasis development and to identify antimetastatic therapies. So far, a large body of evidence supports a role for MKK4 in inhibiting the metastatic spread of the primary tumor, including ovarian, prostate, breast, and pancreatic cancer (Whitmarsh

and Davis, 2007). However, the downstream mediators remain rather elusive and the MKK4-driven molecular output might depend on the environmental context. For example, in ovarian cancer cells, MKK4 suppresses cell invasion through the upregulation of cell cycle inhibitor p21. Induction of p21 is p53-independent and initiates reversible cell cycle arrest, rather than senescence (Lotan et al., 2008). Whether mutant p53-driven attenuation of MKK4 in pancreatic cancer cells result in reduced levels of p21 and escape from cell cycle arrest remains to be tested.

However, this study leaves a few open questions. First, the functional outcome of miR-155 and -181b in invasion seems to be redundant, but how they affect MAP3K10 protein levels, for example by inhibiting its translation or by targeting degradation, remain to be determined. Furthermore, the downstream effectors of MKK4, which mediate the metastasis-suppressive functions, are undefined. Equally important, but also unresolved, is the validation of the mutant p53-MKK4 axis in human pancreatic cancer samples. Do MKK4 and MAP3K10 expression levels correlate with miR151- and -181b status? Do mutations in *p53* co-occur with elevated levels of miR151 and -181b, and attenuated expression of MAP3K10 and MKK4? The answer to these questions will further strengthen our understanding of how mutant p53 exerts its oncogenic function in invasive pancreatic cancer.

In summary, mutant p53 drives the invasive phenotype of pancreatic cancer through two distinct miRNAs, miR151 and -181b, to attenuate MAP3K10 and activated MKK4 levels. Even though each gene or miRNA has been associated to induce invasion, we show for the first time how they interconnect to yield a more aggressive cancer phenotype. Genetic

evidence for a metastasis suppressive function of MKK4 was previously linked to the sporadic presence of *MKK4* mutations in ~5% of cancer cases. However, our data suggest a much more prominent role of MKK4 in tumors expressing mutant p53, which denote the majority of all pancreatic cancers. Therefore, the herein described mutant p53-MKK4 signaling axis broadens our molecular understanding of invasive pancreatic cancer and might reveal biomarkers for early detection and vulnerabilities for new anti-metatsatic therapies. Moreover, our results unravel a novel mechanism of action of p53's gain of function that explains the simultaneous presence of mutations in *p53* and large deletions of the chromosome arm 17p.

3.4. Chapter Contributions

S.W. designed and performed the majority of the experiments. M.S. contributed intellectually to the design of the project. C.A.D. and V.T. conducted and analyzed small RNA sequencing. S.W.L. conceived and supervised the project

3.5. Supplementary Figures

Gene name
Aak1
Dusp10
Ets1
Fbxo33
Foxk1
Gpd1l
Map3k10
Mef2a
Mex3b
Trim2
Ttl
Wnk1

Suppl. Figure 3.1. List of overlapping genes of predicted miR-155 and miR-181b targets with mutant p53-responsive genes as identified by RNAseq

Chapter 4

Cooperative interactions between 17p tumor suppressor genes

4. Chapter 4

Cooperative interactions between 17p tumor suppressors

4.1. Introduction

The cancer genome is characterized by genetic alterations that facilitate uncontrolled proliferation of tumor cells and prevent them from cell death. Genetic changes that drive tumorigenesis, such as particular deletions, amplifications, and somatic mutations, become selected as cancers are initiated and evolve. However, given the genetic instability of many tumors, most of the observed genomic alterations are passenger changes that do not confer a selective advantage to the developing tumor. Despite the abundance of data provided by genome-wide sequencing studies, our knowledge about driver genes and how they contribute to cancer progression remains incomplete. Therefore, functional studies are required for the validation of candidate genes and for the characterization of their actions. Combining genomic data with functional studies holds promise for improving cancer diagnosis, prognosis and therapy.

Large heterozygous deletions occur frequently in the cancer genome, but their biological significance remains elusive (Table 1.1). If only partial loss of gene function can facilitate tumorigenesis, Knudson's "two-hit" hypothesis for the bi-allelic inactivation of tumor suppressors (Knudson, 1971) might not apply to genes located in these regions. Indeed, most cancer-related deletions do not harbor known or putative tumor suppressor genes (Vogelstein et al., 2013), raising the possibility that only the simultaneous partial attenuation of multiple genes might collectively contribute to tumorigenesis. Certainly,

recent studies have confirmed this hypothesis by showing that heterozygous loss of multiple genes, clustered in the same chromosome region, cooperates to enhance tumorigenesis (Xue et al. 2012; Solimini et al., 2012). Formally, the same concept may also apply for large heterozygous deletions where a bona fide tumor suppressor gene has also been disrupted. Large heterozygous deletions encompassing regions on chromosome 17p are very common in human tumors. Here, the presence of the tumor suppressor p53 is widely thought to be the only link to selective pressure that cancer cells undergo to delete this region. However, deletions of the chromosome arm 17p reduce the dosage of many other neighboring genes, which could in principle contribute to tumorigenesis in a haploinsufficient manner, where mono-allelic loss suffices to promote oncogenic progression. The identification of cooperating genes could explain the forces driving selection against the normal homozygous presence of 17p, and in turn, this concept could be further expanded to include effects of deletion of other large chromosomal regions.

Hepatocellular carcinoma (HCC) is characterized by the recurrent, heterozygous loss of chromosome region 17p, and, therefore, represents an appropriate system to study the presence of linked tumor suppressor genes that cooperate during tumor progression. HCC, a common but understudied cancer for which there are few treatment options, has been successfully modeled and studied in mice (Zender et al., 2008; Xue et al., 2012). In particular, the mosaic mouse model of HCC is based on the isolation and ex vivo manipulation of mouse embryonic liver progenitor cells, followed by their retransplantation into recipient mice. The resulting tumors closely resemble the pathology and biology of the human disease. More specifically, the HCC mosaic mouse model utilizes MYC-overexpressing, $p53^{null}$ hepatocytes that are immortalized but not tumorigenic, providing a sensitized

background that is ideal for the identification of additional genetic alterations that might be involved in tumor initiation and progression. Large-scale RNAi screens can be carried out in the HCC ex vivo model, in which pools of shRNAs targeting recurrently deleted genes can be tested for their ability to promote tumorigenesis. This approach has successfully led to the identification of tumor suppressor genes, such as *Xpo4* (Zender at al., 2008) and *Dlc1* (Xue et al., 2008).

The combination of cancer genomics, RNAi, and mosaic mouse models can facilitate the functional annotation of frequently altered genes. One gene that frequently undergoes genetic alterations in HCC is the mitogen-activated protein kinase (MAPK) kinase 4 (MKK4). *Mkk4*, located on chromosome 17p, just 10 cm centromeric to *p53*, is a non-redundant component of stress activated MAPK signaling modules. In response to various stimuli, MKK4 phosphorylates and activates the c-JUN N-terminal protein kinase (JNK) and p38 families of MAPKs. Despite several studies analyzing the role of MAP kinases in cancers, the function of MKK4 in tumorigenesis is not well understood. Some experimental evidence suggests a pro-oncogenic role for MKK4 (Wang et al., 2004; Finegan and Tournier, 2010), whereas others demonstrated a growth-suppressive function (Ahn et al., 2011; Nakamura et al., 2013). Consistent with a growth-suppressive role of MKK4, a wide spectrum of primary cancers shows loss-of-function mutations in *MKK4*, in a LOH background, at a fairly consistent rate (~5%) or harbor homozygous deletions that eliminated coding portions of the *MKK4* locus (Teng et al., 1997; Su et al., 2002).

In this chapter, I aim to explore the hypothesis that frequent heterozygous deletions of chromosome region 17p arise as a single mechanism to facilitate the cosuppression of

multiple genes. By screening pools of shRNAs targeting the mouse orthologs of genes contained in the chromosomal region 17p in vivo, we identified the dual-specific kinase MKK4 as a tumor suppressor gene, whose loss cooperates with *p53* deletion to drive tumorigenesis. Mkk4 suppression significantly enhanced the tumorigenic potential of *p53*-depleted hepatocytes in ex vivo transplantation or transposon-based mouse models of HCC. Hepatocytes with stable RNAi-mediated MKK4 silencing showed increased activation of anti-apoptotic genes and survival genes, which are dependent on upregulation of NF-κB. Therefore, our study identifies and validates MKK4 as a potential haploinsufficient tumor suppressor on 17p and provides evidence that large deletion events target multiple genes that together drive tumorigenesis.

4.2. Results

4.2.1. *In vivo* RNAi screen for the identification of tumor suppressor genes on chromosome region 17p

We have previously established an ex vivo transplantation model of hepatocellular carcinoma (HCC) that is suitable for conducting direct in vivo RNAi screens for genes that positively impact HCC development (Zender et al., 2008). Because (i) chromosome arm deletions might target multiple genes that cooperate to drive tumorigenesis, (ii) 17p is one of the most frequent genomic deletions among multiple human tumor types, we decided to conduct a positive selection screen for tumor suppressor genes on the short chromosome arm 17. To this end, we generated a library of 1,645 shRNAs that targeted all 274 genes contained in the chromosome region 17p (6 shRNAs per gene). The shRNAs were designed using on-chip oligonucleotide synthesis and a bar-coding strategy such that shRNAs could

be amplified from the mixtures in pools of a discrete size (~100 shRNAs per pool). Of note, all of the shRNAs used in this study were cloned into pMLS, a vector optimized for in vivo use, which coexpresses green fluorescent protein (GFP). The pools were individually screened for their ability to enhance tumor potential of immortalized, *p53*$^{-/-}$ liver progenitor cells (LPCs) overexpressing the MYC oncogene at low levels (Figure 4.1.A). These cells provide a ''sensitized'' background where a single additional lesion can trigger tumorigenesis and were therefore suitable for identifying genes that contribute to cancer. As previously described, tumors resulting from the subcutaneous injection of premalignant progenitor cells into recipient mice recapitulated the human disease with solid and trabecular growth pattern (Figure S4.1.A).

To establish efficient screening conditions in our HCC ex vivo mouse model, we tested how knockdown of a potent tumor suppressor gene or a control gene affected the tumorigenic potential of the LPCs. First, we observed that shRNA-mediated depletion of the WNT-pathway regulator APC efficiently accelerated tumorigenesis, even when the library was diluted 1:50 or 1:100 (Figures S4.1B and S4.1.C). By contrast, an shRNA targeting a neutral control gene (Renilla) did not accelerate tumor development and most of the tumors that eventually arose were GFP-negative, indicating that the neutral shRNA did not confer a selective advantage during malignant progression. Dilution studies indicated that a pool of 100 shRNAs can allow for outgrowth and enrichment of shRNAs that target genes with tumor suppressive functions.

Upon subcutaneous injection of LPCs infected with the shRNA pools, tumors developed with varying latency. From a total of 16 analyzed shRNA pools, 4 accelerated

tumor growth and were GFP-positive (Figure S4.1.D). We then used deep sequencing of PCR-amplified shRNAs to analyze the shRNA representation in the preinjected cell pools and the resulting tumors (Figure 4.1.A). According to our hypothesis, enriched shRNAs in the tumor should constitute candidate 'drivers' of the disease. Putative tumor suppressor genes were then prioritized using an enrichment score based on (1) the number of shRNAs targeting the gene retrieved from the tumor, and (2) the extent shRNA enrichment in the tumor (> 15%). We identified 7 potential tumor suppressor genes (~2.6% of total tested genes) (Table 4.1.), most of which have not been recently associated with a tumor suppressive function. However, Mkk4 was the only gene that was targeted by two shRNAs, limiting the possibility of off-target effects (Figure 4.1.B). Therefore, we decided to follow up on Mkk4 and study its tumor suppressive function in HCC.

Candidate TSG	Tumor #	shRNA #	Score
Myh3	2	1	320.2
Mkk4	2	2	302.0
Glp2r	1	1	99.9
Wdr8	1	1	97.4
4933411G11Rik	1	1	35.7
Rap1gab2	1	1	35.3
Ywha	1	1	29.1

Table 4.1. Putative tumor suppressor genes identified in the 17p screen

MKK4 is a dual-specificity kinase with a known role as a central mediator of the Jun N-terminal kinase (JNK) and p38 phosphorylation cascade. *MKK4* is not only frequently deleted but also shows a mutation rate of ~5% in diverse tumor types, such as pancreatic, bile duct, breast, colon and lung cancer (Cunningham et al., 2006) More importantly, *MKK4* is genetically altered in cancer by deletion or mutation events. We confirmed that MKK4 protein levels were efficiently reduced following the transduction of liver progenitor cells

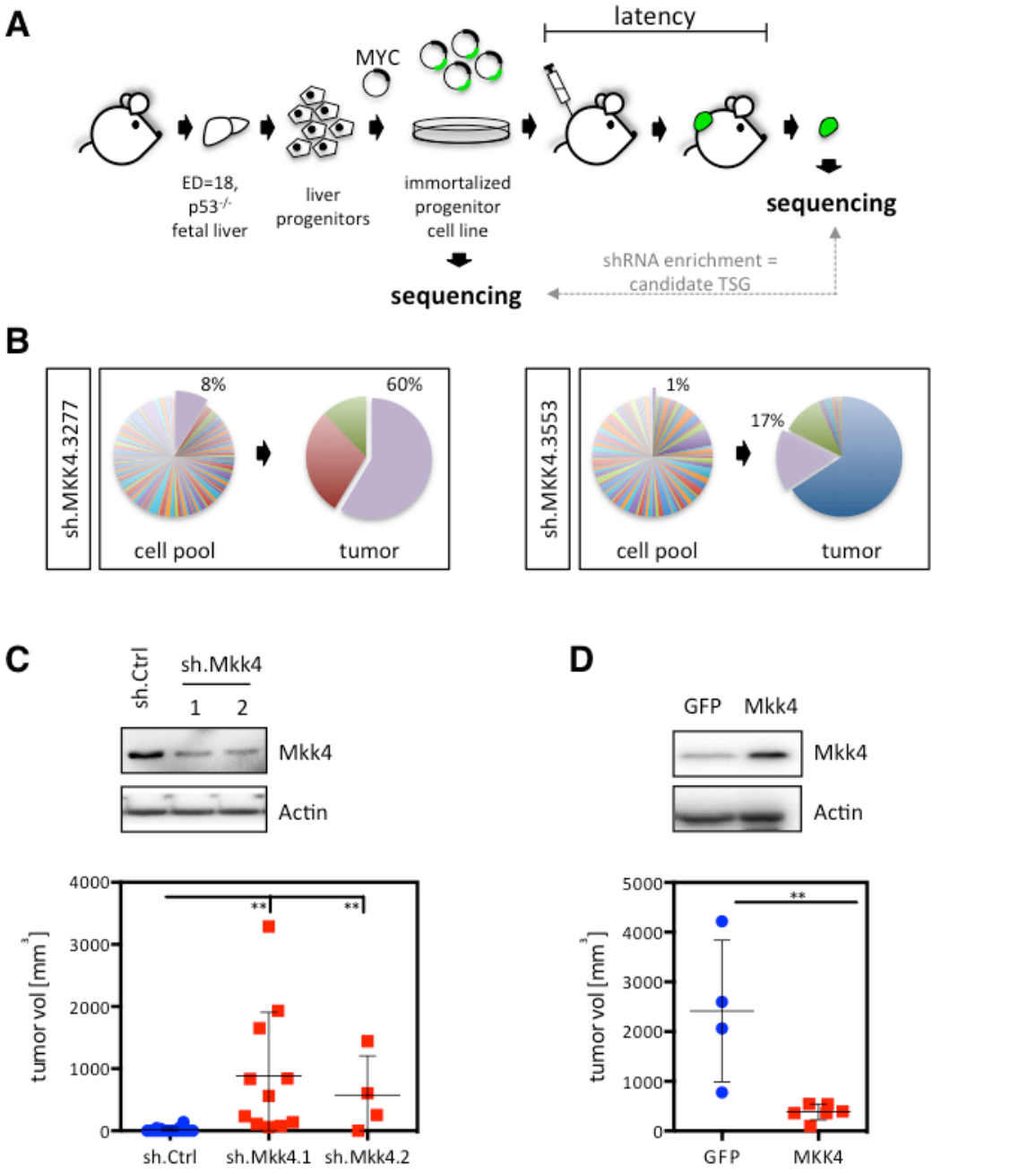


Figure 4.1. *In vivo* RNAi Screen identifies *Mkk4* as a tumor suppressor gene on 17p
(A) Schematic representation of the approach. E18 *p53*$^{-/-}$ liver progenitor cells were isolated and immortalized by overexpressing MYC at low levels. Subsequent infection with shRNA pools and subcutaneous injection allowed tumor growth. (B) Enrichment of two shRNAs targeting MKK4 in tumors compared to their representation in preinjected cell pools. Pie graphs show the representation of each sh.Mkk4 in the total shRNA population analyzed by high-throughput sequencing. (C) Validation of Mkk4 as a tumor suppressor gene. shRNA-mediated knockdown of MKK4 (upper panel, Actin as loading control) increases tumorigenesis of subcutaneously injected *p53*$^{-/-}$;MYC LPC cells (lower panel). (D) Overexpression of *Mkk4* cDNA (upper panel, Actin as loading control) in tumorigenic *p53*$^{-/-}$;MYC liver cells reduces tumor burden upon subcutaneous injection.

with two independent sh.Mkk4 (Figure 4.1.C). Upon injections into the flanks of nude mice, LPCs infected with shRNAs targeting MKK4 (LPC + sh.Mkk4) developed tumors faster and at a higher penetrance than LPCs expressing an shRNA targeting a control gene (LPC + sh.Ctrl) (Figure 4.1.C). We next asked whether MKK4 overexpression in tumorigenic liver cancer cells of the same genotype ($p53^{-/-}$;MYC overexpression at high levels) would attenuate their tumorigenic potential. Whereas tumor cells transduced with a GFP control plasmid consistently developed tumors upon their injection into the flank of nude mice, the tumor burden was significantly reduced in mice injected with tumor cells overexpressing MKK4 (Figure 4.1.D). Together, these data suggest that MKK4 functions as a tumor suppressor gene in liver cancer.

4.2.2. Combined loss of *p53* and *MKK4* cooperates to accelerate tumorigenesis

The results above reveal a tumor suppressive activity of MKK4 in HCC; however, the question remained whether the simultaneous loss of both *Mkk4* and *p53* has a synergistic effect on tumor growth as compared to the depletion of each individual gene alone. To address this question, we used shRNA-encoding transposable elements in conditional *p53* knockout mice ($p53^{fl/fl}$). The system allowed us to disrupt *p53* gene function in a subset of mice upon induction of *Cre* expression and to suppress the function of MKK4 by shRNA-mediated knockdown. Hydrodynamic injection was used to deliver transposable elements together with a sleeping beauty transposase (SB13) into hepatocytes of $p53^{fl/fl}$ mice. Transient expression of SB13 enables stable integration of the transposons in a subset of targeted liver cells. The transposons are engineered to encode a tamoxifen-inducible *Cre* recombinase (*CreERT*) together with cDNAs encoding oncogenes, and/or GFP-linked shRNAs. We

expressed MYC and β-Catenin, two oncogenes frequently altered in HCC, in hepatocytes to create a sensitized background for tumor initiation. Simultaneously, we transduced hepatocytes of these mice with either sh.Mkk4 (1 or 2) or sh.Ctrl and their expression was followed by the detection of the fluorescent protein GFP. Then, we treated on cohort of sh.Mkk4;*p53*$^{fl/fl}$ and sh.Ctrl;*p53*$^{fl/fl}$ mice with tamoxifen to induce *Cre*-mediated excision of the p53 gene or with drug-vehicle to allow the continued expression of p53 (Figure 4.2.A).

After 6 weeks, we quantified the size of each tumor nodule on serial H&E sections of mouse livers of six different genotypes: sh.Ctrl,p53(+/+); sh.Ctrl,p53(-/-); sh.Mkk4.1,p53(+/+); sh.Mkk4.1,p53(-/-); sh.Mkk4.2,p53(+/+); and sh.Mkk4.2,p53(-/-). As expected, we did not observe any liver tumors in mice that expressed both Mkk4 and p53 at endogenous levels. Genetic disruption of either MKK4 or *p53* in hepatocytes overexpressing MYC and β-Catenin resulted in the development of a few liver tumor nodules, indicating that inhibition of MKK4 or p53 alone induces tumorigenesis inefficiently. However, sh.Mkk4-expressing mice with disrupted expression of the *p53* gene developed liver tumors at high frequency (Figures 4.2.B and 4.2.C). Disruption of *p53* in sh.Mkk4.1 mice resulted in an increased tumor burden compared to that in sh.Mkk4.2 mice. This discrepancy was also observed in the ex vivo HCC model and might be due to different knock down efficiencies of Mkk4. Collectively, our data imply that the impact of 17p deletions goes beyond the effects of *p53* mutation or, for that matter, the attenuation of any individual gene.

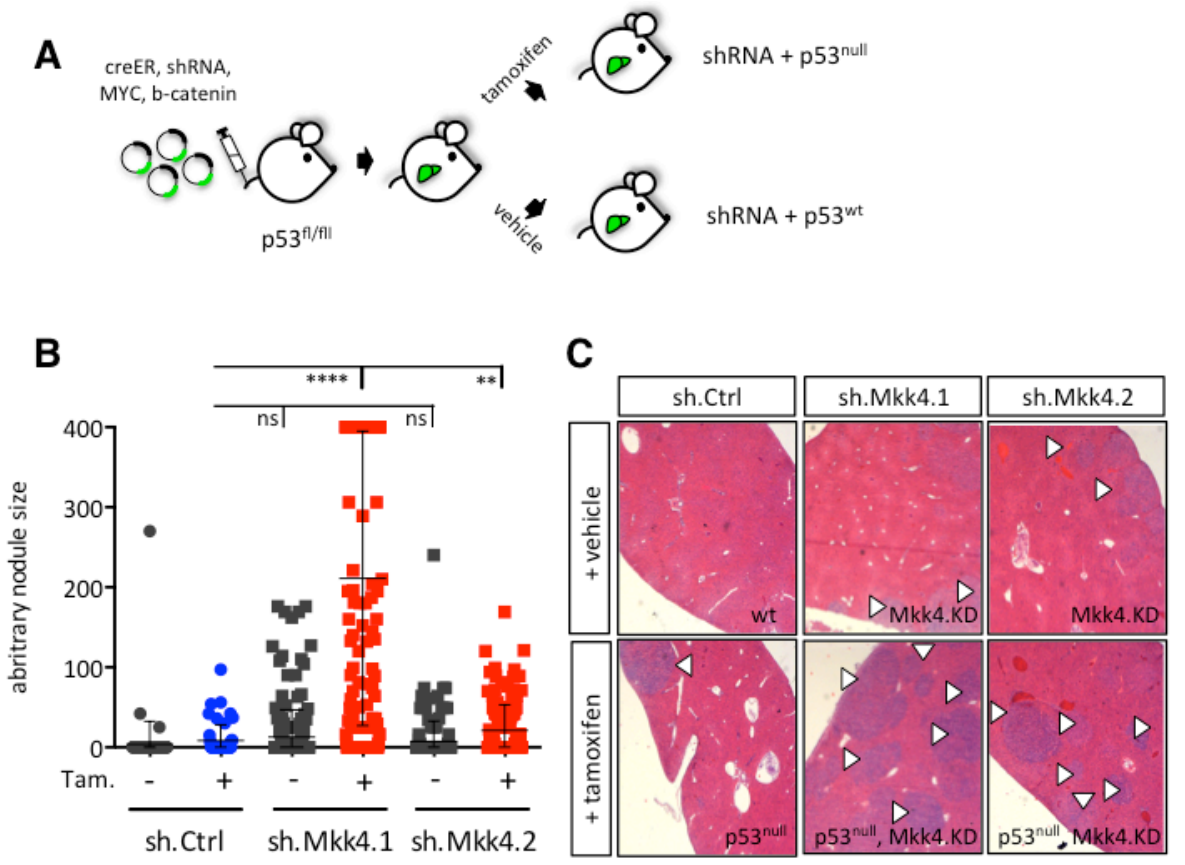


Figure 4.2. Cooperative effects of MKK4 and p53 suppression on tumorigenesis
(A) Schematic representation of suppression of Mkk4 and p53, either simultaneously or individually. Tamixofen treatment induced *Cre* expression to disrupt p53 expression. shRNA expression mediated MKK4 knockdown. Combination of both strategies gives six different genotypes: sh.Ctrl,$p53^{wt}$; sh.Ctrl,$p53^{-/-}$; sh.Mkk4.1,$p53^{wt}$; sh.Mkk4.1,$p53^{-/-}$; sh.Mkk4.2,$p53^{wt}$; and sh.Mkk4.2,$p53^{-/-}$. (B) Quantification of liver tumor size from experimental mice from (A). (C) H&E section of livers. Tumors are marked by white arrows.

4.2.3. Decreased MKK4 activity cooperates with loss of *p53* in hepatocellular carcinoma by triggering an anti-apoptotic response

MKK4 is a dual-specific protein kinase that activates, together with MKK7, the p38 and JNK signaling pathways during development and in response to stress (Davis, 2000; Lee et al., 2002). Recently, it has been proposed that silencing of MKK4 leads to upregulation of MKK7, which potentiates liver regeneration in a JNK-dependent manner (Wuestefeld et al., 2013). However, in our model, MKK4 suppression did not change the expression or activation of MKK7, p38 or JNK as determined by levels of the phosphorylated proteins (Figures 4.3.A and S4.2.C). Because these results suggest that the tumor suppressive functions of Mkk4 do not involve the activation of the canonical pathway, we aimed to understand whether loss of MKK4 triggers a more complex and non-canonical gene signature to drive HCC.

We sought to examine the effects of MKK4 suppression in proliferation and apoptosis resistance, two hallmarks of malignant growth. Using two individual shRNAs targeting MKK4 in *p53*$^{-/-}$ hepatocytes we observed increased levels of anti-apoptotic genes, such as *Ciap1/2, Xiap1* and *Bcl2* (Figure 4.3.B), compared to hepatocytes with normal levels of MKK4 (LPC+sh.Ctrl). NF-κB negatively regulates the apoptotic response by enhancing the expression of anti-apoptotic regulators and by antagonizing essential elements of the apoptotic signaling network. Importantly, we observed that knockdown of MKK4 significantly enhanced the levels of phospho-p65, suggesting that MKK4 suppresses the expression anti-apoptotic genes through the modulation of the NF-κB pathway (Figure 4.3.B).

Apart from its effect to enhance the expression of anti-apoptotic proteins, depletion of MKK4 in liver progenitor cells led to increased levels of activated ERK, as measured by phospho-ERK (Figure S4.2.A). Activation of ERK mediated by depletion of Mkk4 was dependent on NF-κB, as p-ERK levels were rescued in MKK4-depleted hepatocytes upon knockdown of p65 (Figure S4.2.B). Together, these results suggest that suppressive functions of MKK4 in hepatocellular carcinoma rely on its ability to modulate cell proliferation and apoptosis pathways, at least in part, through the regulation of NF-κB.

To gain insight into the anti-apoptotic effects induced by the suppression of MKK4 in hepatocytes in vivo, we subjected mice to a treatment with CD95 (FAS)-activating antibodies, a well-established model of acute liver failure (Ogasawara et al., 1993). First, $p53^{fl/fl}$ mice with stable intrahepatic expression of Mkk4- or control shRNAs, and tamoxifen-inducible *Cre* recombinase were treated with tamoxifen to disrupt *p53* gene function. Hence, we were able to determine the role of Mkk4 suppression in a *p53*-deficient background. Intravenous injection with anti-Fas antibody resulted in death of sh.Ctrl,$p53^{-/-}$ mice within 4 h, due to acute liver failure associated with massive hepatic apoptosis and hemorrhagic necrosis (Figures 4.3.D and 4.3.E). However, MKK4 silencing resulted in protection of hepatocytes from FAS-induced apoptosis and significantly increased the survival of challenged mice. Accordingly, we observed a decreased number of apoptotic hepatocytes in sh.Mkk4-expressing livers as analyzed by cleaved Caspase-3 staining (Figure 4.3.E). These in vivo results confirm our studies in vitro and further support the idea that the anti-apoptotic pathway is induced by MKK4 -loss to potentiate liver tumor growth.

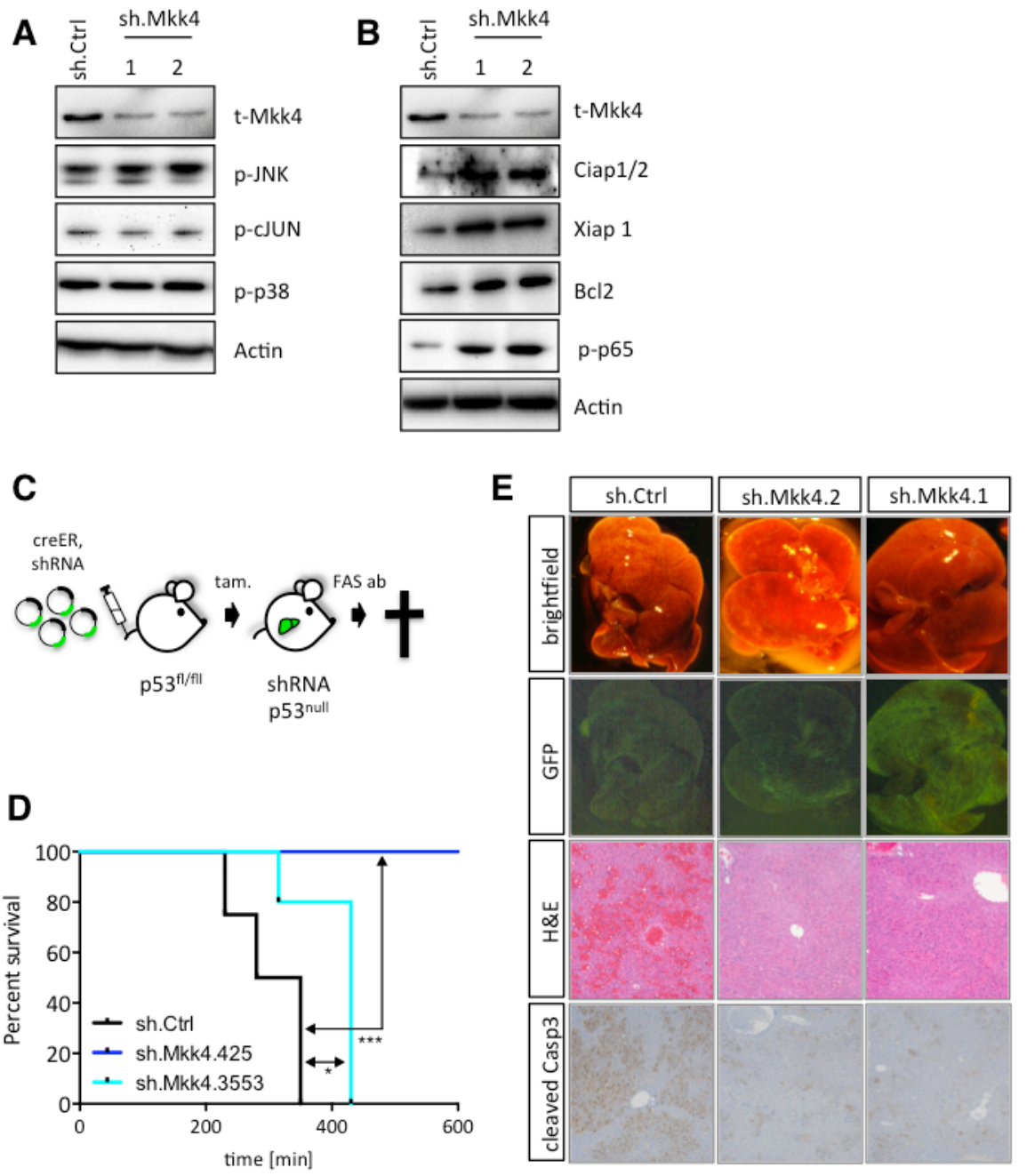


Figure 4.3. MKK4 suppression induces an anti-apoptotic pathway
(A) Protein levels of canonical pathway members p-JNK, p-cJUN, p-p38 in LPCs infected with sh.Ctrl or –Mkk4 (1 or 2). Actin served as a positive control. (B) Protein levels of Mkk4, Ciap1/2, Xiap1, Bcl2, p-p64, and Actin. (C) Schematic outline for the induction of acute liver failure by application of CD95-activating antibody into $p53^{fl/fl}$ mice with sh.Mkk4 (1 or 2) or sh.Ctrl expressing hepatocytes. (D) Survival analysis of mice with stable intrahepatic knockdown of Mkk4 or controls after triggering liver failure as depicted in (C). (E) Brightfield and GFP images of livers expressing sh.Mkk4 (1 or 2) or sh.Ctrl (upper panel). H&E and cleaved caspase 3 staining for apoptotic cells of mouse liver tissue (lower panel). Livers were taken upon mice succumbed to liver failure as described in (C).

4.2.4. 17p deletions are frequently large and target both *MKK4* and *p53*

To better define the frequently occurring 17p deletions in human cancers, we analyzed cancer genome datasets generated by array-based comparative genomic hybridization (aCGH) performed at Cold Spring Harbor Laboratory and the Cancer Genome Atlas (TCGA) project. We analyzed primary tumor samples and cell lines of HCC and colon, lung, ovarian, pancreas, and stomach cancers to determine the extent of chromosome 17p deletions (Figure 4.4.A). Approximately half of these tumors (and as many as 80% of ovarian tumors) harbor deletions of human chromosome 17p, often encompassing a large portion of, or even the entire, chromosome arm. When analyzing the exact chromosomal regions affected by 17p deletions in HCC, we noted that the most frequently deleted region is centered around the *p53* gene and adjacent genes, such as *MKK4*. Together, these data are consistent with the notion that cancers select for chromosome arm 17 deletions at high frequency to target *p53* and *MKK4* loss.

Next, we studied the genetic context of *MKK4* and *p53* loss in more detail. First, we found that *MKK4* and *p53* loss were significantly associated with *MYC* amplifications or gains in the analyzed cancers types, including HCC, colon, lung, ovarian, pancreas, and stomach cancers. This result confirms that mouse models involving MYC overexpression and *p53* loss mimic the human HCC genotype and represent a valid genetic system for exploring the involvement of additional candidate 17p tumor suppressor genes (Figure 4.4.B). More importantly, the analysis revealed that heterozygous deletions of both *MKK4* and *p53* co-occur in a vast majority of cancer patients. In contrast to *p53*, which usually exhibits inactivating mutation of the remaining allele, *MKK4* mutations occurs only in ~5% of tumors, suggesting that MKK4 acts as a haploinsufficient tumor suppressor. In

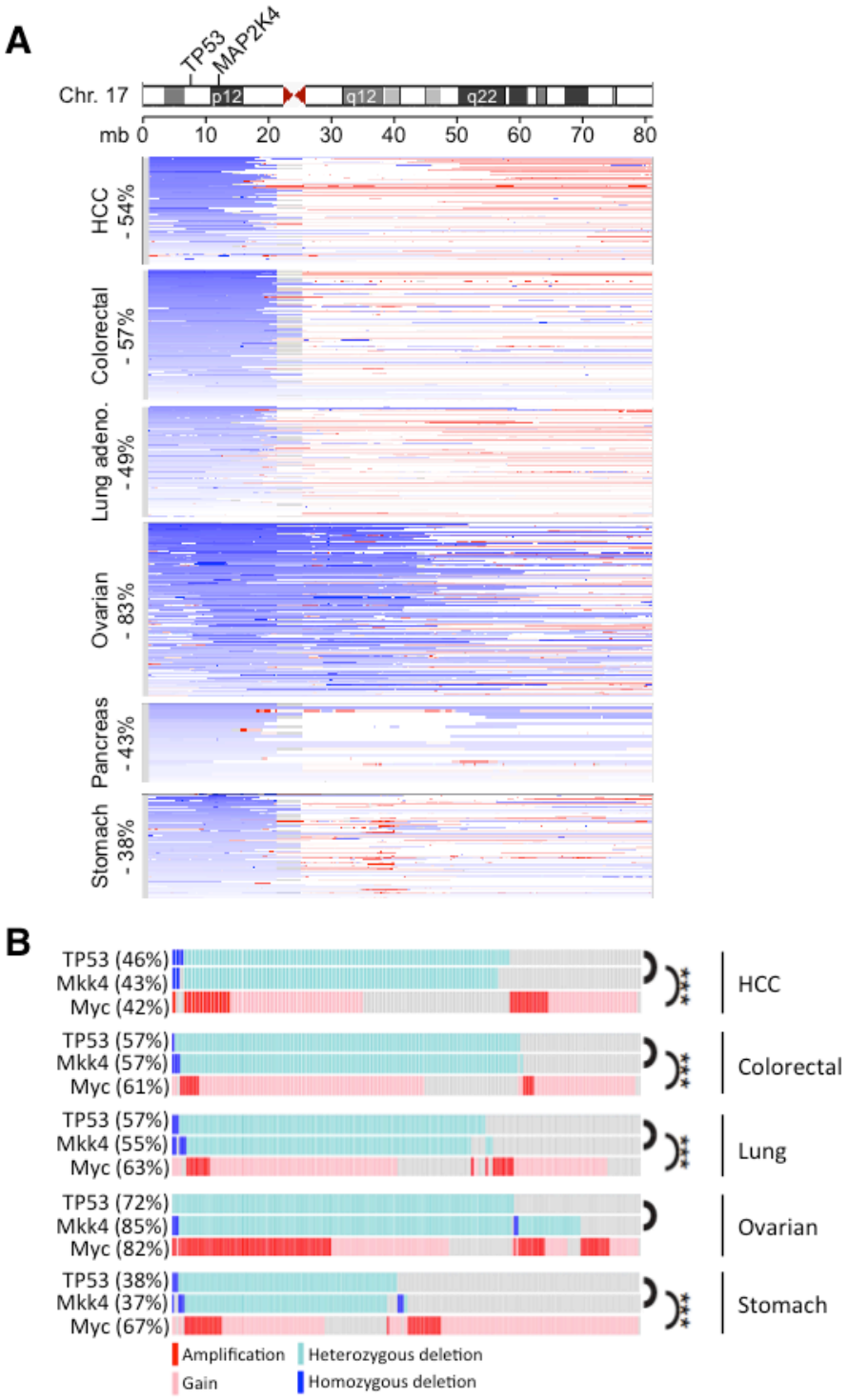


Figure 4.4. Chromosome 17 deletion characteristics and co-occurring genomic aberrations
(A) Size and extent of chromosome 17 deletions (in blue) and amplifications (in red) from individual HCCs, colorectal, lung, ovarian, pancreas, and stomach cancers based on aCGH datal analysis. The location of *p53* and *MKK4* are indicated. (B) *p53* (heterozygous or homozygous) deletions co-occur with *MKK4* deletions and *MYC* amplifications or gains. Each bar represents an individual tumor (from cbioportal.org).

agreement, partial knockdown of MKK4 significantly contributed to malignant progression in our mouse model. Therefore, the gene dosage of *Mkk4* seems to influence the tumor suppressive function of MKK4 in liver cancer.

4.3. Discussion

Cosuppression of multiple genes on chromosome 17p produces additive or cooperative effects on tumor development. Here, we provide an expanded explanation for the frequent occurrence of 17p alterations in pointing to cooperativity between *p53* and *MKK4* inactivation in cancer. We examined the dual impact of attenuating p53, the only functionally validated 17p tumor suppressor gene in HCC so far, and MKK4, the best-scoring candidate tumor suppressor identified in an *in vivo* RNAi screen. Cosuppression of these two genes synergistically accelerated tumor growth compared to knockdown of each gene alone. *MKK4* appears to act as a haploinsufficient tumor suppressor gene that mediates its anti-tumor activities, in part, by suppressing an anti-apoptotic response and modulating cell proliferation. Collectively, we suggest that haploinsufficiency of *MKK4* cooperates with alterations of p53 in the malignant transformation of liver cancer cells, and possible in other forms of cancer.

MKK4 is a dual specificity kinase that functions in the stress-activated kinase-signaling cascade. Extracellular stimuli activate MKK4 to trigger phosphorylation of the JNK and p38 mitogen-activated protein kinases. Various, albeit conflicting, studies support a role for MKK4 dysregulation in tumorigenesis. While some investigations suggest a pro-oncogenic potential of MKK4 (Wang et al., 2004; Finegan and Tournier, 2010), others have

proposed a tumor suppressive function (Yamada et al., 2002; Cunningham et al., 2006). We favor the latter interpretation and show that suppression of MKK4 function results in an increased liver cancer burden.

MKK4 is heterozygously deleted in half of all tumors of different origins, including HCC, colorectal, lung, ovarian, pancreatic, and stomach cancers (Figure 4.4.A), suggesting that the reduction of MKK4 levels positively impacts on tumorigenesis. The presence of *MKK4* loss-of-function mutations at a fairly consistent rate of ~5% in cancers of the pancreas, breast, colon, lung and testis further support its putative tumor suppressive role (Cunningham et al., 2006). Moreover, reduced expression of MKK4 in prostate, ovarian, and pancreatic tumors is linked with more advanced stages of cancer progression and shorter patient survival (Kim et al., 2001; Yamada et al., 2002; Whitmarsh and Davis, 2007). Additionally, a polymorphism in the promoter of the *MKK4* gene (-1304T>G), which increases *MKK4* promoter activity and, therefore, *MKK4* mRNA and protein levels, correlates with reduced risk of AML, lung and colorectal cancer and might therefore play a protective role towards cancer development (Davies and Tournier, 2012).

Given the somewhat ambiguous role of MKK4 in tumorigenesis, it is not surprising that the biological mechanism of MKK4-mediated tumor suppression remains poorly defined. Coexistent mutations of other tumor suppressor genes, such as *p53, BRCA2* and *p16*, suggest that *MKK4* may participate in distinctive tumor suppressive signaling pathways, in addition to its modulation of the canonical downstream mediators, p38 and JNK (Whitmarsh and Davis, 2007). The tumor suppressive role of MKK4 might include the induction of attenuated proliferation as well as increased apoptosis in response to specific

stimuli through the activation of the NF-κB signaling pathway. Similarly, while MKK4 plays a role in the formation of some primary tumors, it may suppress metastasis through interactions with distinct MAP kinases or other non-canonical downstream mediators (Whitmarsh and Davis, 2007). In metastatic human ovarian cancer cells, over expression of MKK4 triggers increased expression of the p53-inducible cell cycle inhibitor p21 (Lotan et al., 2008), pointing to one possible mechanism by which loss of *p53* might complement MKK4 inactivation in tumor cells. Perhaps, distinct context dependent stimuli in different tissues dictate which signaling pathways are targeted by MKK4.

The inactivation of tumor suppressor genes is a key feature of cancer formation and progression. Tumor suppressor genes are inactivated through mutations and subsequent loss of heterozygosity (LOH) or following homozygous deletion. The human *MKK4* gene is located on chromosome 17p11.2 and lies centromeric to the p53 tumor suppressor gene. This arm of chromosome 17 is one of the most frequently deleted in human cancers. However, bi-allelic deletion of *MKK4* is not frequently observed in human tumors. Moreover, despite the high rate of 17p LOH, *MKK4* experiences a low rate of inactivating mutations of only ~5%. Together, the genomic data suggest that complete loss of MKK4 function is not advantageous for a tumor cell. Our experimental data further imply that gene dosage of *Mkk4* might be of importance for its tumor suppressive function. shRNA-mediated knockdown of MKK4 resulted in residual MKK4 mRNA levels and, therefore, a complete knockout was not mimicked. Therefore, we propose that MKK4 functions as a haploinsufficient tumor suppressor gene for which a single copy is insufficient to produce the normal level of gene expression. In *Saccharomyces cerevisiae,* the phenomenon of haploinsufficiency has been relatively well-studied, and more than 180 haploinsufficient

genes have been identified by profiling of heterozygous deletion strains. But why is gene dosage so important for some genes in the genome? Do haploinsufficient genes share genomic or functional attributes? And why do these haploinsufficiency show a wide range of penetrance, expressivity, and phenotypes?

It seems unlikely that the biologically active tumor suppressor gene *MKK4* is subject to a two-hit mutational mechanism in cancer. Therefore, the mechanism by which monolallelic loss drives tumorigenesis remains an interesting open question. For example, in the case of transcription factors, synergistic effects involving cooperative interactions in complexes offer an attractive explanation for haploinsufficiency. A system that requires oligomerization of different subunits is very sensitive to protein dosage and stoichiometric imbalances can affect such synergistic complexes. Moreover, various signals and informational pathways need to be integrated with a limited set of factors (Veitia, 2002). This combinatorial strategy may underlie many cases of haploinsufficiency, including gene expression, regulation and protein-protein interactions. For example, FBW7, a substrate recognition component of the SCF-type ubiquitin ligase, is frequently heterozygously mutated or deleted in human cancers. FBW7 dimerizes, and any single-allelic mutation reduces the amount of wild-type FBW7 by 50% and leads to haploinsufficiency of the remaining wild-type allele with regards to the requirement for dimeric FBW7 functions (Welcker and Clurman, 2008). FBW7 targets $p27^{Kip1}$, which is also haploinsufficient for tumor suppression. $p27^{Kip1}$ targets and inhibits other cycline-dependent kinases to arrest the cell cycle at G_1. By lowering the inhibitory threshold, partial loss of $p27^{Kip1}$ accelerates cell cycle progression (Siu et al., 2012). Furthermore, upon carcinogen treatment or irradiation, heterozygous $p27^{Kip1}$ mice develop more tumors than wild-type mice but fewer than

homozygous mutants (Fero et al., 1998). However, in the case of MKK4, its tumor suppressive function depends on the kinase activity. Most somatic mutations affect protein stability or impair its kinase activity, resulting in rapid degradation of MKK4 through the ubiquitin-proteasome complex (Ahn et al., 2011). Therefore, reduced levels of MKK4 through mono-allelic deletions or mutations might result in lower activated levels of its downstream mediators, that can have a causal effect on tumorigenesis.

Whole chromosome arm deletions are one of the major, but poorly understood, characteristics of the cancer genome. Genome instability can be an explanation for random deletion events; however, it does not explain the high selection pressure for deletion events of the short chromosome arm 17, which is observed across tumor types. The biological relevance of chromosome 17p deletions has not been studied in detail and loss of p53 remains the single explanation of this frequent genomic alteration. Opposing this hypothesis, we have shown experimentally that 17p encompasses at least one additional tumor suppressor gene and that cosuppression of a linked 17p gene promotes tumor formation more potently than suppression of p53 alone. Large deletion events, such as those affecting 17p, might therefore specifically arise from selective pressure to attenuate the activity of multiple genes. The "cancer gene island model" hypothesizes that chromosomal regions encompassing high densities of genes with tumor suppressive functions and low densities of essential genes become targets of frequent heterozygous deletion events (Solimini et al., 2012). The presence of recurrent heterozygous large deletions can therefore be explained by an increase of proliferative fitness through cumulative haploinsufficiency. For example, this phenomenon has been recently observed for chromosome 8p, where the co-deletion of *DLC1, FGL1, FBXO25,* and *TRIM35* accelerated tumor burden in a mouse

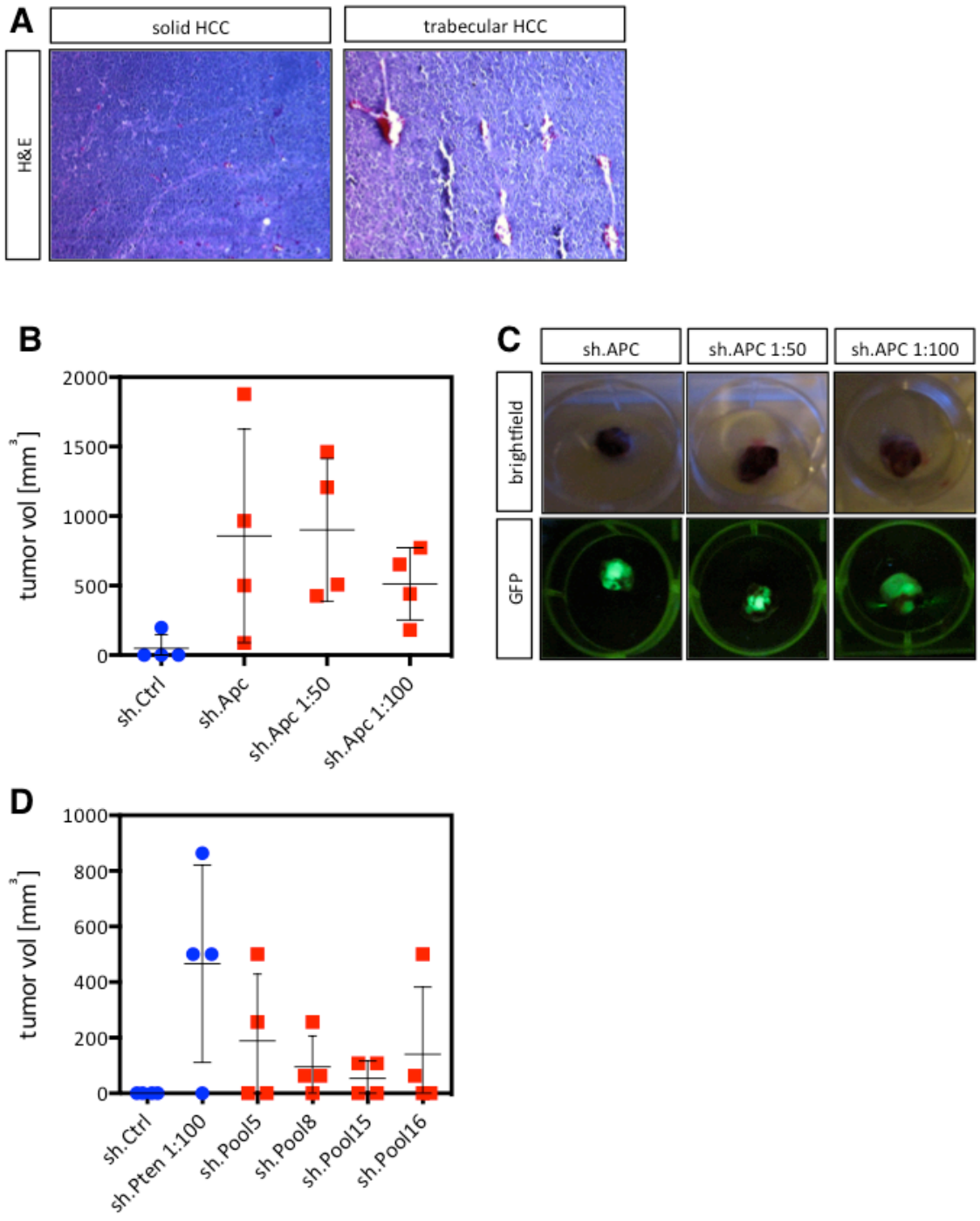
model of liver cancer, and their combined loss correlated with poor survival in human cancer patients (Xue et al., 2012). These studies challenge the prevailing view that bi-allelic inactivation of canonical tumor suppressor genes are critical for tumorigenesis, and instead suggest that mono-allelic disruption of linked cancer genes may collectively have a broad impact on cancer development and progression. Large deletions affecting other chromosomes (e.g., 3p, 5q, 9p) may similarly have wider consequences than what has been suggested to date.

The early embryonic lethality due to anemia and severe hemorrhaging in the liver caused by the deletion of *MKK4* has underscored the critical importance of its function(s) during development (Ganiatsas et al., 1998). However, if MKK4 is essential in liver cells, retention of a copy of the gene should be selected even though mono-allelic deletion of *MKK4* predisposes to cancer. Understanding the physiological requirements for MKK4 activity in signal transduction will be key in deciding whether therapeutic targeting of the MKK4 pathway will prove of clinical benefit, and may possibly explain how best to specifically modulate certain Mkk4-mediated functions but not others.

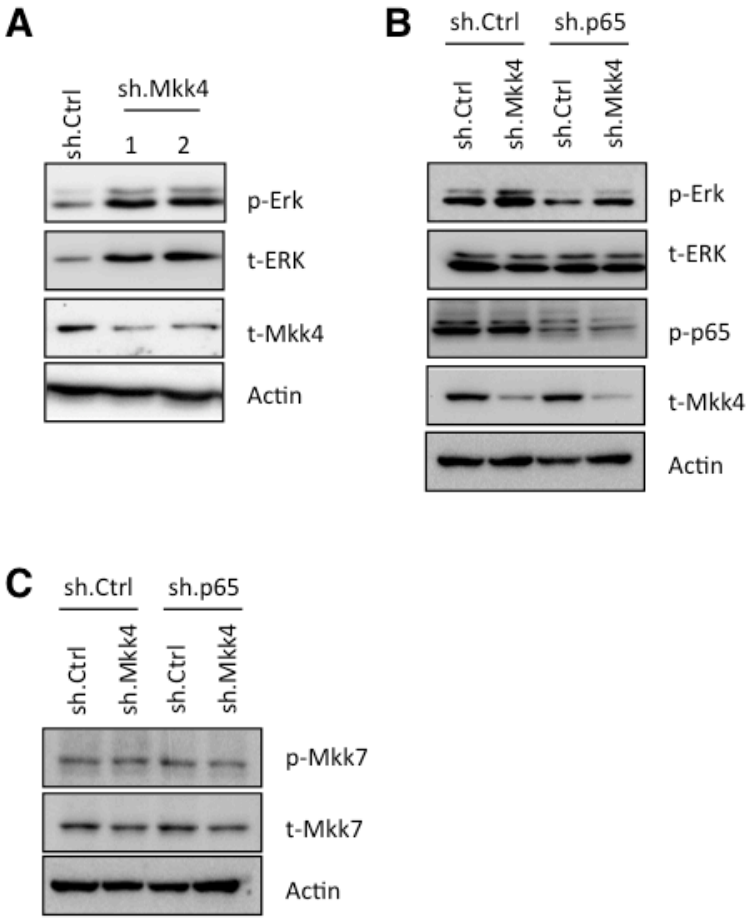
4.4. Chapter Contributions

S.W. and D.F.T designed and performed the majority of the experiments. D.A. contributed intellectually to the design of the project and helped performing western blots. T.K. performed genome data analysis. S.W.L. conceived and supervised the project.

4.5. Supplementary Figures



Suppl. Figure 4.1. Set up of *in vivo* RNAi screen
(A) H&E sections of mouse tumors derived from *p53*$^{-/-}$;MYC LPCs with APC knockdown. Mouse HCCs resemble the histopathological subtypes of HCC, with solid and trabecular growth pattern. (B) Tumor growth after subcutaneous injection of *p53*$^{-/-}$;MYC LPCs expressing sh.Ctrl or -Apc at various dilutions. (C) Brightfield and GFP images of tumors from (B). (D) Screening results. 4 out of 16 tested shRNA pools accelerate tumorigenesis.



Suppl. Figure 4.2. MKK4-suppression induces ERK activation through NF-κB
(A) pERK, tERK, tMKK4, and Actin levels of LPC cells infected with shRNAs targeting Mkk4 (1 or 2), or a nontargeting control (Ctrl) as determined by western blotting. (B) pERK, tERK, p-p65, tMKK4, and Actin levels of LPC+sh.Ctrl and LPC+sh.p65 superinfected with sh.Ctrl or sh.Mkk4. (C) pMKK7, tMKK7, and Actin levels of LPC+sh.Ctrl and LPC+sh.p65 superinfected with sh.Ctrl or sh.Mkk4.

Chapter 5

Conclusions and Future Perspectives

5. Chapter 5

Conclusions and Future Perspectives

Genetic alterations affecting chromosome 17p are one of the most frequent aberrations in human cancers. Such abnormalities usually comprise mutations in the tumor suppressor gene p53 and loss of heterozygosity (LOH) of the remaining chromosome arm 17p, which often affects the 274 protein-coding genes contained in this chromosomal region. My thesis work set out to understand the functional relevance of genetic alterations of chromosome arm 17p in tumorigenesis and how these changes can be exploited for therapeutic benefit.

First, we analyzed the biological consequences induced by mutations in the tumor suppressor p53 and found that mutant p53 enhances the invasive and metastatic potential of pancreatic cancer cells through the regulation of multiple molecular mechanisms. We identified the platelet-derived growth factor receptor b (PDGFRb) as a necessary downstream mediator of mutant p53 action and demonstrated that reduction of PDGFRb activity impaired metastasis in a mouse model of pancreatic cancer. Inhibition of PDGFRb might therefore represent an alternative treatment strategy for metastatic tumors harboring mutations in p53. In addition, we unraveled a miRNA-mediated mechanism by which mutant p53 attenuates the metastasis suppressive functions of MKK4 to potentiate cell invasion. Together, these investigations shed light on the neomorphic functions of mutant p53 in pancreatic cancer.

Besides our efforts to understand the relevance of p53 mutations in pancreatic cancer, we gained insights into the biological significance of large deletions encompassing the chromosome region 17p. We provided evidence that the recurrent large deletions targeting chromosome 17p attenuate the potential tumor suppressor activity of multiple genes rather than acting solely to inactivate p53. To this end, we showed that loss of *Mkk4* enhanced the tumorigenic potential of premalignant p53-null hepatoblasts in a mouse model of hepatocellular carcinoma. A better understanding of the molecular mechanisms underlying these cooperative effects might provide unexpected vulnerabilities that may be relevant to the treatment of malignant diseases.

5.1. Mutant p53 in Cancer: Challenges and Opportunities

Functional inactivation of p53 is an almost universal feature of human cancer. Whereas p53 deletions cause the loss of its tumor suppressive function, p53 mutants additionally acquire dominant negative activity over the wild-type allele as well as oncogenic properties that lead to a gain of function. Given frequent p53 mutations in cancers of different origins, a detailed understanding of neomorphic functions and their underlying mechanism is of high importance to understand the nature of all cancers. Even though a large number of studies performed on mutant p53 have revealed a wealth of information, only one decisive conclusion has emerged: there is no singular remedy that impedes the gain of function activities of mutant p53.

Given the many cellular functions that are initiated by wild type p53, it would have been naïve to assume that the properties of gain of functions of p53 mutants are not equally

diverse. Indeed, multiple neomorphic functions have been ascribed to mutant p53, including its ability to promote cell invasion and metastasis, increased cell proliferation and survival, drug and apoptosis resistance, genomic instability, enhanced angiogenesis, and inflammation (Muller and Vousden, 2014). More recent studies have also reported a role of mutant p53 in cell reprogramming or in regulating the interaction of cancer cells with tumor stroma. In contrast to wild type p53, inactivation or mutation of p53 facilitates the dedifferentiation of somatic cells into pluripotent stem cells and, for example, initiates tumor formation by promoting the generation and expansion of the mammary epithelial stem cells (Lu et al., 2013). Therefore, as for wild type p53, mutant p53 affects many different cellular outcomes that most likely depend on the tumor-type and cellular context.

Many important biological processes are regulated through different, but parallel, mechanisms in mammalian cells. Here, we have identified multiple mechanisms through which mutant p53 drives invasion and metastasis in pancreatic cancer: by regulating PDGFRB levels in a transcriptional manner and by modulating the miRNA profile to cause inactivation of MKK4. Moreover, previous studies have identified additional downstream mediators of mutant p53 that drive invasion and metastasis, such as integrin recycling (Muller et al., 2009), the mevalonate pathway (Freed-Pastor et al., 2012) or miRNA biogenesis (Su et al., 2010). Hence, the molecular mechanisms governed by mutant p53 to drive invasion seem versatile and redundant.

How does mutant p53 promote its different neomorphic functions? A single base substitution in the DNA binding domain of p53 generally cancels its ability to bind to consensus DNA binding regions of target gene promoters. Whereas a few forms of mutant

p53 can recognize specific DNA structures to directly drive gene expression, most of them affect transcription of target genes through binding and modulating other transcription factors. Differently from wild type p53, mutant p53 binds to its family members p63 and p73. By inhibiting p63, mutant p53 regulates expression of Dicer, DEPDC1, Cyclin G2, and SHARP1, whereas inhibition of p73 leads to gene expression changes of PDGFRb. Moreover, mutant p53 also interacts with a wide variety of proteins, such as MRE11, SREBP, VDR, or NFR2, to modulate their cellular pathways and contribute to a more aggressive cancer phenotype (Muller and Vousden, 2014). Therefore, one of the main challenges in the field is the identification of interaction partners and the downstream pathways that are modulated by them.

Single base substitutions in the p53 gene that induce neomorphic functions mainly cluster in the DNA binding region, with only a few affecting other domains (Leroy et al., 2013). Common hotspot mutations are clustered at codons 175, 245, 248, 249, 273, and 282; yet, their spectrum and frequency depends on the tumor type. The type of mutation classifies the mutant p53 protein into either a (i) contact mutant, or (ii) conformational mutant (Cho et al., 1994). Despite these oversimplified classifications, each mutation generates subtly different alterations in the structure and conformational stabilities of the p53 protein (Joerger and Fersht, 2007). Complicating experimental studies, different mutants are not alike in respect to their ability to inhibit wild type p53 and in their range of gain of functions Moreover, contrasting phenotypes generated by the same mutant in different tissue types could potentially reflect differential expression of mutant p53 targets, including p63 and p73. For example in breast and lung cancer cells, mutant p53 attenuates Dicer and global miRNA expression in a p63-dependent manner, whereas in pancreatic cancer cells,

mutant p53 induces the expression of distinct miRNAs and does not affect Dicer levels (Muller et al, 2014; Chapter 3). Remarkably, not only the position but also the type of the mutation influences the spectrum of p53 outputs (Muller and Vousden, 2014). Whereas the $p53^{R280K}$ mutant enhances invasion, $p53^{R280T}$ promotes proliferation and cell cycle progression. Hence, it remains a challenge to identify the mechanistic and functional consequences of each p53 mutation. Moreover, how these mutations affect disease progression and therapeutic responses remain a difficult question.

But why is the mutation pattern of p53 so versatile? The distribution of mutations in well-studied oncogenes and tumor suppressor genes are highly characteristic and nonrandom. Therefore, it is the pattern of mutations, rather than the mutation rate, that classifies driver genes into oncogenes or tumor suppressor genes. Oncogenes exhibit recurrent missense mutations at the same amino acid positions, whereas mutations in tumor suppressor genes are inactivating, cause protein-truncation and occur throughout the length of the gene (Vogelstein et al., 2013). For example, nearly all mutations in *KRAS* are at the identical amino acid, codon G12, and classify that gene unambiguously as an oncogene. Such mutations enhance the wild type activity of KRAS and make it constitutive active. On the other hand, in squamous cell carcinomas, mutations affecting the *NOTCH1* gene are not recurrent and usually inactivating, therefore suggesting a tumor suppressive role. In the case of p53, however, missense mutations occur throughout the DNA binding domain, affect different amino acids, but do not cause protein-truncations. Therefore, the paradoxical distribution of mutations in the p53 gene does not fit the typical criteria for other well-documented cancer genes. The presence of some hotspot mutations is accompanied by many other mutations throughout the p53 gene and, therefore, indicates a tumor

suppressive function. Consistently, mutations prevent DNA binding of p53 and cause a loss p53's tumor suppressive function. Paradoxically, the missense mutations also induce a gain of function; however, they might not be under evolutionary selection. This assumption is supported by the fact that different mutations cause different types of gain of functions and activate varying downstream mediators. Therefore, a p53 mutation does not, as in the case of oncogenes, enhance wild type activity to cause oncogenic potential but rather induces a gain of function that by itself might not be enough to initiate tumor growth but only in combination with the loss of the tumor suppressive functions. Whereas cancer cells select for the loss of p53's tumor suppressive function, the gain of functions might be an advantageous, but not necessary, addition to the aggressive phenotype.

The impact of mutant p53 on tumorigenesis makes it an attractive therapeutic target. The most efficient therapeutic strategy would be to target mutant p53 directly. Given that the accumulation of mutant p53 is a requisite for its gain of function activities, enhancing the turnover of the mutant protein by inducing its proteosomal degradation represents one potential approach. Indeed, SAHA, a HDAC inhibitor, has shown promising results in destabilizing mutant p53 by preventing the interaction between HDAC6 and Hsp90 (Li et al., 2011). However, whether a decrease of mutant p53 levels and the simultaneous degradation of wild type p53, if still expressed, are sufficient for a therapeutic benefit, remains debatable. Given the antitumor effects observed upon reactivation of wild-type p53 in several models (Xue et al., 2007), an alternative would be to restore wild type function of p53 mutants in order to induce apoptosis or senescence. This idea relies on the concept of reverting the conformational changes introduced by a given mutation. Small molecules, such as PRIMA-1 or PhiKan083, bind to the DNA binding domain of specific mutants to

promote correct folding and to restore wild type function (Boeckler et al., 2008). PRIMA-1 is currently in phase 1 clinical trials (Lehman et al., 2012). Despite these directed strategies, targeting downstream mediators in tumors expressing mutant forms of p53 may also hold promise. This approach requires the identification of commonalities of mechanisms through which mutant p53 proteins function, possibly by identifying interacting partners that exert relevant outcomes. For example, the small molecule RETRA inhibits the interaction between p73 and mutant p53, resulting in decreased cell survival and suppressed xenograft growth. Alternatively, the inhibition of downstream signaling pathways that are activated by mutant p53 might be exploited for therapeutic intervention. Some examples have proven successful in *in vitro* or *in vivo* mouse models and include the PDGFRb pathway by imatinib (Chapter 2), blocking the cholesterol synthesis pathway by statins (Freed-Pastor et al., 2012), and the EGFR pathway by EGFR inhibitors (Mueller et al, 2009). Another, yet underexplored, area is the identification mutant p53-dependent vulnerabilities, which would help utilizing synthetic lethality as a therapeutic strategy.

On occasion, single mutants become highly sensitive to specific second mutations, resulting in cell death (synthetic lethality) or a decrease in fitness (synthetic sickness). Therefore, synthetic lethality might be a promising strategy for specific targeting of tumor cells. Uncovering synthetic interactions, however, is not trivial because they are rare and not always directly conserved between organisms. The first synthetic lethal interaction was discovered in breast and ovarian tumor cells with *BRCA1* and *BRCA2* mutations, which are impaired for effective DNA double-strand break repair and, thus, sensitive to the inhibition of PARP enzymatic activity, resulting in chromosomal instability, cell cycle arrest and subsequent apoptosis (Farmer et al., 2005). Whether the same phenomenon can be applied

in tumors expressing mutant p53 is not clear. Identifying synthetic lethal genes is mostly based on genome-wide or kinome-wide RNAi screening, which has been extensively utilized to identify sensitizing targets to chemotherapeutic agents. In the case of mutant p53, such a screen would be carried out in isogenic cells lines in which mutant p53 either expressed or depleted by, for example, CRISPR-mediated modulation of the p53 gene locus. Such screens have not been carried out yet; however, a number of genes and pathways that may result in synthetic lethality in mutant p53-expressing tumors were recently identified using a computational approach (Wang and Simon, 2013). PLK1 was proposed to be a candidate for mutant p53 synthetic lethality due to its higher expression in tumors expressing mutant p53 and its inhibitory role in the transactivation of p53 function. However, PLK1 is an essential gene and the causal effect of its inhibition on normal cells might be equally deleterious. Therefore, the functional identification of synthetic lethal interaction with p53 mutants that cause a proliferative defect has yet to be carried out. Alternatively, there might be less obvious vulnerabilities created by mutant p53. As most neomorphic functions are pro-metastatic, a synthetic sickness screen might identify mutant p53-dependent interactions that cause invasion and metastasis. Here, one would compare the shRNA distribution in metastatic clones of distant organs to the primary tumor, carrying a mutant p53 or characterized by p53 knock down. Other vulnerabilities created by mutant p53 might be in the DNA damage pathway or cell metabolism.

5.2. Dissecting large deletions – the orchestral performance of concurrent loss of haploinsufficient genes

The cancer genome is characterized by amplifications, deletions, rearrangements, point mutations, and loss of heterozygosity (LOH) that collectively result in tumorigenesis. In the case of chromosome 17p, common genetic alterations not only include p53 mutations, but also the concomitant loss of the other chromosome arm not affected by mutational events. In most cases, such deletions target the entire short chromosome arm of 17 and its 274 genes. This observation opens the question whether large deletions target only one driver gene but simultaneously cause the deletion of many passenger genes without any causal effect on tumorigenesis or, alternatively, whether they target multiple genes that cooperate to drive tumor outgrowth.

Whether the loss of multiple genes from chromosome 17p contributes to the tumorigenic phenotype remains unclear. Our data from Chapter 4 suggest that the recurrent 17p deletion events reflect the selective advantage of simultaneously targeting the two tumor suppressors MKK4 and p53. The combined loss of both genes maximizes proliferative fitness through cumulative effects. Others have functionally verified the presence of physically "linked gene clusters" on chromosome 6q and 8p (Scuoppo et al., 2012; Xue et al., 2012). Moreover, sequencing studies investigating the contribution of recurrent heterozygous deletions to tumorigenesis indicate that large deletion events preferentially target regions of clustered genes that negatively regulate proliferation but spare essential genes (Solimini et al., 2012). The biased distribution of proliferation-inhibitory genes over proliferation-inducing genes in these regions suggests a selective advantage for a cancer cell with large deletions. In addition to deletions, amplifications of

extended chromosome arm regions are also frequently observed and may result in the enhanced expression of multiple genes that induce proliferation (Zender et al., 2006). The idea of "linked gene clusters" challenges the prevailing view that a single gene in a region is the driver and that neighboring genes become altered simply as passenger events. Therefore, because hundreds to thousands of genes per tumor are affected by large deletions, it might be the combined loss of multiple growth control genes that affect tumorigenesis across many cancer types. Cumulative effects of gene loss may play an important role and might change the way in which we think about cancer evolution.

The realization that large deletions or amplifications target multiple genes is a rather new concept underlying tumor evolution. Albeit exciting, it confronts us with many challenges, as functional studies with a single gene affected by a amplification or deletion will miss the causal consequences of linked genes on tumorigenesis. Moreover, regarding large deletions, only 22% of recurrent large deletions target known tumor suppressor genes, suggesting that potential tumor suppressive functions of many genes remains to be identified. However, the presence of cryptic tumor suppressor genes in large deletions might not be the only answer. For example, loss of regulatory long non-coding RNAs or altered 3D chromosome structures that impair binding of enhancers to promoters over great distances (or even across different chromosomes) could contribute to the impact of large chromosomal deletions on tumorigenesis. These events are not pinpointed by conventional sequencing analysis and, thus, the identification of their presence and function requires new experimental approaches.

Large deletions often affect one allele only. Heterozygous loss of substantial chromosomal regions affects different chromosomes, occurs frequently in cancer patients, and is present in tumors of different origins. Therefore, the question becomes whether they harbor bona fide tumor suppressor genes for which single-copy loss has a functional role in driving tumor growth. Solimini et al. argue that most recurrent heterozygous deletions are not caused randomly, but that they impact tumorigenesis in a haploinsufficient manner (Solimini et al., 2012). Therefore, the impact on tumorigenesis of large deletions that occur heterozygously cannot be explained by Knudson's "two-hit hypothesis" and, instead, questions the dogma that all important tumor suppressors behave according to the two-hit inactivation model.

Some classical two-hit tumor suppressors also display haploinsufficiency, supporting the idea that haploinsufficiency carries a functional role in cancer. Notably, p53 itself exhibits haploinsufficiency in that mice with heterozygous p53 deletions ($p53^{+/-}$) show an intermediate survival compared to p53-null and wild-type mice, and some tumors that develop in the $p53^{+/-}$ animals retain an intact, functioning wild-type allele (Venkatachalam et al., 1998). Other classical cancer susceptibility genes that show haploinsufficiency are *BRCA1* and *BRCA2*. *BRCA1/2* carriers develop premalignant lesions that drive tumorigenesis by promoting loss of the second allele or, alternatively, of other tumor suppressor genes (Bellacosa et al., 2010). Other convincing examples of haploinsufficient genes include $p27^{Kip1}$, *DMP1*, *PTEN*, *RB*, *SMAD4*, *LKB1*, *NKX3.1*, *MSH2*, and *MAD2* (Santarosa and Ashworth, 2004). Therefore, the idea that Knudson's two-hit model of inactivation is more complex for most tumor suppressor genes is reasonable. One would need to determine whether loss of the second allele is at the normal cellular rate for such

events or whether it is facilitated by haploinsufficiency. Careful comparison of the genetic composition of resulting tumors in mice carrying either heterozygous or homozygous mutations in the gene of interest might hint at the answer. Such studies would help to find whether the haploinsufficient nature of classical tumor suppressor genes contributes to loss of the wild-type allele and in which cases this event is random.

It is believed that the functional and molecular outcome of haploinsufficient tumor suppressor genes is, just like the classical tumor suppressor genes, highly tissue-specific and context-dependent. For example, gene dosage effects of heterozygous *PML* mutations become only apparent in mice carrying the translocation between human chromosomes 15 and 17, creating a PML-RARα fusion protein, but not in a wild-type background (Rego et al., 2001). Thus, the tumorigenic advantage conferred by haploinsufficiency of a tumor suppressor gene is too low to be selected for but a second mutation in another tumor suppressor gene can synergies with the first. As a result, this cell could gain a phenotype either of proliferative increase or genomic instability; thereby, become selected for to drive tumorigenesis.

But what are the contributions of haploinsufficient genes on tumorigenesis? Based on the nature of haploinsufficiency that includes the partial reduction of gene function only, one might hypothesize that haploinsufficient genes produce a "sensitized background" for tumorigenesis to occur. This hypothesis might hold true given the fact that tumors initiated by alterations in haploinsufficient gene exhibit later onset and milder phenotypes than the corresponding tumor with homozygous gene loss (Santarosa and Ashworth, 2004). Therefore, haploinsufficiency that causes small proliferative advantages or modest

abnormalities in DNA repair mechanisms could increase the mutation rate. For example, haploinsufficiency of the *NF1* gene is required to establish the *NF1* homozygous state that initiates tumor growth. However, such phenomena are difficult to prove experimentally and whether they are of general applicability is unknown. Here, only the analysis of the sequence of events affecting each allele and their individual contribution to tumorigenesis could give the definitive answer. Moreover, whether the cooperative effects of multiple haploinsufficient genes together yield a stronger effect that cannot only sensitize but initiate tumor growth remains to be elucidated. Generating mouse models with large heterozygous deletions that are believed to carry multiple haploinsufficient genes, such as 17p, might give an answer to that question.

Haploinsufficiency has a well-established role in numerous developmental disorders, such as the Grieg's syndrome (caused by haploinsufficiency of *GLI3*) (Kalff-Suske et al., 1999) or the type 2 Waardenburg's syndrome (caused by haploinsufficiency of *MITF*) (Nobukuni et al., 1996). The notion that a partial decrease in gene dosage is relevant to cancer enjoys increasing acceptance, but is held back due to inadequate evidence. Convincing validation of a haploinsufficient gene would comprise evidence for retention of the wild-type allele, absence of deleterious mutations, continued expression of the protein product, as well as phenotypic effects on tumorigenesis. Developing quantitative assays that can assess and score partial differences in expression levels will be helpful in the future. Questions remain whether haploinsufficiency, just like transcriptional or post-translational regulation, can be harnessed for cancer prevention and therapy. Certainly, directly or indirectly targeting haploinsufficient tumor suppressor genes would require a quantitative analysis of their expression levels in the tumor of the patient, since the sole assessment of

their presence or absence would not be enough. Moreover, how the partial loss of certain genes changes vulnerabilities and dependencies of cancer cells has not been explored. Therefore, whether the activation or inhibition of alternative pathways or synthetic lethality approaches can be considered, as in the case with classical tumor suppressor genes, is not defined.

In conclusion, the concept of physical linkage of haploinsufficient tumor suppressor genes from one heterozygous deletion event seem to have a more prevalent and impacting role in tumorigenesis than was previously assumed. The prevalence of heterozygous large deletions represents a new mechanism for cancer evolution and needs further investigation addressing the following points: Does the combined loss of genes affect the same or different cellular pathways? How do their molecular functions synergize? Is the cumulative reduction of dosage of genes with tumor-suppressive properties enough to initiate tumor growth or does it occur to particularly promote or enhance tumor progression? Does haploinsufficiency occur in the context of specific genetic or environmental insults? Does the concomitant loss of multiple genes create unexpected vulnerabilities and dependencies that can be harnessed for cancer therapies? If large deletions create cancer cell vulnerabilities, these might have been missed in studies of single genes. Therefore, chromosome arm deletions can create phenotypes unique from those arising through loss of a single tumor suppressor gene, and thus should be considered and studied as distinct events.

Collectively, my thesis work illustrates how a well-studied tumor suppressor locus can impact on tumorigenesis that exceeds the simple loss or gain and, moreover, how such a locus can cooperate with other genes affected by genetic alterations. The situation is

complex: p53 mutations lead to gain of function activities and combined haploinsufficiencies that drive cancer in unique and unanticipated ways. The many genetic modifications affecting 17p contribute to tumorigenesis in different ways. This situation is reminiscent of the *INK4a/ARF* locus, where single deletion events can target at least three tumor suppressors that interact to influence tumorigenesis (Sharpless et al., 2003). Such events were expected to be the exception; however, our results, combined with emerging data from others, suggest that this might rather be the rule. If so, our view about mechanisms underlying of cancer evolution and therapeutic strategies might need to be expanded. If haploinsufficient genes act combinatorial and in a dosage-dependent manner, how do they affect cancer susceptibility and resistance? Such questions can only be addressed after identifying the causal genes and their effects, but the lack of mouse models with such complex genotypes prevents us, to date, from experimental studies. Mouse models of cancer susceptibility could be generated by enriching for combinations of alleles that are frequently genetically altered in the cancer genome. Despite the fact that the mapping of the number and chromosomal location of genes involved in various forms of cancer is time and labor intensive, rapid advances in mouse genome projects will help to accelerate the progress in detecting modifier loci.

Chapter 6

Materials and Methods

6. Chapter 6

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Retroviral Constructs, Antibodies and Reagents

All vectors were derived from the Murine Stem Cell Virus (MSCV, Clontech) retroviral vector backbone. PDGFRb cDNA (Addgene, #23893) was subcloned into MSCV-PGK-Puro-IRES-GFP (MSCV-PIG) (Hemann et al. 2003). miR30-based shRNAs were designed and cloned as previously described (Zuber et al. 2011) and sequences are available upon request. shRNAs were cloned in the MLP vector (MSCV-mir30-PGK-Puro-IRES-GFP) for constitutive expression and inducible shRNAs were cloned into the TRMPV-Neo vector (pSIN-TRE-dsRed-miR30-PGK-Venus-IRES-NeoR) as previously described (Zuber et al. 2011). All constructs were verified by sequencing.

p53 was detected using mAb NCL-P53-505 (Novocastra) and hAB OP43 (Calbiochem). Anti-Actin (A3854) was purchased from Sigma. Anti-PDGFRa (3164) and –PDGFRb (3169) antibodies were purchased from Cell Signaling. Alexa Fluor 568-Phalloidin (A12380) was purchased from Invitrogen. Crenolanib (CP-868596) and imatinib (I-5508) were purchased from Selleckchem and LC Laboratories, respectively.

Cell Culture and Drug Treatments

All cell lines were maintained in DMEM + 10% FBS, at 37° C in 5% CO_2. Stable cell lines expressing shRNAs were generated by retroviral mediated gene transfer. Briefly, Phoenix or Ampho packaging cells (for murine or human cell lines, respectively) were transfected by

the calcium phosphate method with vectors expressing NF-YA, NF-YB, p53, p73, PDGFRa, PDGFRb or Renilla-control shRNAs, and the generated viruses were harvested to infect KPC, $KP_{fl}C$, or a series of human pancreatic, breast, lung and colon cancer cell lines. Infected cells were selected with puromycin and experiments were carried out on derived cell populations. To generate cells stably expressing mutant p53, p73 or PDGFRb, $KP_{fl}C$ cells were infected with MSCV-p53-R175H, -R273H, PDGFRb or pcDNA3-HA-p73□ and selected in puromycin and G418 respectively to yield stable pools. Cultured cells were treated with 300 nm crenolanib and 3 μM imatinib. These concentrations were selected after determining the concentration required to fully inhibit the phosphorylation of PDGFRb without any effects on cell proliferation.

Wound healing and Invasion Assays

Wound healing assays were conducted as previously described (Goulimari et al., 2005). Briefly, cells were seeded in 6-well plates, grown until confluent, and wounding was performed with a 10-□l microtiter tip that was cut longitudinally. Three-dimensional invasion assays were carried out as previously described (Kitzing et al., 2007). In brief, 24-well transwell inserts (Greiner bio-one) were lined with collagen type 1 (BD Biosciences), and cells were seeded on the inverted inserts. The lower chambers were filled with medium containing 0.5% FBS and upper chambers containing the collagen matrix were filled with medium containing 20% FBS and the chemoattractant HGF.

Immunostaining and Microscopy

Live cell recordings were performed immediately after wounding for 18 h at 37 °C using a Zeiss observer Microscope. Pictures were acquired every 10 min with a motor-controlled

Leica DC 350 FX camera, which enables simultaneous recordings from multiple wells. For statistical analysis, the wound distance from each well was measured in duplicate at three randomly defined wound gap locations per frame recorded per experiment. Invasion assay inserts were fixed using 4% formaldehyde and stained using DAPI and Alexa568 phalloidin (Invitrogen) before confocal microscopy (Perkin Elmer Spinning Disk) was conducted. Images were analyzed using Imaris software.

RT–qPCR

Total RNA was isolated using TRIZOL (Invitrogen), and cDNA was obtained using the TaqMan reverse transcription reagents (Applied Biosystems). Real-time PCR was carried out in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) on the ViiA™ 7 Real-Time PCR System (Invitrogen). GAPDH or □-actin served as endogenous normalization controls.

RNA sequencing and Data Analysis

RNA of KPC samples was isolated and separated by size. Nucleotide sequencing of RNAs of >200 nt and <200 nt length was carried out as previously described (Djebali et al., 2012). Directional (stranded) libraries for Paired End (PE) sequencing on the Illumina platform were generated as described previously (Parkhomchuck et al., 2009). Primary data processing and library mapping was completed using the Spliced Transcripts Alignment to a Reference (STAR) software (Dobin et al., 2013). Differential expression analysis for sequence count data (FPKM values) was conducted using DESeq as described previously (Anders and Huber, 2010).

PDGFRb Luciferase Reporter Assay

The promoter assay was performed as previously described (Hackzell et al., 2002). In brief, cells were seeded in 24-well plates and transiently transfected with 0.5 ug of expression plasmid, 2.0 ug of reporter plasmid, and 20 ng of renilla-luciferase vector (PGL4.74, Promega). After 36 h, cells were lysed (Dual-Luciferase Reporter Assay System, Promega), and firefly luciferase and renilla luciferase activities were measured (Varioskan Flash Multimode Reader, Thermo Scientific). Results shown were normalized to renilla activity and are representative of at least three independent replicates.

Co-Immunoprecipitation

To detect p53/p73 and p73/NF-Y binding, sub-confluent $KP_{fl}C$ cells were infected with mutant p53 or an empty control construct and transiently transfected with HA.p73 (Addgene) using Lipofectamine 2000 (Invitrogen). Thirty-six hours post-transfection, cells were lysed in RIPA Buffer (20 mM Tris pH 7.4, 1% TritonX-100, 37 mM NaCl, 2 mM EDTA, 1% SDS, 0.5% NP-40, 10% Glycerol, phosphatase inhibitors [2.5 mM Sodium pyrophosphate, 1 mM β-Glycerophosphate, 1 mM Na_3VO_4], and protease inhibitors [Roche]). Whole cell lysates (0.5 mg protein) were pre-cleared with A/G Sepharose beads (Invitrogen), incubated at 4° C with either anti-HA (Covance, 16B12) or anti-p53 antibody (Calbiochem, OP43) overnight and subsequently with protein A/G beads for 2 h. The bead pellet was extensively washed in lysis buffer three times and then electrophoresed on 10% SDS-PAGE gels followed by immunoblotting using anti-p53, anti-HA, anti-GFP (Cell Signaling, 2555) and anti-NF-YB (Santa Cruz, FL-207) antibodies.

To measure levels of endogenously phosphorylated PDGFRb, KPC cells were treated with the drug as described and harvested in phospho-lysis buffer (50 mM Tris pH 7.5, 1% Tween-20, 200 mM NaCl, 0.2% NP-40, phosphatase inhibitors [2.5 mM Sodium pyrophosphate, 1 mM β-Glycerophosphate, 1 mM Na_3VO_4], and protease inhibitors [Roche]). Anti-PDGFRb (Santa Cruz, 958) was used to immunoprecipitate PDGFRb from whole cell lysate samples containing 0.5 mg protein. Washed immunoprecipitates were subjected to SDS-Page and immunoblotted with anti-PTyr-100 antibody (Cell Signaling, 9411).

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) experiments were carried out as previously described (Beckerman et al., 2009). Briefly, KPC cells were treated with 1% formaldehyde prior to lysis in RIPA Buffer and sonication to yield 500 bp fragments. Protein A/G Sepharose beads were conjugated to anti-NF-YB antibody (Santa Cruz, 13045) which were subsequently used to immunoprecipitate NF-YB from 1 mg whole cell lysate. Quantitative ChIP was carried out on an ABI StepOne Plus using SYBR green dye. Genomic Location of the NF-YB site within the promoter of *PDGFRB* was located using a literature search (Hackzell et al., 2002).

Immunohistochemistry and Immunofluorescence

Tissues were fixed overnight in formalin, embedded in paraffin, and cut into 5-□m thick sections. Sections were subjected to hematoxylin and eosin staining, and immunohistochemical and immunofluorescent staining following standard protocols. The following primary antibodies were used: mouse anti-GFP (Cell Signaling), rabbit anti-p-

PDGFR-□ (Tyr 1021) (Santa Cruz Biotechnology), mouse anti-p53 (OP43, Calbiochem) and rat anti-CK8 (DSHB). For immunofluorescence Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 568 goat anti-rat were used as secondary antibodies, and DAPI was used as a chromogen. Images were acquired using a Zeiss Axio Imager D1 scope.

shRNA Library Cloning, Recovery, and Determination of Representation

The miR30 design based shRNA library was described before (Zender et al., 2008). Targeting sequences were selected on the basis of the DSIR algorithms. The library was subcloned into the MLS vector by a pooled approach. During the cloning procedure it was ensured by deep sequencing that a 1000- fold overrepresentation of each shRNA was maintained.

Genomic DNA was isolated from liver tumors as indicated and the shRNA sequences were amplified using primers (Dow et al., 2012) flanking the miR30 cassette harboring the Illumina adaptor sequence. The same PCR approach was applied to the library pool plasmid DNA. Deep sequencing analyses were done using the Illumina GA IIx with a 46 bp single end run. Base calling and export of the sequencing results were done with multiple FASTA files via the Illumina Pipeline Vers. 1.8. Data analysis was done with a Perl-Script. The sequences were aligned to the shRNA library data (only 100% matches) and summarized.

Mouse Studies

All animal experiments were performed in accordance with a protocol approved by the Memorial Sloan-Kettering Institutional Animal Care and Use Committee. For colonization studies, KPC cells ($1x10^5$) were resuspended in 200 μl PBS and injected intravenously into

the tail vein of 8 week-old female athymic nude mice. Lungs were harvested 7 days post injection and analyzed for colonization by GFP positivity (Nikon SMZ1500) and histology.

Pdx1-Cre (Hingorani et al., 2003), *LSL-Kras*^{G12D} (Jackson et al., 2001) and *LSL-p53*^{R172H} (Olive et al, 2004) mouse strains were previously described: Male *Pdx1-Cre*$^{+/+}$, *LSL-p53*$^{R172H/R172H}$ mice were bred with female *LSL-Kras*$^{G12D/+}$ mice to generate KPC mice. Strains were maintained on mixed background. Mice were genotyped by polymerase chain reaction analysis as described previously (Hingorani et al., 2006). Mice were dosed twice daily by oral gavage with 50 mg/kg Imatinib in ddH_2O or with ddH_2O only. Organs and tumors were removed and fixed in 10% buffered formalin and tumor and metastatic burden was assessed by gross pathology and histology.

Generation of Subcutaneous Tumors

The indicated cell lines (LPC, MIT) were transduced by retroviruses expressing single (sh.Ctrl, sh.MKK4, sh.Apc) or pooled shRNAs (17p library, ~100 shRNA per pool). 1×10^6 cells were injected subcutaneously (rear flanks) on nu/nu mice. Subcutaneous tumor volume was monitored by caliper measurements.

Hydrodynamic Injection and FAS treatment

Vectors for hydrodynamic tail vein injection were prepared using the QIAGEN EndoFree Maxi Kit (QIAGEN). For transposon-mediated gene transfer, animals received a 5:1 molar ratio of transposon to transposase- encoding plasmid (25 mg total DNA, unless otherwise stated). DNA was suspended in saline solution at a final volume of 10% of the animal's body weight and injected via the tail vein in less than 10 s. Progression of liver cancer was

monitored at different time points by whole-liver GFP imaging using a Hamamatsu Imaging system.

The Fas (CD95)-activating antibody Jo2 (Becton Dickinson Biosciences) (Ogasawara et al., 1993) was applied by intraperitoneal injection in a dose of 0.5 μg/g body weight.

Human Data Sets

The gene expression data and survival analyses of PDAC patients is from The International Cancer Genome Consortium (ICGC) pancreatic cancer project, which is stored at Gene Expression Omnibus (GEO) with accession number GSE50827. It includes gene expression data from 103 primary tumor samples, 89 of which contain disease-free survival and clinicopathological annotations, and used in the survival analysis according to previously described methods (Biankin et al., 2012).

Gene expression data of ovarian, colorectal, and pancreatic cancer patients with annotated clinical outcomes were downloaded from GEO (GSE9899 (Tothill et al., 2008), GSE17537 (Smith et al., 2010), and GSE28735 (Zhang et al., 2012)). Preprocessed data were downloaded as provided in the data matrix files (GCRMA/RMA normalized Affymetrix expression microarray data) and gene set enrichment analysis (GSEA) was performed on each set after mean-centering across samples. Median of probes per gene was used to account for differential representation on the chip. GSEA analysis was used to evaluate 40-gene signature. For survival analyses, gene expression data was clustered into groups using kmeans and Kaplan-Meier analyses was performed. Significance for these plots was determined using the logrank test.

For the Mkk4 project, we analyzed the aCGH data produced using representational oligonucleotide microarray analysis for the frequency and size of deletions in a series of human HCCs and breast, colon, and lung cancers available at Cold Spring Harbor Laboratory (Zender et al., 2008; Xue et al., 2012). We used this method to study gene dosage alterations in human HCC as described recently (Xue et al., 2012). Copy number aberrations (CNAs) were visualized from the individual representational oligonucleotide microarray analysis aCGH plots of the specific HCC samples using Integrated Genomics Viewer software (Broad Institute; http://www.broadinstitute.org/igv/home). In addition, available CNA from SNP6 arrays from the Cancer Genome Atlas (http://cancergenome.nih.gov/) for HCC and breast, colon, and lung adenocarcinomas were visualized using Integrated Genomics Viewer software and analyzed for the occurrence of chromosome 8p deletion.

Chapter 7

References

7. Chapter 7

References

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