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Investigation of long noncoding RNAs in the p53 response to oncogenic stress

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Abstract

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The tumor suppressor protein p53 plays a central role in sensing and responding to cellular stress, and loss of normal p53 activity is a feature of most human cancers. P53, functioning as a transcription factor, regulates a wide network of genes that coalesce to drive many diverse tumor suppressive cellular processes. Despite three decades of extensive study, it is unclear which p53 targets are most critical for these tumor suppressive functions and under which biological contexts. More recent studies have revealed that the p53 network is not solely comprised of protein coding genes but also includes many long noncoding RNAs (lncRNAs), which are an abundant but poorly characterized class of heterogenous RNA molecules. This work aimed to expand our understanding of the role of lncRNAs in the p53 transcriptional network and the importance of these diverse RNAs in various p53-regulated processes and/or tumor suppression.

We focused on characterizing the p53 response to stress generated by hyperactive oncogenes (*i.e.* oncogenic stress), which is an important but understudied facet of p53-mediated tumor suppression. By performing genome-wide analysis of p53 regulation across a panel of cancer cell lines

established from a genetically-engineered mouse model of p53-driven lung adenocarcinoma, sarcoma, and lymphoma, we uncovered oncogenic stress-specific, tumor type-specific, and p53 outcome-specific patterns of p53 gene activation and repression, including the activation of several novel and previously described lncRNAs. We interrogated the contributions of many of these lncRNAs to p53 pathway function, using a number of locus-specific genetic and molecular approaches to down- or upregulate lncRNA expression *in vitro*. These characterization efforts revealed that local gene regulation, also known as *cis*-regulation, is the dominant mode of action for lncRNAs within the p53 network, in contrast to most previous reports on individual p53-regulated lncRNAs. We show that this regulation is largely in the positive direction, suggesting that lncRNAs do not significantly contribute to p53-mediated gene repression, and demonstrate that lncRNA *cis*-regulation can be important for p53-dependent growth arrest in certain tumor contexts.

Altogether, this work provides novel insights into the p53 response across different cellular contexts and clarifies the contributions of lncRNAs to the p53 tumor suppressor network. Namely, we propose that lncRNAs primarily serve as an important fine-tuning regulatory mechanism. As such, this work has broader implications on the mechanisms by which lncRNAs as a class may influence other complex transcriptional networks important in human health and disease.

Investigation of Long Noncoding RNAs in the P53 Response to Oncogenic Stress

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Table of Contents

Abstract i

Acknowledgements v

Table of Contents vii

Nonstandard Abbreviations ix

List of Figures and Tables xii

Chapter 1: Background 1

Long noncoding RNAs and the p53 tumor suppressor network

Sifting through the "junk" 1

Long noncoding RNAs in cancer 9

The p53 tumor suppressor pathway 17

LncRNAs in the p53 pathway 22

Conclusion 36

Chapter 2: Project Framework 38

Overview 38

Model systems 42

Tools for the study of lncRNA loci 44

Chapter 3:

Cis-*regulatory lncRNAs in the p53 response to oncogenic stress* ... 49

Introduction 49

Results 50
Data Summary and Conclusions 74

Chapter 4:

Characterization of novel p53-activated lncRNA 2310008N11Rik.. 77
Introduction 77
Results 77
Data Summary and Conclusions 93

Chapter 5:

Senescence-associated lncRNAs in p53 pathway function 94
Introduction 94
Results 95
Data Summary and Conclusions 120

Chapter 6: Summary and Perspectives 122
Final remarks 134

Chapter 7: Materials and Methods 135
Supplementary Tables 146

References 150

Nonstandard Abbreviations

-as	Antisense
ASO	Antisense oligonucleotide
Bahcc1	BAH domain and coiled-coil containing 1
Bfsp2	Beaded filament structural protein 2
BrdU	5-bromo-2'-deoxyuridine
CC3	Cleaved caspase 3
ChIP	Chromatin immunoprecipitation
CHR	Chromatin
Con	Control
CP	Cytoplasm
cPARP	Cleaved poly(ADP-ribose) polymerase
CPAT	Coding potential assessment tool
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR-activation
CRISPRi	CRISPR-inhibition
CRISPR-SAM	CRISPR-Synergistic Activation Mediator
DAPI	4’,6-diamidino-2-phenylindole
Dhx15	DEAH-Box helicase 15
Doxo	Doxorubicin
Doxy	Doxycycline
DREAM	Dimerization partner, RB-like, E2F and multi-vulval class B
E1A	Adenovirus early region 1A
ER	Estrogen receptor
EV	Empty vector
dCas9	Endonuclease-dead Cas9
dRNA	‘Dead’ RNA
FACS	Fluorescence-activated cell sorting
FC	Fold change

FITC	Fluorescein isothiocyanate
Gadd45γ	Growth arrest and DNA damage gene 45 gamma
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
Geno	Genotoxic (stress)
GFP	Green fluorescent protein
GOF	Gain-of-function
gRNA	Guide RNA
GRO	Global run-on
GSEA	Gene set enrichment analysis
IRES	Internal ribosome entry site
KPR	*$Kras^{LA2\text{-}G12D/+}$; $p53^{LSL/LSL}$; $Rosa26\text{-}CreER^{T2}$*
KRAB	Krüppel-associated box
Kras	Kirsten rat sarcoma viral oncogene homolog
LA	Lung adenocarcinoma
LncRNA	Long noncoding RNA
LincRNA	Long intergenic noncoding RNA
LOF	Loss-of-function
LSL	*loxp*-STOP-*loxP*
Ltc4s	Leukotriene C4 synthase
LY	Lymphoma
MBP	MS2 binding protein
Malat1	Metastasis associated lung adenocarcinoma transcript 1
Maml1	Mastermind like transcriptional coactivator 1
MEF	Mouse embryonic fibroblast
ncRNA	Noncoding RNA
Neat1	Nuclear enriched abundant transcript 1
NP	Nucleoplasm
OE	Overexpression
Onco	Oncogenic (stress)
ORF	Open reading frame
sORF	Short open reading frame
p53RE	p53 response element

PAM	Protospacer adjacent motif
PI	Propidium iodide
Pvt1	Plasmacytoma variant translocation 1
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RAPT1	RNA activator of p53 translation 1
Rhod	Ras homolog family member D
RNA pol II	RNA polymerase II
-s	Sense
SA	Sarcoma
SA β-Gal	Senescence-associated β-galactosidase
smRNA-FISH	Single molecule RNA fluorescence *in situ* hybridization
Spag9	Sperm associated antigen 9
SpCas9	*Streptococcus pyogenes* Cas9
TAD	Transactivation domain
Tam	4-hydroxytamoxifen
TIDE	Tracking of indels by decomposition
TSS	Transcription start site
UTR	Untranslated region
Zmat3	Zinc finger matrin-type 3

List of Figures and Tables

Figure 1: LncRNAs are highly heterogenous but can be grouped by gene structure and/or broad regulatory modes of action..... 6

Figure 2: Characterization of the p53 response to oncogenic stress..... 53

Figure 3: Pathway analysis of p53-responsive transcriptome reveals core and outcome-specific p53 signatures..... 57

Figure 4: LncRNAs activated by p53 in response to oncogenic stress are chromatin-enriched and positively correlated with neighboring mRNAs..... 62

Figure 5: LncRNA p53 response element (p53RE) mutagenesis reveals co-regulatory relationships..... 66

Figure 6: *LincRNA-Gadd45γ* positively regulates *Gadd45γ* in *cis*..... 70

Figure 7: Contributions of lncRNA-mRNA co-regulation to p53's anti-proliferative response to oncogenic stress.....73

Figure 8: *LncRNA 7 (2310008N11Rik)* is a novel, p53-regulated lncRNA..... 81

Figure 9: *LncRNA 7* exogenous OE does not affect cellular proliferation or apoptosis under genotoxic or oncogenic stress..... 86

Figure 10: Epigenetic silencing of *lncRNA 7* locus does not affect p53 pathway function in p53-restorable MEFs..... 88

Figure 11: Assessing *lncRNA 7* CRISPRi and CRISPRa in *KPR* LA cells..... 92

Figure 12: Several p53-activated lncRNAs have senescence-associated expression patterns..... 97

Figure 13: *LincRNA-RAPT1 (9230114K14Rik)* locus and development of knockout mouse models..... 101

Figure 14: Characterization of *lincRNA-RAPT1*..... 105

Figure 15: CRISPRi and CRISPRa strategies of senescence-associated lncRNA LOF and GOF..... 109

Figure 16: CRISPRi of *lncRNA 7* or *Ltc4s-as* does not affect proliferation or survival in LA *KPR* cells..... 112

Figure 17: CRISPRa of *lncRNA 7* or *Ltc4s-as* does not affect proliferation or survival in LA and SA *KPR* cells..... 116

Figure 18: *Ltc4s-as* positively regulates *Ltc4s*, but not *Maml1*, in *cis*..... 119

Table 1.1: LncRNAs reported to be directly activated by p53..... 37

Table 2.1: Survey of genome-wide p53 ChIP-seq and RNA profiling studies.... 48

Table 3.1: LncRNAs activated by p53 during the oncogenic stress response.... 59

Table 3.2: Descriptions of lncRNA-associated p53 response elements..... 64

Supplementary Table 1: Key plasmids used in this work

Supplementary Table 2: gRNA, dRNA, and ASO information

Supplementary Table 3: qRT-PCR, PCR, and sequencing primer information

Chapter 1: Background

Long noncoding RNAs and the p53 tumor suppressor network

Sifting through the "junk"

One of the most surprising revelations of the early 21st century, aided by the advent of modern genomics, was the prodigious amount of transcription present in the mammalian genome. The discovery that as much as 75% of the human genome is transcribed, despite less than 2% of it encoding for proteins, challenged the traditionally held view that in the flow of genetic information, RNAs primarily serve as intermediary messengers for dictating protein production (with a few important regulatory exceptions) (Djebali et al. 2012; Rinn and Chang 2012). One interpretation of this unexpected abundance of expressed RNAs is that many of these transcripts represent non-functional transcriptional noise, conceptually progressing from "junk DNA" to "junk RNA." However, a growing body of work suggests that noncoding RNAs (ncRNAs) of many different classes can contribute to biology in important, versatile ways that are continuously being expanded upon.

Long noncoding RNAs (lncRNAs) are one class of ncRNAs, defined by two simple criteria: a length exceeding 200 nucleotides and a lack of protein coding potential, as determined by various bioinformatic and/or experimental strategies (Mercer et al. 2009; Rinn and Chang 2012). LncRNAs have garnered much interest in recent years, in part due to the sheer number of lncRNA transcripts that have been uncovered in the human and mouse transcriptomes. According to NONCODE

v5 (2018), which is an integrated database of published ncRNA datasets, the human genome could contain as many as 95,000 lncRNA genes, corresponding to roughly 170,000 transcripts, while the mouse genome encodes around 90,000 lncRNA genes, producing roughly 130,000 transcripts (Fang et al. 2018). Thus, in both humans and mice, the number of lncRNA genes and transcripts greatly surpasses the number of protein coding genes and mRNAs.

Furthermore, transcriptome profiling studies have shown that many lncRNAs have expression patterns that correlate with specific cell- or tissue-types, biological processes, or disease states (Cabili et al. 2011; Rinn and Chang 2012; Iyer et al. 2015; Hung et al. 2011; Guttman et al. 2009). While these associations are tantalizing, determining whether they reflect functionality remains a massive on-going endeavor. Encouragingly, large scale efforts to tackle this question on a genome-wide scale have suggested that dozens, if not hundreds of lncRNA loci could contribute to essential cellular processes (Liu et al. 2017). Additionally, several recent studies have revealed important roles for a myriad of individual lncRNAs, including in cell cycle regulation (Dimitrova et al. 2014; Marín-Béjar et al. 2013; Marchese et al. 2016), genome maintenance (Lee et al. 2016; Hu et al. 2018), pluripotency (Loewer et al. 2010; Jain et al. 2016), genomic imprinting (Mancini-Dinardo et al. 2006; Latos et al. 2012), nuclear organization (Sunwoo et al. 2009; Hacisuleyman et al. 2014; Chen et al. 2016), cell death and survival (Hung et al. 2011; Li et al. 2017; Chaudhary et al. 2017), differentiation (Kretz et al. 2013; Yin et al. 2015; Ritter et al. 2019), and immune signaling (Petermann et al. 2019; Mineo et al. 2020), to name a few examples [also reviewed more extensively in (Fatica and Bozzoni 2014; Yao et al. 2019)]. However, the rate of lncRNA detection

has far out-stripped the rate of lncRNA characterization, leaving the question of whether this ever-expanding catalogue of lncRNA transcripts consists mostly of functional “wheat” or biologically insignificant “chaff” an important unanswered question.

Modes of lncRNA function

LncRNAs greatly increase the complexity of the mammalian genome and can be expressed from a multitude of genic configurations, including overlapping, sharing promoters with, or being entirely enclosed within other genes. LncRNAs can also be expressed from regulatory DNA or noncoding portions of coding regions, such as promoters, enhancers, and introns (Rinn and Chang 2012) (Fig. 1A). Thus, lncRNAs are highly heterogenous at the level of genomic anatomy as well as function. Despite this frequent complexity in gene structure, the biogenesis of most lncRNAs closely resembles that of mRNAs, though this may also be a reflection of a sequencing-based sampling bias towards the detection of polyadenylated transcripts (Uszczynska-Ratajczak et al. 2018). Nonetheless, most identified lncRNA transcripts are produced by RNA polymerase II and are capped, spliced, and polyadenylated. LncRNAs are, however, in general less efficiently spliced, less abundant, and less conserved at the primary sequence level than mRNAs (Derrien et al. 2012; Quinn and Chang 2015), and lncRNA examples with exceptional processing do exist [*e.g.* unique 3’ end processing (Wilusz et al. 2012; Yang et al. 2011a), circularization (Li et al. 2018)].

So, what do lncRNAs do, and how do they do it? Perhaps unsurprisingly, it is impossible to productively answer this question with a single answer, in part due to the inclusive definition of a lncRNA. While this definition is useful for distinguishing lncRNAs from protein coding genes and other small regulatory ncRNAs with well-defined functions (*e.g.* microRNAs, tRNAs, snRNAs), it is less helpful in guiding the study of lncRNAs themselves (Rinn and Chang 2012). However, regardless of the inherent heterogeneity of lncRNAs as a class, all lncRNAs are fundamentally large RNA molecules. Accordingly, it is predicted that any lncRNA functions are principally mediated through the formation of complex, modular RNA structures that interact with other molecules (*e.g.* protein, RNA, DNA) in versatile, combinatorial ways (Goff and Rinn 2015) (Fig. 1B).

One way to further classify lncRNAs is based on where in the cell a lncRNA transcript is normally found. A broad but conceptually useful distinction is between nuclear and cytoplasmic lncRNAs. This is because lncRNA localization patterns can vary greatly and have important implications on feasible functions. Most lncRNAs are nuclear-enriched (Derrien et al. 2012; Cabili et al. 2015), but many lncRNAs are also found in the cytoplasm. Cytoplasmic lncRNAs have been reported to play a variety of roles, many related to post-transcriptional gene regulation. Examples of this include binding to and competing away microRNAs from target mRNAs (Chen et al. 2015; Faghihi et al. 2010; Hu et al. 2018), serving as microRNA precursors (Keniry et al. 2012), and directly influencing mRNA stability (Kretz et al. 2013; Gong and Maquat 2011) or translation (Carrieri et al. 2012; Zhang et al. 2013a) through specific RNA-protein interactions. Nuclear

lncRNAs have been linked to regulating gene expression through a variety of mechanisms, discussed further below.

LncRNAs can also be categorized according to broad modes of action. While such classifications cannot be made *a priori* functional interrogation of a lncRNA locus, limiting their usefulness in predicting lncRNA function, they can prove helpful in elucidating patterns of function that subsequently expand our understanding of the various ways lncRNAs can contribute to biology. One such distinction is between lncRNAs that act near their site of transcription, or in *cis*, versus those that exert a function elsewhere, deemed *trans*-acting lncRNAs (Sun et al. 2018; Gil and Ulitsky 2020). While cytoplasmic lncRNAs are *trans*-acting by definition, nuclear lncRNAs can function in either *cis* or *trans* to regulate gene expression (Sun et al. 2018) (Fig. 1B). Many well-characterized lncRNAs fall into the category of nuclear, *cis*-acting lncRNAs and have served as useful paradigms for how lncRNAs in the nucleus may function.

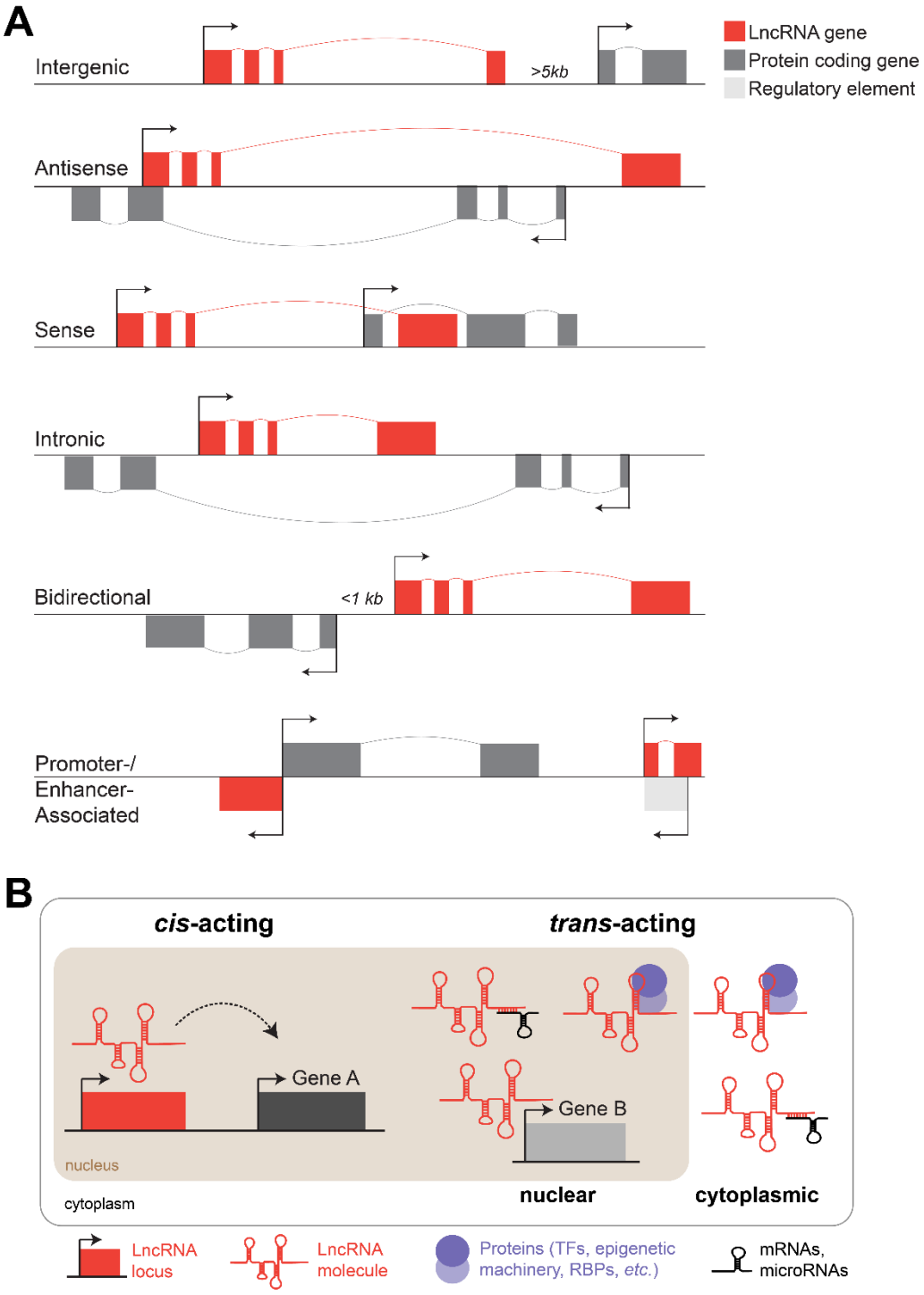


Figure 1: LncRNAs are highly heterogenous but can be grouped by gene structure and/or broad regulatory modes of action. (A) Examples of the various genomic architectures lncRNA loci can exhibit. LncRNA genes are often categorized by their relationship to nearby (intergenic, bidirectional) or overlapping (antisense, sense, intronic) protein coding genes or regulatory DNA elements (promoters, enhancers). **(B)** LncRNAs can also be categorized by modes of action, whether acting locally (*cis*) or distally (*trans*) from their sites of transcription to influence gene expression. Both *cis*-acting and *trans*-acting lncRNAs are thought to fundamentally

function through the formation of RNA-protein, RNA-RNA, and/or RNA-DNA interactions. TF= transcription factor. RBP= RNA binding protein.

One important example is *XIST (X-Inactive Specific Transcript),* which is amongst one of the first lncRNAs described (Brown et al. 1991). *XIST* plays a central role in orchestrating dosage compensation in mammals by driving the heterochromatinization of one of the two X chromosomes present in females in a process called X chromosome inactivation (XCI) (Sahakyan et al. 2018). *XIST* establishes XCI through multiple mechanisms involving specific interactions between XIST RNA and various protein complexes. Several chromatin modifying complexes interact with *XIST*, including PRC2 (Polycomb Repressive Complex 2) and SHARP (SMRT- and HDAC-Associated Repressor Protein), and contribute to epigenetically silencing the chromosome *XIST* is expressed from (Loda and Heard 2019). In addition to facilitating epigenetic regulation, XIST also promotes *cis* repression by interacting with nuclear matrix components to recruit the silenced X chromosome to the transcriptionally repressive nuclear laminar compartment (Loda and Heard 2019; Chen et al. 2016). Thus, *XIST* is able to regulate gene expression in *cis* through recruiting and/or otherwise interacting with proteins that modulate gene expression. Though *XIST* is unique in its ability to drive chromosome-wide gene repression, this basic mechanism is broadly applicable to many nuclear acting lncRNAs, including lncRNAs that act in *cis* to activate gene expression (Werner et al. 2017; Gil and Ulitsky 2020), as well as those that regulate gene expression in *trans*.

Nuclear lncRNAs are uniquely poised to regulate gene regulation, as illustrated by *XIST*'s localization being intrinsically linked to its function in organizing XCI. The lncRNA *Airn* (*Antisense Igf2r ncRNA*) is another well-studied example of lncRNA-mediated *cis* regulation that drives this point home further. *Airn* is expressed from the *Igf2r* imprinted cluster and is important for the paternal imprinting of several protein coding genes in this locus, which *Airn* accomplishes through multiple mechanisms (Quinn and Chang 2015). *Airn* is oriented antisense to and overlapping *Igf2r*. The act of *Airn* being transcribed through *Igf2r*'s promoter is sufficient to repress *Igf2r* expression via transcriptional interference, strongly suggesting that Airn RNA is unlikely to be important for the silencing of *Igf2r* (Latos et al. 2012). Thus, *Airn* exemplifies one RNA-independent mechanism by which lncRNA loci can still exert *cis*-regulation: lncRNA biogenesis. However, Airn RNA may be important for silencing other genes in the *Igf2r* imprinted cluster, illustrating the potential multifunctionality of lncRNA loci. One proposed model for the silencing of *Slc22a3*, located upstream of *Airn* in the same cluster, is that *Airn* promotes epigenetic silencing through the recruitment of the H3K9 histone methyltransferase G9a (Nagano et al. 2008). Therefore, by virtue of both its genomic architecture and localization, the *Airn* lncRNA locus is able to modulate local gene expression in both RNA molecule-dependent and RNA molecule-independent ways.

It is important to note that these broad modes of lncRNA locus-mediated *cis*-regulation are not restricted to gene silencing, and have in fact been more widely described for lncRNA loci that positively influence the expression of their neighboring genes (Anderson et al. 2016; Engreitz et al. 2016; Werner et al. 2017;

Joung et al. 2017; Gil and Ulitsky 2020). Thus, there is a growing appreciation for the unique ability of nuclear lncRNAs to enact local gene regulation. Less clear is the broader impacts of lncRNA-mediated *cis*-regulation on gene regulatory networks as a whole, and, ultimately, how important lncRNA *cis*-regulation is for the myriad of biological processes lncRNAs have been implicated in.

LncRNAs in cancer

Historically, efforts to elucidate the molecular mechanisms underlying cancer pathogenesis have focused on genetic and epigenetic alterations in protein-coding genes. However, several pieces of evidence suggest that lncRNAs may also be important contributors, discussed below and also recently reviewed in (Olivero and Dimitrova 2020).

First, many lncRNA genes have been found to associate with disease-associated genomic alterations, including single nucleotide polymorphisms (SNPs) and copy number variations (CNVs). One comparison of over 900 GWAS (Genome-Wide Association Studies) revealed that more than 90% of disease-associated SNPs fall within noncoding regions (Maurano et al. 2012), though this is to some degree expected given the predominance of noncoding regions in the human genome. Nevertheless, multiple recent efforts to characterize noncoding transcription across the human genome have found that genomic regions encoding lncRNAs are enriched for trait-associated SNPs over background intergenic regions (Hangauer et al. 2013; Iyer et al. 2015). These observations suggest a possible link between lncRNA loci and disease-associated SNPs, though the significance of most of these associations remains to be determined.

A few examples of cancer-associated SNPs connected to lncRNA expression have been described. One such lncRNA locus is *NBAT1 (Neuroblastoma Associated Transcript 1)*, in which a germline SNP located in *NBAT1*'s second intron is associated with high risk neuroblastoma and correlated with *NBAT1* expression (Pandey et al. 2014). Another example is the lncRNA *ANRIL* (*Antisense Noncoding RNA in the INK4 Locus*; also known as *CDKN2B-AS*), which is transcribed antisense to the *CDKN2A/B* locus (encoding the tumor suppressive proteins ARF, CDKN2A, and CDKN2B) (Kong et al. 2018). This complex locus harbors several cancer risk-associated SNPs, some of which interestingly associate with *ANRIL* expression levels, but not with those of its overlapping protein coding genes (Cunnington et al. 2010). As *ANRIL* expression is increased in many different cancers and associated with aggressive disease (Kong et al. 2018), exploring the ways these variants influence the regulation of this locus could be informative as a model for how such cancer-associated SNPs may mechanistically contribute to pathophysiology.

Cancer genomes are marked by genomic instability and frequently undergo amplifications or deletions leading to somatic CNVs (SCNVs) that can contribute further to malignancy. Common alterations in cancer cells include amplifications of oncogenes, such as *MYC*, and loss of tumor suppressor genes, such as those encoded by the *CDKN2A/B* locus (Beroukhim et al. 2010). Several lncRNAs have also been observed to exhibit SCNVs linked to tumorigenesis (Huarte 2015; Hu et al. 2014). *LncRNA FAL1 (Focally Amplified lncRNA on Chromosome 1)* was identified as one of nearly 3,000 lncRNA loci frequently exhibiting copy number alterations across thousands of patient tumor samples and is overamplified in

multiple cancers. Moreover, shRNA knockdown of *lncRNA FAL1* was shown to restrict proliferation *in vitro* and restrict tumor growth in xenograft experiments *in vivo*, demonstrating the oncogenic consequences of overamplifying this lncRNA (Hu et al. 2014).

PVT1 (Plasmacytoma Variant Translocation 1) is another lncRNA locus that is frequently over-amplified in cancer, most often with its nearest neighbor, the proto-oncogene *MYC*, which is located 70 kb upstream (Guan et al. 2007; Tseng et al. 2014). In one study, addition of a single extra copy of the genomic region spanning and including *PVT1* and *MYC* but not *MYC* alone was shown to promote tumorigenesis in a breast cancer mouse model, whereas deleting the entire 300 kb *PVT1-MYC* locus in HCT116 cells reduced cellular proliferation and xenograft tumor growth (Tseng et al. 2014), suggesting that *PVT1* cooperates with *MYC* to drive cancer. However, the complexity of this locus, which includes several regulatory DNA elements (Cho et al. 2018; Fulco et al. 2016) and multiple RNA isoforms of *PVT1* (Olivero et al. 2020), make it somewhat difficult to interpret these data and say whether amplification of PVT1 RNA outright drives or suppresses tumorigenesis. It is likely that both tumor suppressive and oncogenic mechanisms are at play at this lncRNA locus.

Lastly, it has long been appreciated that lncRNA expression patterns are often associated with many biological phenomena and/or cellular states. In one recent comprehensive analysis of over 7,000 RNA-seq datasets, spanning 27 distinct human tissues and cancer states, nearly 60,000 human lncRNAs were identified. Of these lncRNAs, over 10,000 were found to be significantly

dysregulated (*i.e.* downregulated or upregulated) in various cancers compared to matched normal tissues. Furthermore, the authors were able to segregate cancers of similar tissues of origin by their lncRNA expression patterns, further demonstrating the exquisite disease-specificity lncRNAs can have (Iyer et al. 2015). In some cases, lncRNA expression patterns can further be associated with specific features of cancer prognosis, such as recurrence (Zhou et al. 2018), metastasis (Ji et al. 2003), invasiveness (Lim et al. 2020), and overall survival (Gupta et al. 2010; Lim et al. 2020).

Functional studies of cancer-associated lncRNAs

Despite these key observations, the broader role of lncRNAs in cancer remains poorly understood, primarily due to fact that the majority of these cancer-associated lncRNAs have not yet been functionally dissected using robust loss-of-function (LOF) and gain-of-function (GOF) models. Whether lncRNA expression patterns are the drivers or transcriptional by-products of oncogenic transformation remains an open-ended question in the field (Olivero and Dimitrova 2020). Still, a handful of lncRNAs have been suggested to functionally contribute to cancer development, demonstrating the value of careful case-by-case interrogation of lncRNA loci. The following is a discussion of three illustrative examples.

One well-studied example *is* ***MALAT1*** *(Metastasis Associated Lung Adenocarcinoma Transcript 1). MALAT1* is a highly abundant, conserved lncRNA that was initially identified in a screen for potential biomarkers for metastatic lung

cancer, in which high *MALAT1* expression was found to be highly predictive of metastasis and overall poor prognosis in non-small cell lung cancer (NSCLC) patients (Ji et al. 2003). *MALAT1* has since been observed to be upregulated in many human cancers and has been at the center of efforts to understand the ways in which lncRNAs can contribute to tumorigenesis [reviewed in (Zhang et al. 2017)]. Early investigations into *Malat1* function using mouse models of lung and breast cancer revealed that exogenous overexpression of *Malat1* led to increased tumor growth and metastasis, whereas *Malat1* loss [using antisense oligonucleotides (ASOs) or promoter deletion strategies (Zhang et al. 2012)] impaired these processes (Gutschner et al. 2013; Arun et al. 2016). These data provided some of the earliest functional evidence for a lncRNA directly influencing tumorigenesis *in vivo*. However, in more recent work, *Malat1* knockout was shown to promote metastasis in the same murine breast cancer model. Even more remarkably, this pro-metastasis phenotype was rescued in a targeted *Malat1* overexpression transgenic mouse, suggesting a provocative tumor suppressive role for this lncRNA (Kim et al. 2018). Notably, this latter study used a different genetic model for *Malat1* knockout, in which a LacZ and synthetic polyadenylation signal (LacZ+PAS) cassette was inserted roughly 100 bp downstream of *Malat1*'s endogenous transcription start site (TSS), leading to premature transcriptional termination of *Malat1*-Lacz without disrupting *Malat1*'s promoter. However, it is not immediately apparent why these two genetic *Malat1* LOF models would yield completely opposing phenotypes.

Mechanistically, *Malat1* localizes to nuclear speckles (Tripathi et al. 2010), where various roles in regulating transcription have been proposed. These include

modulating alternative splicing through interactions with splicing factors (Tripathi et al. 2010) and interacting with chromatin modifying complexes to promote the relocalization of genes from transcriptionally repressive to transcriptionally active nuclear bodies (Yang et al. 2011b). Moreover, mapping the genomic binding sites of MALAT1 RNA in human breast cancer cells using capture hybridization analysis of RNA targets (CHART) revealed that *MALAT1* associates with sites of active transcription (West et al. 2014). Thus, while *Malat1*'s exact cellular functions remain unclear, it is likely that *Malat1* acts as a nuclear RNA scaffold to coordinate gene expression, possibly in a tissue or otherwise context-specific manner. Equally unresolved is the precise role of *Malat1* in tumorigenesis, perhaps in part due to this functional context-specificity.

The lncRNA ***HOTAIR*** *(HOX Transcript Antisense RNA)* is another well-studied transcript with altered expression across several cancers compared to normal tissues (Iyer et al. 2015; Kogo et al. 2011; Gupta et al. 2010). *HOTAIR* was initially found in a search for ncRNAs contributing to the temporal and spatial collinearity of vertebrate *HOX* gene expression and is expressed antisense to the *HOXC* gene cluster (Rinn et al. 2007). Similar to *MALAT1*, the molecular details of *HOTAIR* function remain incompletely understood, despite clear associations between *HOTAIR* expression and cancer. Early siRNA knockdown experiments and *in vitro* RNA pull down assays suggested that *HOTAIR* functions in *trans* to direct the localization of repressive chromatin modifying complexes (*i.e.* PRC2 and CoREST), leading to the epigenetic silencing of genes in the distally located *HOXD* cluster and elsewhere in the genome (Rinn et al. 2007; Tsai et al. 2010). *HOTAIR* has thus been proposed to contribute to tumorigenesis through driving aberrant

epigenetic alterations, and indeed high *HOTAIR* expression correlates with aggressive disease and/or poor prognosis in colorectal and breast cancers (Kogo et al. 2011; Gupta et al. 2010). Furthermore, exogenous overexpression of *HOTAIR* in murine breast cancer cells was shown to drive the metastatic potential of these cells in xenograft experiments and lead to global gene dysregulation, possibly through PRC2 (Gupta et al. 2010). However, more recent work has called into question the specificity of this proposed lncRNA-PRC2 interaction (Davidovich et al. 2013), or its necessity for *HOTAIR*'s ability to drive transcriptional repression (Portoso et al. 2017).

Genetic dissections of *HOTAIR* function have also proven perplexing. In one mouse model in which murine *Hotair* had been deleted, *HoxD* gene derepression and apparent homeotic transformations consistent with dysregulated *HoxD* expression were observed (Li et al. 2013). This remarkable phenotype starkly contrasted with the essentially unchanged *HoxD* expression patterns observed in a second knockout mouse in which the entire *HoxC* cluster, including *Hotair*, had been ablated (Schorderet and Duboule 2011). Further characterization of these and additional *Hotair* knockout alleles (Lai et al. 2015) conclusively demonstrated that *Hotair* loss does not significantly affect mouse development or *HoxD* expression (Amândio et al. 2016). Nonetheless, these data suggesting the dispensability of *Hotair* in normal development do not preclude the possibility of *HOTAIR* dysregulation contributing to tumorigenesis, either through PRC2-linked epigenetic dysregulation or through other yet-to-be uncovered mechanisms.

One additional, well-studied lncRNA with ties to cancer is ***XIST***. Correlative links between defects in XCI and cancer development have long been noted, such as the loss of Barr bodies seen in some advanced breast and ovarian cancers (Pageau et al. 2007). However, it wasn't until the development of a conditional knockout mouse in which *XIST* was deleted in hematopoietic progenitor cells during embryogenesis, but after the establishment of XCI, that a direct link between *XIST*-mediated dosage compensation and tumor suppression was demonstrated *in vivo*. In these mice, hematopoietic *XIST* depletion leads to global gene dysregulation, including notable X-linked gene reactivation, and the rapid onset of female-specific hematologic cancer with 100% penetrance (Yildirim et al. 2013). The potential significance of aberrant XCI in other cancers remains to be seen.

In summary, several lines of evidence suggest that lncRNAs may contribute to cancer-related processes, in both oncogenic and tumor suppressive capacities. However, the number of lncRNAs demonstrated to be important in modulating tumorigenesis remains only a small fraction of the thousands of lncRNA transcripts that are expressed in the human and mouse transcriptomes (Iyer et al. 2015; Djebali et al. 2012). More careful *in vitro and in vivo* dissections of candidate cancer-associated lncRNAs will be needed to determine whether correlative links to disease reflect true functional involvement, and ideally, opportunities for therapeutic intervention.

The p53 tumor suppressor pathway

One approach to find functional lncRNAs involved in cancer that our group and others have adopted has been to look for lncRNAs regulated by key oncogenes [*e.g*. Myc (Kim et al. 2015; Marchese et al. 2016)] or tumor suppressors, such as p53 (reviewed below).

p53, encoded by the *TP53* gene in humans and *Trp53* in mice, is an important tumor suppressor in the mammalian genome. One commonly used moniker for p53 is the "guardian of the genome," due to its central role in sensing and responding to various cellular stressors (Kastenhuber and Lowe 2017; Vousden and Prives 2009). Mice deficient for p53 (*Trp53*$^{-/-}$ or *Trp53*$^{+/-}$) are significantly more prone to spontaneous tumorigenesis (Donehower et al. 1992; Jacks et al. 1994), and human patients with germline mutations in *TP53* suffer Li Fraumeni Syndrome, which is a disease marked by dramatically increased susceptibility to a broad spectrum of cancers (Olivier et al. 2010). Furthermore, p53 is the most frequently somatically mutated gene in human cancer and p53 loss is often associated with poor clinical indicators, such as advanced tumor grade and decreased survival (Kandoth et al. 2013).

Key to p53's tumor suppression is its function as a stress-stabilized transcription factor (Brady et al. 2011; Kaiser and Attardi 2018), though transcription-independent roles for cytosolic p53 in regulating mitochondrial permeabilization and apoptosis have also been observed (Speidel 2010). Indeed, most p53 mutations that arise in cancer are missense mutations that fall within p53 protein's DNA binding domain (Kastenhuber and Lowe 2017), further

suggesting that the ability of p53 to bind to and regulate target genes is needed for tumor suppression. In unstressed cells, p53 protein is constitutively expressed but rapidly degraded. However, upon exposure to various types of stress (*e.g.* DNA damage, oncogene activation, hypoxia, nutrient-starvation), p53 protein accumulates. This is primarily due to the acquisition of post-translational modifications that disrupt interactions with its main negative regulator, Mdm2 (Mouse double minute 2), which is an E3 ubiquitin ligase that promotes the proteasomal degradation of p53. The activation of *ARF* (*Alternate Reading Frame of CDKN2A*; also known as *p14* in humans and *p19* in mice) in response to oncogenic signaling is another central mechanism by which p53 protein is stabilized, as ARF binds to Mdm2 and further inhibits Mdm2-p53 interactions (Horn and Vousden 2007). Once stabilized, p53 transcriptionally regulates a wide network of genes by binding to specific DNA motifs known as p53 response elements (p53REs) found in the promoters or gene bodies of targets (Riley et al. 2008).

Activation of the p53 transcriptional network by stress can lead to a myriad of cellular outcomes and can paradoxically promote both survival and death, depending on various context-specific factors that are still incompletely understood. A non-exhaustive list of biological processes in which p53 has been implicated includes the so-called “canonical” p53 responses of apoptosis and cell cycle arrest, as well as more recently discovered roles in regulating autophagy, cellular metabolism, pluripotency, DNA repair, ferroptosis, and genome maintenance, to name a few examples [reviewed in (Kaiser and Attardi 2018;

Kruiswijk et al. 2015)]. Elucidating how the many different functions ascribed to p53 synergize to culminate in tumor suppression is a major area of research. Another unresolved question related to the remarkable functional diversity of p53 is *how* this one transcription factor specifies so many different cellular outcomes, or in other words, what are the molecular determinants of p53 cell fate decision-making? Perhaps unsurprisingly, decades of research have yet to uncover a single universally-applicable answer to this question (Kastenhuber and Lowe 2017).

One setting this question has been explored in is comparing the p53 outcomes of senescence and apoptosis, which are two alternative, permanent ways by which p53 limits aberrant growth to suppress tumorigenesis (Childs et al. 2014; Kaiser and Attardi 2018). Apoptosis, or programmed cell death, can be triggered by severe DNA damage (*e.g*. genotoxic compounds, irradiation) in many cell types, but can also be preferentially initiated in certain cell types in a more stress-agnostic manner (Lowe et al. 1993b; Ventura et al. 2007; Childs et al. 2014). *In vivo*, apoptosis can suppress tumor growth through the physical elimination of tumor cells (Ventura et al. 2007; Symonds et al. 1994). Senescence is a stable state of cell cycle arrest that is morphologically, epigenetically, and metabolically distinct from temporary or reversible cell cycle arrest (Campisi and d'Adda di Fagagna 2007). Multiple stress stimuli can promote senescence, most of which are tied to DNA damage signaling. For example, elevated levels of reactive oxygen species (ROS), genotoxic compounds, and, in human cells, natural telomere shortening (leading to what is referred to as "replicative senescence") can all trigger senescence (Muñoz-Espín and Serrano 2014). Persistent oncogenic signaling can also trigger

senescence (sometimes referred to as “oncogene induced senescence” or OIS), likely through the activation of similar DNA damage signaling pathways by oncogene-driven defects in DNA replication or ROS production (Bartkova et al. 2006; Di Micco et al. 2006; Muñoz-Espín and Serrano 2014). Senescence can have pleiotropic effects on tumorigenesis, at times promoting preneoplastic growth (Krtolica et al. 2001) or triggering tumor-permissive immune suppression through the secretion of a characteristic host of inflammatory molecules, extracellular matrix components, and growth factors known as SASP (senescence-associated secretory phenotype) (Ruhland et al. 2016; Muñoz-Espín and Serrano 2014). In other situations, senescence can serve to restrict hyperproliferation (Ventura et al. 2007) or signal the immune clearance of tumor cells (Xue et al. 2007), contributing to tumor suppression. Thus, apoptosis and senescence are two p53-regulated cellular processes that are important for tumor suppression, though what factors govern the initiation of one versus the other in any given situation are unclear.

Much effort has been exerted towards characterizing the p53 regulated transcriptome, hoping to identify either a core set of universal p53 targets central to tumor suppression or specific subsets of targets important for specific p53 functions (discussed more in **Chapter 2**). One early proposed model for how the latter could come about was based on biochemical observations that p53 bound with higher affinity to the promoters of target genes involved in cell cycle arrest than those involved in apoptosis, suggesting that different thresholds of p53 levels could lead to the preferential activation of either high or low affinity targets, leading to cell cycle arrest or apoptosis, respectively (Weinberg et al. 2005; Hafner

et al. 2019). However, this line of thinking has been thrown into question as an increasing number of genome-wide analyses of p53-dependent gene regulation show that p53 outcome-specificity does not correlate with the activation of protein coding genes important for specific p53 functions (Fischer 2017; Andrysik et al. 2017; Verfaillie et al. 2016). For example, p21 (encoded by the gene *Cdkn1a*) and Puma (encoded by *Bbc3*) are key mediators of p53-dependent cell cycle arrest (Brugarolas et al. 1995) and apoptosis (Nakano and Vousden 2001), respectively. However, both are consistently activated by p53 across many studies, irrespective of cell type, stressor, or ultimate p53 outcome initiated (Fischer 2017). Thus, one main take-away from these global analyses has been that p53 outcome-specificity is most likely driven by many different mechanisms and contingent upon the type, intensity, and/or duration of stress encountered, as well as by cell- or tissue-specific factors (Hafner et al. 2019).

Related to these context-specific determinants could be the activation of individual p53 targets with context-specific functions. Such targets may be overlooked in large-scale metanalyses of "universal" p53 targets due to either tissue- or stressor-specific activation. While the first p53 targets identified were protein coding genes, as our understanding of the breadth of the noncoding genome grows, so too has our appreciation for the numerous noncoding genes regulated by p53, first with the identification of microRNA targets of p53 (Raver-Shapira et al. 2007; Vousden and Prives 2009) and more recently, lncRNAs. Elucidating how these newly emerging lncRNA targets fit into the broader p53 network is an important, on-going area of research.

LncRNAs in the p53 pathway

Several recent studies have employed a variety of transcriptome profiling techniques to identify p53-regulated lncRNAs, many of which will be discussed shortly. Most focus has been spent on lncRNAs that are transcriptionally activated by p53, though there is also some evidence for the repression of lncRNA loci by p53 having physiological importance. For example, *lncPRESS1 (p53 Regulated and Embryonic Stem cell Specific)* is highly expressed in human embryonic stem cells and repressed by p53 during differentiation (Jain et al. 2016). shRNA-mediated knockdown of *lncPRESS1* leads to loss of pluripotency and gene expression changes consistent with differentiation (Jain et al. 2016), suggesting that repression of *lncPRESS1* may be an important aspect of p53's general role in restricting pluripotency (Li et al. 2012; Kastenhuber and Lowe 2017). Nonetheless, most p53-regulated lncRNAs that have been shown to contribute to p53 pathway function are transcriptionally activated by p53.

Below is a detailed catalogue of p53-activated lncRNAs, with a focus on lncRNA examples for which evidence of direct regulation by p53 has been experimentally provided (*e.g.* p53 binding at lncRNA associated p53REs, p53RE reporter assays). A comprehensive summary of direct p53-activated lncRNAs can also be found in **Table 1.1**.

Nuclear-acting p53-activated lncRNAs

Most p53-activated lncRNAs have been reported to play various roles in the nucleus. ***LincRNA-p21*** was amongst the earliest identified p53-activated

lncRNAs, found to be upregulated in a p53-dependent manner in both genotoxic and oncogenic stress conditions, and is named after its genomic proximity to *p21*, located ~15 kb upstream in the mouse genome (Huarte et al. 2010). *LincRNA-p21* is conserved, and early siRNA knockdown studies suggested *trans*-acting roles for both mouse and human *lincRNA-p21*: driving global gene repression and apoptosis in mouse embryonic fibroblasts (MEFs) and repressing translation in Hela cells (Huarte et al. 2010; Yoon et al. 2012). However, subsequent experiments using either a genetically engineered mouse in which the *lincRNA-p21* promoter had been deleted or ASO-mediated RNA knockdown suggested that *lincRNA-p21* acts solely to promote p21 expression in *cis* and that the global changes in gene expression observed upon *lincRNA-p21* loss can be attributed to decreased p21 expression (Dimitrova et al. 2014). It is worth noting that this more limited, local role for *lincRNA-p21* is easier to reconcile with its low copy number per cell and spatial restriction to its site of transcription in MEFs. However, it is conceivable that human *lincRNA-p21*, which is exported into the cytoplasm, may have additional roles.

Mechanism-wise, both lncRNA-mediated recruitment of hnRNP-K (Dimitrova et al. 2014) and DNA regulatory elements in the *lincRNA-p21* locus (Groff et al. 2016) have been reported to contribute to the *cis*-activation of p21. Importantly, MEFs in which *lincRNA-p21* has been either genetically or co-transcriptionally depleted have a growth advantage (Dimitrova et al. 2014), consistent with defects in the G1/S checkpoint, demonstrating the potential for lncRNA locus-mediated *cis*-regulation to functionally contribute to p53-regulated cellular processes.

Pvt1b is a p53-induced isoform of *Pvt1* and another lncRNA our group has recently demonstrated to regulate gene expression in *cis* downstream of p53 activation. However, unlike *lincRNA-p21*'s reinforcing effect on *p21* expression, *Pvt1b* activation leads to the repression of *Myc*, located roughly 50 kb upstream in mice. In MEFs and murine cancer cells, *Pvt1b* knockdown by ASOs or genetic disruption of *Pvt1b*'s p53RE leads to *Myc* derepression and increased proliferation *in vitro*. Most strikingly, *in vivo* targeting of *Pvt1b*'s p53RE leads to increased tumor growth in an autochthonous mouse model of lung cancer, suggesting that *Pvt1b* is an important lncRNA component of p53's tumor suppressive transcriptional program (Olivero et al. 2020). Precisely how *lincRNA-p21* and *Pvt1b* mediate positive and negative *cis*-regulation, respectively, remains incompletely understood and is likely locus-specific, involving both RNA-dependent and -independent mechanisms (Olivero et al. 2020; Cho et al. 2018; Dimitrova et al. 2014; Groff et al. 2016).

PANDA *(p21 Associated NcRNA DNA Damage Activated)* was originally identified in a tiling array screen for novel ncRNAs associated with cell cycle regulation and is transcribed roughly 5 kb upstream of and antisense to *CDKN1A* (*p21*) (Hung et al. 2011). In human fibroblasts, *PANDA* expression is induced by both DNA damage and oncogenic stress (Hung et al. 2011; Puvvula et al. 2014). Interestingly, *PANDA* does not seem to be conserved between humans and mice, despite the syntenic conservation of other p53-regulated lncRNAs encoded in the *CDKN1A/Cdkln1a* locus [*e.g. LincRNA-p21, DINO (discussed below)*]. *PANDA* has been reported to play multiple nuclear roles following p53 activation, mediated through interactions with transcription factors and chromatin modifying

complexes. In human fibroblasts exposed to DNA damage, *PANDA* was observed to limit DNA damage-induced apoptosis by sequestering the transcription factor NF-YA (Hung et al. 2011).

In more recent work, *PANDA* was shown to regulate proliferation as well through distinct, context-specific mechanisms. In unstressed, proliferating human fibroblasts, *PANDA* interacts with hnRNP-U (Heterogeneous Nuclear Ribonucleoprotein U) and BMI1 (BMI1 proto-oncogene, Polycomb Ring Finger), which is a component of the repressive PRC1 complex. siRNA knockdown of *PANDA* in unstressed cells led to the derepression of several genes involved in cell cycle regulation and the onset of cellular arrest, suggesting that *PANDA*-hnRNP-U-BMI1 interactions may be important for epigenetically silencing pro-arrest genes (Puvvula et al. 2014). In senescent human fibroblasts, *PANDA* was observed to interact with NF-YA and prevent NF-YA-mediated activation of pro-proliferation genes (Puvvula et al. 2014). Interestingly, either siRNA knockdown of *PANDA* or dysregulation of NF-YA protein levels was sufficient to allow escape from senescence, although *p21* levels were also reduced by *PANDA* knockdown. As *p21* has a well-established role in promoting senescence (Serrano et al. 1997; Campisi and d'Adda di Fagagna 2007), it is difficult to disentangle the contributions of *PANDA* from this effect on p21 RNA. siRNAs have been previously shown to be able to reduce lncRNA transcription at the chromatin (Stojic et al. 2016), posing the possibility that *PANDA* regulates *p21* expression in *cis* (being located only 5 kb downstream). However, it is important to note that Hung *et al.* also used siRNAs to knockdown *PANDA* and did not see a concomitant effect on p21 RNA levels. Thus, this discrepancy highlights the difficulties of interpreting

siRNA experiments targeting nuclear lncRNAs. Unfortunately, genetic dissection of the *PANDA* locus is currently lacking, making it impossible to determine whether *PANDA* truly regulates *p21* in *cis* during cellular stress, similar to *lincRNA-p21*.

DINO *(Damage Induced Noncoding)* is a conserved lncRNA also expressed from the *CDKN1A/Cdkn1a* locus. In humans, *DINO* is transcribed divergently from the *CDKN1A* promoter, while mouse *Dino* is transcribed immediately upstream of *Cdkn1a* on the same strand. Though induced by both genotoxic and oncogenic stress, *DINO* levels are most drastically elevated in response to DNA damage, reaching several hundred copies per cell in human fibroblasts treated with Doxorubicin (Doxo) (Schmitt et al. 2016). Consequently, *DINO* has thus far been best characterized in the context of the p53 response to acute DNA damage. In Doxo-treated cells, *DINO* depletion by RNAi led to reduced p53 transcriptional activity and impaired damage-induced cell cycle arrest, suggesting that *DINO* was required for full activation of the p53 network. This contribution to p53's transcriptional activity was further supported by experiments demonstrating that *DINO* directly interacts with and stabilizes p53 protein and that DINO RNA localizes to the p53REs of several direct p53 targets, perhaps reflecting a role in directing p53's localization as well. Importantly, these data were further supported by experiments performed in MEFs isolated from a *Dino* promoter deletion mouse. *Dino* knockout MEFs treated with Doxo had less p53 protein, decreased p53 transactivity, and increased cellular proliferation, consistent with *Dino* expression being important for robust p53 pathway function (Schmitt et al. 2016). Intriguingly, *DINO* levels were recently shown to also influence p53 protein

stability in primary human foreskin keratinocytes expressing viral oncogene HPV16 E7 (Sharma and Munger 2020). Whether *DINO* regulates p53 levels during oncogenic stress through mechanisms similar to or distinct from those employed during the DNA damage response remains to be investigated.

Similar to *DINO*, ***PURPL*** (*p53 Upregulated Regulator of p53 Levels*) is a p53-activated lncRNA that in turn regulates p53 levels. *PURPL* was identified by RNA-seq as a nuclear-enriched, DNA damage-induced lncRNA in three human colorectal cancer cell lines (Li et al. 2017). Either genetic disruption of a *PURPL*-associated p53RE in clonal HCT116 cells or ASO knockdown of *PURPL* in multiple human colorectal cancer cell lines led to increased p53 protein levels (Li et al. 2017). These effects were observed both in the presence and absence of DNA damage, suggesting *PURPL* may be important for maintaining appropriately low levels of p53 in conditions of low stress. RNA pulldown and RNA immunoprecipitation experiments demonstrated that *PURPL* interacts with MYBBP1A and HuR, leading to the disruption of nucleoplasmic MYBBP1A-p53 protein interactions and subsequent p53 protein destabilization. Functionally, *PURPL* loss was observed to decrease proliferation and increase apoptosis *in vitro* and limit tumor growth in xenograft experiments *in vivo*, consistent with increased p53 protein in the absence of *PURPL* (Li et al. 2017). Thus, *PURPL* is a p53-activated lncRNA that negatively regulates p53 levels, perhaps to restrict improper p53 pathway activation.

Linc-Pint (*p53-Induced Transcript*; also known as *lincRNA-Mkln1*) is a nuclear-enriched intergenic lncRNA that was first identified as a direct p53 target

induced by genotoxic and oncogenic stress in the same microarray profiling experiment that uncovered *lincRNA-p21* (Huarte et al. 2010; Marín-Béjar et al. 2013). Under conditions of genotoxic stress, ASO knockdown of *linc-Pint* in MEFs and lung cancer cells led to decreased proliferation and increased apoptosis. Moreover, exogenous overexpression of *linc-pint* from a retroviral transgene rescued this phenotype, suggesting *linc-pint* acts in *trans* downstream of p53 activation to modulate growth and survival (Marín-Béjar et al. 2013). Mice in which *linc-Pint* expression was ablated through a LacZ reporter knockin were significantly smaller than controls, further suggesting a role for this lncRNA in growth regulation (Sauvageau et al. 2013). Murine *linc-Pint* and human orthologue *LINC-PINT* have both been reported to interact with PRC2 and regulate the repression of gene networks related to proliferation, survival, and invasion (Khalil et al. 2009; Marín-Béjar et al. 2013, 2017). In line with this, ASO knockdown of *LINC-PINT* also increased proliferation in multiple human cancer cell lines, while exogenous overexpression decreased proliferation and invasion *in vitro* and decreased tumor growth and metastasis *in vivo* in xenograft and liver metastasis mouse models, respectively (Marín-Béjar et al. 2017). Intriguingly, a short, highly conserved region located within exon 2 of *LINC-PINT* was shown to be necessary and sufficient for this anti-invasion phenotype in human colorectal cancer cells (Marín-Béjar et al. 2017). However, these data must be considered in the context of recent reports of an exon 2-encoded micropeptide with anti-proliferative effects expressed from a circular isoform of *LINC-PINT* (Zhang et al. 2018). Nevertheless, these studies suggest that the *linc-Pint/LINC-PINT* locus may play a tumor suppressive role downstream of p53.

NEAT1 *(Nuclear Enriched Abundant Transcript 1)* is a highly abundant, conserved lncRNA and is best known as an integral architectural component of nuclear paraspeckles. The functional role of *Neat1* in these nuclear bodies is still incompletely understood but likely related to the regulation of gene expression (Sunwoo et al. 2009; Clemson et al. 2009; West et al. 2014). *Neat1* knockout mice are viable and develop largely normally, barring subtle defects in mammary gland and corpus luteum development (Nakagawa et al. 2011; Standaert et al. 2014; Nakagawa et al. 2014). These data suggest that *Neat1* and/or paraspeckles may not be functionally important under normal physiological conditions but do not preclude their importance during stress.

In support of this hypothesis, *Neat1/NEAT1* is a direct target of p53 and is induced by DNA damage in multiple mouse and human primary and transformed cell lines (Mello et al. 2017; Adriaens et al. 2016). In one study, *Neat1* knockout mice were observed to be resistant to carcinogen-induced skin cancer and skin keratinocytes in carcinogen-exposed knockout mice were seen to proliferate more slowly than WT controls. These data suggest that *Neat1* acts as an oncogene in this context, likely by preventing the accumulation of DNA damage that would lead to p53 stabilization and tumor cell death in WT mice (Adriaens et al. 2016). Tumor suppressive functions have also been ascribed to *Neat1* post-p53 activation. In MEFs expressing the oncogenes *E1A* and *HRasV12*, *Neat1* knockout promoted cellular transformation, while exogenous overexpression of *Neat1* decreased transformation, similar to the effects of p53 loss and overexpression in these cells, respectively (Mello et al. 2017). *Neat1* loss also promoted *in vivo* pancreatic cancer initiation in a genetically engineered mouse model of pancreatic ductal

adenocarcinoma, further suggesting that *Neat1* may be an important downstream mediator of p53 tumor suppression in some settings (Mello et al. 2017). Thus, like many lncRNAs, the contributions of *Neat1* to stress responses downstream of p53 activation are likely to be context-specific.

TUG1 *(Taurine Upregulated Gene 1)* was originally characterized as a regulator of retinal development (Young et al. 2005) but has since been linked to many biological processes, including tumorigenesis. *TUG1* has been reported to regulate gene expression through multiple mechanisms, including sponging miRNAs (Zhou et al. 2019) and recruiting repressive chromatin modifying complexes to genomic loci (Khalil et al. 2009; Zhang et al. 2014; Zhou et al. 2019). Within the p53 network, *TUG1* is activated by p53 under conditions of genotoxic stress in multiple primary and transformed cell lines (Guttman et al. 2009; Zhang et al. 2014). RNAi knockdown of *TUG1* in human NSCLC cells led to increased proliferation *in vitro* and increased tumor growth in xenograft mouse experiments, possibly through the epigenetic derepression of *HOXB7* (Zhang et al. 2014). While these data suggest that *TUG1* plays a tumor suppressive role downstream of p53 in these cells, it is important to note that both oncogenic and tumor suppressive roles have been observed for this lncRNA in other human cancer cell lines (Zhou et al. 2019), suggesting that *TUG1* function may be highly context-specific. *TUG1* is conserved in mice as well, and interestingly, a LacZ replacement *Tug1* knockout mouse was shown to have impaired spermatogenesis with no other obvious gross morphological defects (Lewandowski et al. 2020; Sauvageau et al. 2013), though it is difficult to say whether this spermatogenesis phenotype stemmed from the loss of *Tug1* expression or the genetic disruption of its neighboring gene, *Morc2a*, with

which *Tug1* shares a promoter. Nevertheless, this study illuminated the complexity of the *Tug1* genomic locus, which was revealed to contain previously unappreciated *cis*-regulatory DNA elements and a conserved, expressed micropeptide (Lewandowski et al. 2020). Employing similar genetic strategies in the context of cellular stress will shed further light on the ways these *trans*- and *cis*-acting components of the *Tug1/TUG1* locus contribute to cellular processes within the p53 pathway and beyond.

A recent genome-wide assessment of HCT116 cells treated with DNA damage identified several p53-regulated (PR) lncRNAs, including two that were further characterized and named ***PR-lncRNA 1*** and ***PR-lncRNA 10*** (Sánchez et al. 2014). Both PR-lncRNAs were shown to be required for robust p53 transactivity in response to genotoxic stress, as ASO knockdown of either led to decreased induction of several p53 targets. Consistent with decreased p53 activity, PR-lncRNA depleted HCT116 cells grew more quickly and were more resistant to DNA damage-induced apoptosis (Sánchez et al. 2014). The mechanisms by which these two lncRNAs regulate p53 activity are still unclear, though it is worth noting that our own closer examination of *PR-lncRNA 1's* sequence revealed the presence of two highly conserved sORFs, one conserved amongst primates and a second conserved through mice at both the amino acid and nucleotide levels. This raises the possibility of *PR-lncRNA 1* representing a micropeptide-encoding lncRNA, as has been described at the *Tug1* locus and other lncRNA loci (Lewandowski et al. 2020; Anderson et al. 2015; Zhang et al. 2018). The development of additional, genetic LOF models could greatly facilitate addressing this and other questions

regarding how these lncRNA loci contribute to p53 pathway function, as well as directly test their importance in tumor suppression.

In addition to binding to promoters and gene bodies, p53 also frequently associates with enhancers (Melo et al. 2013; Allen et al. 2014; Younger et al. 2015). This enhancer binding activity can be important for modulating the expression of distally located protein coding and lncRNA genes (Melo et al. 2013, 2016). ***Linc-475*** is one lncRNA regulated by a p53 bound enhancer and is induced by genotoxic stress in human breast cancer and melanoma cell lines. Both siRNAs and CRISPR-Cas9 deletion of the p53 bound enhancer element in clonal MCF7 cells led to depleted *linc-475* levels and cell cycle defects that could be attributed to decreased *p21* levels (Melo et al. 2016). Further experiments demonstrated that less RNA Pol II and p53 were present at p21's promoter in the absence of *linc-475*, suggesting that this lncRNA is important for optimal *p21* induction, and subsequently a robust cell cycle arrest response to DNA damage. How *linc-475* exerts this apparent *trans* nuclear function is unclear.

LED *(LncRNA Activator of Enhancer Domains)* is another lncRNA associated with p53 activity at enhancers. *LED* is activated by DNA damage in a p53-dependent manner in multiple human cancer cell lines (Léveillé et al. 2015; Allen et al. 2014). siRNA knockdown of *LED* in MCF7 cells led to the dysregulation of hundreds of genes and increased proliferation. This could be explained in part by significantly less p21 levels in *LED* depleted cells. In a transcriptional reporter assay, *LED* knockdown was shown to decrease the transcriptional activity of a p53 bound enhancer associated with *p21*, suggesting that *LED* promoted *p21*

expression by affecting the activity of this p53 bound enhancer, possibly through regulating H3K9 acetylation (Léveillé et al. 2015). As LED RNA also associated with hundreds of sites in the human genome and *LED* knockdown correlated with decreased enhancer RNA (eRNA) production from several other p53-responsive enhancers, *LED* seems to be an important lncRNA mediator of p53-dependent enhancer activation through yet-to-be fully fleshed out mechanisms (Léveillé et al. 2015). Interestingly, LED's promoter is often hypermethylated in cancer, particularly in acute lymphocytic leukemia, suggesting that epigenetic dysregulation of this lncRNA is associated with disease (Léveillé et al. 2015).

PINCR *(p53-Induced Noncoding RNA)* is another lncRNA directly activated by p53 and linked to enhancer activity (Chaudhary et al. 2017). *PINCR* was identified as a lncRNA induced by DNA damage in a panel of human colorectal cancer cell lines, and ASO knockdown or genetic *PINCR* knockout in clonal cell lines was observed to sensitize cells to stress-induced apoptosis. Interestingly, exogenous overexpression of *PINCR* was able to partially rescue this phenotype, suggesting a *trans*-acting mechanism for *PINCR*. Transcriptomic analysis revealed that *PINCR* depletion impaired the DNA damage-dependent induction of several p53 target genes involved in cell cycle arrest and survival. Mechanistic studies revealed that *PINCR* interacts with nuclear matrix component Matrin 3, likely facilitated by six Matrin 3 binding motifs present in PINCR RNA, and that *PINCR*-Matrin 3 interactions were important for p53 binding to enhancers associated with three tested p53 target genes (Chaudhary et al. 2017). Thus, *PINCR* appears to act as an RNA scaffold to facilitate interactions between some p53 regulated genes and distal enhancers during the p53 response to DNA damage. How pervasive this

transcriptional co-activator role is throughout the genome or its importance in other biological contexts remains an interesting open-ended question.

Cytoplasmic-acting p53-activated lncRNAs

Given that most p53-regulated lncRNAs identified thus far are predominantly nuclear localized and appear to exert nuclear functions, less is known about the role(s) p53-activated lncRNAs play in the cytoplasm. Below are two examples for which we have some mechanistic understanding.

GUARDIN is a lncRNA activated by p53 during the DNA damage response and has been implicated in modulating p53's genome maintenance functions (Hu et al. 2018). In multiple human cancer cell lines, shRNA knockdown of *GUARDIN* led to decreased proliferation and increased cell death *in vitro*, and *GUARDIN* depleted HCT116 cells contributed to significantly smaller tumors in xenograft mouse experiments. Interestingly, *GUARDIN* knockdown only affected cell viability in p53-proficient cells, suggesting that *GUARDIN* tempers the cytotoxic effects of p53 stabilization in some cancer cells. In line with this, exogenous overexpression of *GUARDIN* was sufficient to reverse this p53-dependent cytotoxicity in one human NSCLC cell line (Hu et al. 2018). Mechanism-wise, *GUARDIN* was shown to promote genome stability and cell survival under conditions of genotoxic stress in multiple ways. These include stabilizing TERF2 (Telomeric Repeat Binding Factor 2) by sequestering *mir23a* (TERF2 being an important component of the telomere protecting Shelterin complex) and stabilizing BRCA1 (Breast Cancer Type 1), which plays a central role in DNA repair,

by facilitating interactions between BRCA1 and its stabilizing binding partner BARD1 (BRCA1 Associated RING Domain 1) (Hu et al. 2018). Thus, *GUARDIN* acts in the cytoplasm to post-transcriptionally regulate multiple targets involved in genome maintenance and repair.

LincRNA-RoR *(Regulator of Reprogramming)* was first identified in a microarray screen for lincRNAs involved in pluripotency, where it was demonstrated to positively regulate reprogramming through RNAi LOF and exogenous overexpression GOF experiments (Loewer et al. 2010). Later work revealed that *lincRNA-Ro*R is directly activated by p53 in human cancer cell lines treated with DNA damage and that, intriguingly, *lincRNA-RoR* and p53 protein levels are negatively correlated (Zhang et al. 2013a). siRNA knockdown and transgene overexpression experiments demonstrated that *lincRNA-RoR* negatively regulates DNA damage-induced p53 translation, likely through interacting with phosphorylated hnRNP-I (also known as PTBP1) in the cytoplasm (Zhang et al. 2013a). hnRNP-I has been previously shown to bind to p53 mRNA and regulate cap-independent p53 translation (Grover et al. 2008), which is an important aspect of p53's activation during stress (Halaby et al. 2015), suggesting that *lincRNA-RoR* may interfere with this stress-driven p53 activation. Indeed, *lincRNA-RoR* overexpression reduced apoptosis and prevented cell cycle arrest in cells exposed to genotoxic stress, suggesting that *lincRNA-RoR* GOF prevented robust p53 pathway activation. Thus, *lincRNA-RoR* represents yet another p53-activated lncRNA that adjusts the p53 response to DNA damage by tuning p53 levels, here at the level of p53 protein translation.

Conclusion

Despite decades of research, many questions regarding how p53 senses and responds to such a wide range of stress inputs to suppress tumorigenesis remain incompletely answered. Studying lncRNAs in the p53 network can provide valuable new insights into these old questions, as well as provide perspective on the roles lncRNAs may play in cancer more broadly. The above examples illustrate the many ways by which p53-activated lncRNAs can contribute to p53 pathway function. In reviewing *in vitro*, and when available, *in vivo* functional characterization data regarding these lncRNAs, it becomes readily apparent that p53-activated lncRNAs can perform many diverse functions through a myriad of mechanisms of action. One over-arching theme that emerges, however, is that lncRNA loci often modulate the transcriptional output of the p53 network, whether globally (*e.g.* by modulating p53 protein levels or p53 transactivity), at a limited subset of p53 targets, or on an individual locus basis. Another commonality that emerges is that p53-activated lncRNAs often have tissue- or stress-specific functions. To this point, it is important to note that most searches for p53-activated lncRNAs have focused on lncRNAs induced in response to DNA damage. The functional characterizations of lncRNAs in other stressor contexts has the potential to expand our understanding of the ways in which the p53 network integrates and responds to different stresses. Nevertheless, this growing body of work suggests that p53-activated lncRNAs add an important regulatory layer to the p53 network and may represent an underexplored pool of "effectors" of p53 pathway function(s) and/or tumor suppression.

Table 1.1: LncRNAs reported to be directly activated by p53

LncRNA	Cons	Context studied	Proposed function(s) in p53 pathway	Ref(s)
DINO	m & h	Geno	**Nuclear:** Promote p53 transactivation; (Unknown): Promote p53 protein stability → ensure robustness of p53 response to DNA damage	(Schmitt et al. 2016)
GUARDIN	h	Geno	**Cytoplasmic:** Stabilize TERF2 by sponging *mir23a;* stabilize BRCA1-BARD1 interactions → promote genome stability and survival	(Hu et al. 2018)
LED	h	Onco	**Nuclear:** Promote enhancer activity at a subset of p53-responsive enhancers; promote *p21* expression → regulate proliferation	(Léveillé et al. 2015)
Linc-475	h	Onco	**Nuclear:** Promote *p21* expression → regulate proliferation and oncogene-induced senescence	(Melo et al. 2016)
Linc-Pint	m & h	Geno; Onco	**Nuclear:** Recruit PRC2 to epigenetically silence a network of genes → restrict proliferation and invasion	(Huarte et al. 2010; Marín-Béjar et al. 2017, 2013)
LincRNA-p21	m & h	Geno	**Nuclear:** Positive *cis*-regulation of *p21* → G1/S check-point reinforcement. **Cytoplasmic:** (human) Repress translation	(Dimitrova et al. 2014; Yoon et al. 2012; Huarte et al. 2010)
LncRNA-RoR	m & h	Geno	**Cytoplasmic:** Repress p53 translation during stress → restrict p53 pathway activation	(Zhang et al. 2013a)
Loc285194	h	Geno	**Cytoplasmic:** Negatively regulate *miR-211* → restrict cellular proliferation	(Liu et al. 2013b)
Neat1	m & h	Geno; Onco	**Nuclear:** (1) Dampen DNA damage response → restrict p53 stabilization; promote tumorigenesis *in vivo* (2) Regulate global gene expression→ restrict transformation *in vitro* and tumorigenesis *in vivo*	(Adriaens et al. 2016; Mello et al. 2017)
PANDA	h	Geno; Onco	**Nuclear:** (1) Sequester NF-YA → promote survival or restrict proliferation; (2) Recruit hnRNP-U and BMI1 to repress a subset of genes → promote proliferation	(Hung et al. 2011; Puvvula et al. 2014)
PR-lncRNA 1	m & h	Geno	**Nuclear:** Promote p53 transactivation → ensure robustness of p53 response to DNA damage	(Sánchez et al. 2014)
PR-lncRNA 10	h	Geno	**Nuclear:** Promote p53 transactivation → ensure robustness of p53 response to DNA damage	(Sánchez et al. 2014)
PINCR	h	Geno	**Nuclear:** Promote promoter-enhancer interactions at a subset of p53 targets through Matrin 3 interactions→ promote survival	(Chaudhary et al. 2017)
PURPL	h	Geno; Onco	**Nuclear:** Destabilize p53 protein in low stress conditions → regulate proliferation and promote survival	(Li et al. 2017)
Pvt1b	m & h	Geno; Onco	**Nuclear:** Negative *cis*-regulation of *Myc* → restrict proliferation *in vitro* and tumorigenesis *in vivo*	(Olivero et al. 2020)
TUG1	m & h	Onco	**Nuclear:** Recruit epigenetic machinery to repress *HOX7B* → restrict proliferation	(Zhang et al. 2014)
TRMP	h	Onco	**Cytoplasmic:** Suppress IRES-dependent *p27* translation → regulate proliferation	(Yang et al. 2018)

Cons= conservation (m= mouse; h= human); Geno= genotoxic stress; Onco= oncogenic stress

Chapter 2: Project Framework

Overview

LncRNAs are increasingly being implicated in a wide array of complex cellular and organismal processes [see **Chapter 1**; also recently reviewed in (Yao et al. 2019)]. Most lncRNAs have been proposed to function by regulating gene expression through a variety of mechanisms, including influencing transcription (Dimitrova et al. 2014; Marín-Béjar et al. 2013; Melo et al. 2016), post-transcriptionally regulating mRNA localization, stability, or translation (Hu et al. 2018; Kretz et al. 2013; Carrieri et al. 2012), or post-translationally modulating protein localization or stability (Li et al. 2017; Schmitt et al. 2016; Hung et al. 2011; Lee et al. 2016; Hu et al. 2018). Additionally, many lncRNAs are dysregulated in numerous diseases, including several human cancers (Iyer et al. 2015; Olivero and Dimitrova 2020), suggesting that lncRNA expression could be tied to cancer pathogenesis. However, the importance of lncRNA-mediated gene regulation in human health and disease is incompletely understood.

P53 is a universal tumor suppressor that transactivates a wide network of protein coding and noncoding target genes in response to stress to restrict tumorigenesis (Vousden and Prives 2009). As reviewed in **Chapter 1**, several studies within the past decade have identified lncRNAs that are transcriptionally activated by p53 and important for various p53-regulated processes. However, comparisons across studies in which individual p53-activated lncRNAs are characterized make it difficult to gain an accurate sense of the true extent to which

lncRNAs contribute to the p53 pathway. Additionally, a comprehensive understanding of the mechanisms by which p53-activated lncRNAs modulate this important transcriptional network is also lacking. This is in part due to the fact that most genome-wide searches for p53-regulated lncRNAs have been performed in the context of genotoxic stress (*i.e.* stress induced by DNA damaging agents) (see **Table 1.1**). p53 can also be activated by the aberrant expression of oncogenes (*e.g.* over-amplification or the acquisition of constitutively activating mutations) (Serrano et al. 1997; Horn and Vousden 2007), which leads to cellular hyperproliferation and a multifaceted stress called oncogenic stress. Key characteristics of oncogenic stress that lead to p53 stabilization include the persistent activation of mitogenic and DNA damage response pathways (Bartkova et al. 2006; Di Micco et al. 2006; Meek 2009; Collado and Serrano 2010).

Seminal work from the Attardi lab (Stanford) suggests that the p53 transcriptional response to oncogenic stress is likely distinct from that initiated by acute DNA damage. p53 regulates transcription through two transactivation domains (TADs) (Sullivan et al. 2018). Simultaneous mutation of both TADs significantly impairs the ability of mouse embryonic fibroblast (MEFs) to respond to either genotoxic or oncogenic stress, as p53's ability to activate a robust p53 network is severely compromised. Double TAD mutated p53 is also unable to suppress tumorigenesis *in vivo* in cancers of multiple tissues of origin, reinforcing the importance of p53-dependent transcriptional regulation in tumor suppression (Brady et al. 2011; Jiang et al. 2011).

However, the cellular consequences of mutating either TAD independently are surprisingly dependent on the type of stressor faced, despite both TAD mutations compromising p53 activation to varying extents (Brady et al. 2011). In MEFs exposed to genotoxic stress, loss of TAD1 is sufficient to significantly impair DNA damage-induced cell cycle arrest and apoptosis. In contrast, TAD1 mutation does not impede the initiation of oncogene-induced senescence in MEFs, suggesting that the antiproliferative p53 response to oncogenic signaling does not rely on the same p53 targets as the response to genotoxic stress (Brady et al. 2011). Interestingly, the lncRNA *Neat1* is one p53 target that has been reported to function in a similar, stress-specific manner, namely in that *Neat1* activation downstream of genotoxic stress is dispensable for the p53 response to DNA damage but essential for p53 pathway function in response to oncogene-induced cellular transformation (Mello et al. 2017). These observations beg the question whether a distinct cohort of lncRNAs are activated by p53 during the response to oncogenic stress.

Another set of related, important questions in the p53 field is what determines the spectrum of targets that are activated by p53 in any given setting and how target selection relates to the distinct downstream cellular outcomes of p53 activation. In an effort to address these questions, recent years have seen many research groups employ genome-wide p53 binding and RNA profiling to characterize the p53 transcriptional response across a number of biological contexts. These include the response to genotoxic stress (Kenzelmann Broz et al. 2013; Younger et al. 2015; Li et al. 2012; Catizone et al. 2019; Zhang et al. 2013b), as well as the p53 response to oncogenic signaling, either by pharmacologically

stabilizing p53 in cancer cell lines or driving the overexpression of specific oncogenes (Allen et al. 2014; Tonelli et al. 2017; Andrysik et al. 2017; Menendez et al. 2013). **Table 2.1** provides a detailed catalogue and cross-study comparison of these genome-wide profiling experiments.

However, despite the variety of biological settings sampled, disentangling the contributions of cell type-intrinsic versus stimuli-intrinsic effects of p53 activation has proven extremely challenging. Some studies have concluded that p53 function is primarily determined by the type of stress stimuli encountered (Menendez et al. 2013). Others have suggested that cell identity plays a pivotal role in determining where p53 binds in the genome (Nikulenkov et al. 2012; Botcheva and McCorkle 2014) and/or which genes are transactivated (Tonelli et al. 2017; Catizone et al. 2019; Zhang et al. 2013b), regardless of stress stimuli or ultimate cellular outcome. In contrast to both models, one recent metanalysis of fifteen human p53 ChIP-Seq datasets observed that p53 binding is largely invariant, irrespective of either cell or stimuli identity (Verfaillie et al. 2016). However, eleven of the datasets analyzed in this study were collected under genotoxic conditions, highlighting the need for further investigation of the p53 response to oncogenic stress. Especially lacking in the field are comparisons across diverse tumor types.

In order to answer these important questions related to p53-regulated lncRNAs and broader p53 pathway function, my research broadly aimed to **(1)** characterize the p53 transcriptional response to oncogenic stress, and **(2)** identify and functionally interrogate the contributions of p53-activated lncRNAs in a number of cellular responses triggered by oncogenic stress. In **Chapter 3**, I work

towards addressing these aims through surveying the p53-regulated transcriptomes of three different tumor types, revealing important principles of gene regulation in the p53 pathway and implications for lncRNAs in these processes. In **Chapter 4** and **Chapter 5**, I focus on the careful molecular dissection of several p53-activated lncRNA loci identified in **Chapter 3** to further address aim **(2)**, namely asking whether lncRNAs function downstream of p53 to modulate the decision between initiating apoptosis or senescence in response to oncogenic signaling.

Model systems

Oncogenic stress

Central to this work were two *in vitro* model systems for the study of p53 pathway function. To model the p53-dependent response to oncogenic stress, we took advantage of a well-established genetically engineered mouse model of p53-driven tumorigenesis: the *Kras$^{LA2\text{-}G12D/+}$; p53$^{LSL/LSL}$; Rosa26-CreER^{T2}* (*KPR*) mouse (Ventura et al. 2007; Feldser et al. 2010). In this mouse, p53 has been genetically inactivated by the insertion of a *loxP*-flanked transcription-translation STOP cassette into the first intron of both *Trp53* alleles, leading the complete loss of p53 expression (Ventura et al. 2007). Additionally, *Kras^{G12D}* (*i.e.* a constitutively active oncogenic mutant of *Kras*) is spontaneously activated *in vivo* through somatic recombination at a targeted *Kras$^{LA2\text{-}G12D}$* allele. This leads to the heterozygous expression of *Kras^{G12D}* in a subset of cells throughout the mouse (Johnson et al. 2001). Importantly, somatic *TP53* and *KRAS* mutations are often

concurrent in many human cancers, especially in adenocarcinomas of the lung and other tissues, making this model of oncogenic stress clinically relevant (The Cancer Genome Atlas).

Loss of p53 and gain of oncogenic *Kras*^{G12D} leads to the rapid development of a spectrum of cancers in *KPR* mice, including lung adenocarcinomas, thymic lymphomas, and sarcomas (Johnson et al. 2001), from which cell lines can be established. Importantly, p53 expression can be restored in *KPR* mice or tumor-derived cell lines by administering 4-hydroxytamxifen (Tam), which drives the nuclear translocation of *CreER*^{T2} expressed from the *Rosa-26* locus, leading to the excision of the STOP cassette (Ventura et al. 2007; Feldser et al. 2010). Thus, the *KPR* mouse represents an ideal model for studying p53-dependent gene regulation and p53 pathway function downstream of oncogenic stress. The use of this model in probing p53 outcome-specificity is discussed further in **Chapter 3**.

Genotoxic stress

As **Table 2.1** illustrates, p53 has been most extensively studied in the context of genotoxic stress. While this is undoubtedly an important area of research in its own right, this predominance is also likely in part due to the fact that many of the earliest insights into p53's cellular functions were made in the context of the DNA damage response (Kastan et al. 1992; Lowe et al. 1993b; Brugarolas et al. 1995). MEFs exposed to various genotoxic insults (*e.g.* ionizing irradiation, UV, pharmacological compounds) represent a long-established *in vitro* system for studying novel components of the p53 pathway. As such, MEFs

have well-characterized p53 cellular responses and well-established assays for testing various p53 functions (Attardi et al. 2004; Lowe et al. 1994). MEFs exposed to the Doxorubicin (Doxo; a chemotherapeutic topoisomerase II inhibitor) served as a complementary *in vitro* system for probing the functions of multiple newly-identified p53-activated lncRNAs within this work.

Tools for the study of lncRNA loci

As the study of lncRNAs matures as a field, it is becoming increasingly clear that experimental approaches to manipulate lncRNA expression must be tailored to the specific lncRNA locus under study, often more so than for protein coding genes. The following is a brief discussion of the merits and caveats of common lncRNA loss-of-function and gain-of-function strategies, many of which were employed in this work.

LncRNA loss-of-function (LOF)

While the ease of genetic manipulation has been greatly augmented in recent years by CRISPR-Cas9 technology, effective genetic inactivation of lncRNA loci is still challenging. This is due to the fact that our understanding of the RNA sequences important for lncRNA function remains imprecise. Genetic lncRNA knockouts have, by necessity, most often consisted of large-scale promoter or gene body deletions (Bassett et al. 2014; Kopp and Mendell 2018), unlike the targeting of protein coding genes which can be functionally inactivated by introducing small insertions and deletions that disrupt open reading frames (ORFs). One important

caveat of these large-scale deletion models is that potentially important DNA elements are also ablated along with lncRNA expression. Additionally, deletions targeting annotated promoters may not completely abrogate the use of alternative lncRNA transcription start sites. Thus, while large-scale deletions can be an effective LOF strategy for the initial characterization of a novel lncRNA locus, more precise genetic strategies are more ideal and, increasingly, more common. Two such strategies are inserting premature transcription termination signals or deleting more limited sequences (*e.g.* splice sites, DNA regulatory motifs). CRISPR-Cas9 based epigenetic inhibition (CRISPRi) (Gilbert et al. 2013) has also proven to be a useful alternative or complementary approach for stably inactivating lncRNA loci (Kopp and Mendell 2018).

Another vital consideration for performing lncRNA LOF experiments is endogenous lncRNA localization. RNA interference (RNAi) is one common strategy for lncRNA LOF and has been historically used to target both cytoplasmic and nuclear lncRNAs. However, the efficacy of RNAi in targeting nuclear lncRNAs (or nuclear subpopulations of lncRNAs) has been called into question (Lennox and Behlke 2016), making the results of some of these lncRNA studies difficult to interpret. Antisense oligonucleotides (ASOs) are a commonly used alternative to RNAi and work to deplete RNA levels by triggering endogenous nuclear RNase H activity, leading to co-transcriptional RNA degradation and potentially premature transcriptional termination (Lai et al. 2020; Lee and Mendell 2020). Thus, depending on ASO design, ASO-mediated knockdown alone can be insufficient to determine whether an observed phenotype is through the loss of a lncRNA transcript or disrupted lncRNA biogenesis (*i.e.* perturbed transcription, splicing).

Nonetheless, ASOs function in the nucleus and thus can be an effective tool for knocking down both nuclear and cytoplasmic lncRNAs.

A third developing technology for lncRNA knockdown is CRISPR-Cas13 RNA targeting, Cas13 being a type VI CRISPR-associated RNA-guided RNase. A number of CRISPR-Cas13 platforms have recently been reported to be both more specific and more effective at knocking down both nuclear and cytoplasmic RNAs than position-matched shRNAs (Abudayyeh et al. 2017; Cox et al. 2017; Konermann et al. 2018). The ability to target nuclear lncRNAs in a genetically encodable system such as CRISPR-Cas13 would be invaluable, as ASOs are transient and thus less-suited for use in longer-term functional assays. However, CRISPR-Cas13 has not been widely adopted as a tool for RNA knockdown yet, likely due to still enigmatic gRNA design rules. Once a better understanding of what constitutes an effective Cas13 guide has been established, CRISPR-Cas13 is certain to greatly facilitate lncRNA characterization efforts.

LncRNA gain-of-function (GOF)

The most commonly employed lncRNA GOF stratagem is transgene overexpression, in which full length or mutant lncRNA sequences are introduced into cells through either stable or transient means. However, it is important to note that such transgene overexpression systems can only be deemed true GOF (*i.e.* informative about the endogenous function of a lncRNA) for lncRNAs that act in *trans*. In fact, rescuing lncRNA LOF through the introduction of a lncRNA transgene is a crucial experiment in proving true *trans* lncRNA functionality. Such

approaches are less appropriate for the study of lncRNA-mediated *cis*-regulation, as *cis*-acting lncRNAs, by definition, exert their functions at their sites of expression. Driving lncRNA expression from elsewhere in the genome would not be expected to positively or negatively affect expression from its endogenous locus. To circumvent this issue, molecular tools to recruit ectopically expressed lncRNAs to their endogenous genomic loci have been developed (Shechner et al. 2015) but have not been as widely employed as means to locally manipulate gene regulation. One such approach is CRISPR-Cas9 based gene activation (CRISPRa) (Gilbert et al. 2013). CRISPRa is suitable for both *trans*- and *cis*-acting lncRNA GOF, as lncRNA upregulation is achieved by increasing the transcriptional activity of the endogenous lncRNA locus.

In summary, efficiently and specifically manipulating the expression of lncRNAs can be technically challenging. For lncRNA LOF in particular, it can be vital to employ multiple, orthogonal strategies in order to elucidate the functional feature(s) of a lncRNA locus. Additionally, the appropriateness of traditional (*i.e.* protein coding gene-oriented) LOF and GOF tools should be judiciously assessed on a lncRNA-by-lncRNA basis. In this work, multiple locus-specific strategies were employed to functionally characterize the many diverse lncRNAs under study. Specific experimental details and rationales can be found in **Chapters 3-5**.

Table 2.1: Survey of genome-wide p53 ChIP-Seq and RNA profiling studies

Ref	RNA profiling	Cell line(s)	Stressor	Category
(Akdemir et al. 2014)	RNA-seq	hESCs	Doxorubicin	Geno
(Catizone et al. 2019)	RNA-seq	IMR90	Etoposide	Geno
(Huarte et al. 2010)	Microarray	MEFs	Doxorubicin	Geno
(Kenzelmann Broz et al. 2013)	RNA-seq	MEFs	Doxorubicin	Geno
(Lee et al. 2010) *	Microarray	mESCs	Doxorubicin	Geno
(Li et al. 2012)	Microarray	mESCs	Doxorubicin	Geno
(McDade et al. 2014)	Microarray	Primary human keratinocytes	Cisplatin	Geno
		Primary human keratinocytes	Doxorubicin	Geno
(Smeenk et al. 2011)	Microarray	U2OS	Actinomycin D	Geno
		U2OS	Etoposide	Geno
(Younger et al. 2015)	RNA-seq	MEFs; Human fibroblasts	Doxorubicin	Geno
(Zhang et al. 2013b) *	Microarray	MEFs; mESCs	Doxorubicin	Geno
(Li et al. 2017)	RNA-seq	HCT116; RKO; SW48	Doxorubicin	Onco; Geno
(Menendez et al. 2013)	Microarray	U2OS	Doxorubicin	Onco; Geno
(Sánchez et al. 2014)	RNA-seq	HCT116	5-FU	Onco; Geno
(Tonelli et al. 2017)	RNA-seq	*Eμ-Myc; Arf*$^{-/-}$ lymphoma	Doxorubicin	Onco; Geno
(Allen et al. 2014)	GRO-seq	HCT116	Nutlin-3	Onco
(Andrysik et al. 2017)	RNA-seq; GRO-seq	HCT116; MCF7; SJSA	Nutlin-3	Onco
(Brady et al. 2011)	Microarray	MEFs	Exogenous *HrasV12*	Onco
(Chaudhary et al. 2017)	Microarray	HCT116; RKO; SW48	Nutlin-3	Onco
(Huarte et al. 2010)	Microarray	*Kras*^{G12D}; *p53*$^{LSL/LSL}$ lung cancer	Endogenous *KrasG12D* (p53 activation)	Onco
(Léveillé et al. 2015)	GRO-seq	MCF7	Nutlin-3	Onco
(Menendez et al. 2013)	Microarray	U2OS	Nutlin-3	Onco
(Nikulenkov et al. 2012)	Microarray	MCF7	Nutlin-3	Onco
(Tonelli et al. 2017)	RNA-seq	*Eμ-Myc; p53ER*$^{TAM/+}$ lymphoma	Endogenous *Eμ-Myc* (p53 activation)	Onco
		Eμ-Myc; Arf$^{-/-}$ lymphoma	Nutlin-3	Onco
(Yang et al. 2018)	Microarray	*p53-Tet-ON* H1299	p53-induction	Onco

*= ChIP followed by microarray analysis employed; all other studies used high throughput sequencing; Geno= genotoxic stress; Onco= oncogenic stress

Chapter 3:

Cis-*regulatory lncRNAs in the p53 response to oncogenic stress*

Part of the work described in this chapter is being prepared as a manuscript for publication as follows: Tesfaye, E., Bendor, J., Martínez-Terroba, E., Zamudio, J., and Dimitrova, N. Core and outcome-specific signatures of p53 restoration in cancer modulated by cis-*regulatory lncRNAs. Co-author contributions that have also been included in this thesis are specified in figure legends and/or text.*

Introduction

The ability of p53 to sense and respond to oncogenic signaling is an important facet of its potent tumor suppressive capabilities (Brady et al. 2011; Kaiser and Attardi 2018). Yet, the p53 response to oncogenic stress is poorly understood, in part due to the dearth of global analyses comparing said response across diverse tumor types (discussed in **Chapter 2**; **Table 2.1**). Especially under-explored are the contributions of p53-activated lncRNAs to how p53 responds to oncogenic signalling across different cancer contexts. Detailed in this chapter is some of our work towards a more comprehensive understanding of the p53 transcriptional response to oncogenic stress and the regulatory roles previously described and novel lncRNAs play in it, achieved by performing a global survey of three different tumor types derived from the same genetically engineered mouse model of cancer.

Primarily discussed here are my main contributions to this project, namely the identification of stressor-specific, lineage-specific, and p53 outcome-specific transcriptional programs across our mouse model of cancer and the characterization of a cohort of newly-identified p53-activated lncRNAs (continued in **Chapters 4** and **5**).

Results

Genome-wide profiling of the p53 response to oncogenic stress

In collaboration with Jesse Zamudio (J. Zamudio; UCLA), a quantitative biologist, we performed genome-wide p53 ChIP-seq and RNA-seq across a panel of lung adenocarcinoma (LA), sarcoma (SA), and lymphoma (LY)-isolated cell lines from the *KPR* mouse model of cancer, which is an established model for studying the p53 pathway (Ventura et al. 2007; Feldser et al. 2010) and described in detail in **Chapter 2**. In brief, in this model, oncogenic stress is derived from an activated $Kras^{G12D}$ allele, and p53 expression is inactivated by the homozygous insertion of a *loxP*-flanked transcriptional STOP cassette into the endogenous p53 locus. Importantly, p53 expression can be restored by the addition of Tam, which removes the STOP cassette, thus allowing the analysis of events downstream of p53 in the presence of oncogenic stress (Fig. 2A).

We utilized a panel of six cell lines derived from three distinct *KPR* tumor types: two LA, two SA, and two LY. CreER-mediated recombination of the p53 locus in these isolated cell lines was confirmed at 24h post Tam treatment by genotyping (Fig. 2B), and restoration of p53 expression was confirmed by qRT-PCR (Fig. 2C). Following p53 restoration, p53 protein is stabilized by the presence

of oncogenic stress, as evidenced by immunoblotting (Fig. 2D) and the activation of transcriptional targets of p53, such as p21 (El-Deiry et al. 1993) (Fig. 2C, D).

In addition to these molecular indicators of p53 restoration, *KPR* cell lines also displayed cellular outcomes consistent with previously described *in vivo* p53 responses. p53 activation has been reported to lead to tumor regression via senescence in murine LA and SA tumors (Feldser et al. 2010; Ventura et al. 2007) and apoptosis in lymphoma (Ventura et al. 2007). Following p53 restoration, we observed induction of apoptosis in LY cells, but not LA and SA cells (Fig. 2D), and growth arrest in LA and SA cells, as expected. Positive staining for β-galactosidase activity at 168 hours post p53 restoration in LA and SA cells was consistent with these cells having underwent oncogene induced senescence (Serrano et al. 1997) (Fig. 2E). Thus, these data established *KPR*-derived cells as a suitable model to investigate oncogenic-stress specific, tumor type-specific, and p53 outcome-specific transcriptional programs.

We first queried where in the genome p53 binding occurred across the three tumor types by performing p53 ChIP-seq in one LA (LA2), one SA (SA1), and one LY (LY1) cell line. In parallel to our p53 ChIP-seq analysis, we analyzed RNA-seq data from the full panel of two LA (LA1 and LA2), two SA (SA1 and SA2) and two LY (LY1 and LY2) cell lines, allowing us to determine how p53 binding events related to transcriptional changes. Both p53 ChIP-seq and RNA-seq experiments were performed at 24 hours following mock treatment or Tam-mediated p53 restoration by Nadya Dimitrova (N. Dimitrova). A schematic of this experimental pipeline can be found in (Fig. 2F). Bioinformatic analyses to identify regions bound by p53 and expressing transcripts differentially regulated in response to p53

restoration were performed by N. Dimitrova and J. Zamudio, yielding a list of peak-associated transcripts that N. Dimitrova and I interrogated further. 4,391 p53-bound peaks were identified across the three tumor types, corresponding to 4,745 individual peak-associated transcripts. Notably, 1,036 peaks did not have any detectable transcripts, consistent with previous reports of p53 frequently associating with intergenic regions, such as enhancers and repetitive elements (Léveillé et al. 2015; Wang et al. 2007). Overlap analysis of both transcript-associated and intergenic peaks was performed by J. Zamudio and revealed that 94% of p53 binding events were tumor type-specific, with only 44 sites (1%) being bound by p53 across all three tumor types, suggesting that p53 binding is perhaps driven more by cell- or tissue-specific factors than stressor type, as has been previously suggested (Tonelli et al. 2017; Catizone et al. 2019; Smeenk et al. 2011; Nikulenkov et al. 2012).

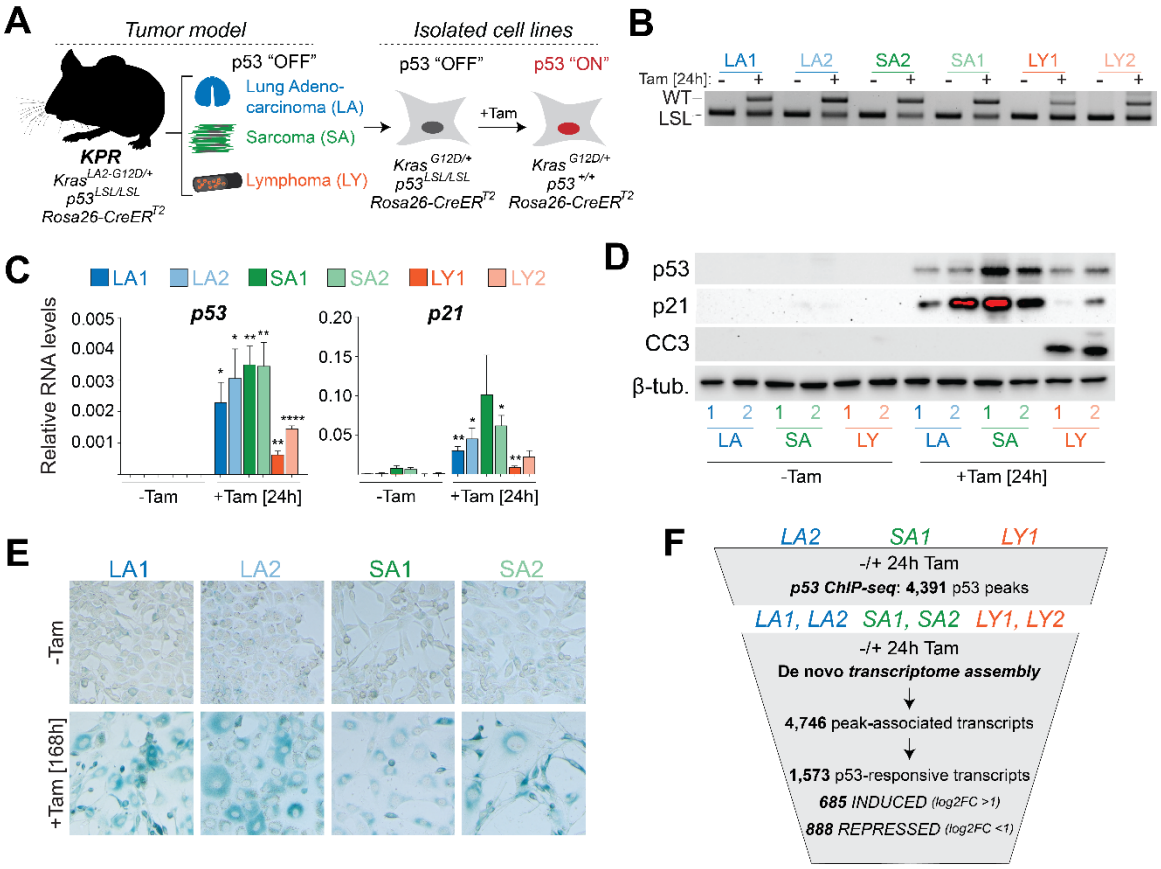


Figure 2: Characterization of the p53 response to oncogenic stress. (A) Schematic of the *Kras*$^{LA2\text{-}G12D/+}$*; p53*$^{LSL/LSL}$*; Rosa26-CreER*^{T2} (*KPR*) mouse model and isolated tumor-derived cell lines. Tam-mediated p53 restoration (p53 "ON") and subsequent p53 stabilization by oncogenic stress is indicated by a red nucleus. Six independent *KPR* cell lines, two per indicated tumor type, were used in this work. **(B)** Genotyping of indicated *KPR* cell lines and treatments. Expected bands: WT (p53 "ON"/restored) = 364 bp; LSL (p53 "OFF"/ unrestored) = 278 bp. **(C)** qRT-PCR analysis of p53 and p21 mRNA relative to endogenous control RNA *Gapdh*. Data show mean ±SEM (n=3 biological replicates). *p <0.05, ** p<0.01, and **** p<0.0001, unpaired t test. **(D)** Immunoblotting for p53, p21, and CC3 protein. β-tubulin served as a loading control. **(E)** Senescence-associated β-galactosidase staining in indicated LA and SA *KPR* cells and treatments. **(F)** Diagram of p53 ChIP-Seq and RNA-seq experiments, including summary of identified p53-bound peaks and *de novo* assembled transcriptome results. p53 ChIP-seq was performed in LA2, SA1, and LY1 cells, whereas RNA-Seq was performed in all *KPR* cell lines. p53-responsive transcripts were called based on log2FC (FDR >0.05).

In (F), original ChIP-seq and RNA-seq experiments were performed by N. Dimitrova. Transcriptome assembly and p53 ChIP-Seq peak-transcript associations were performed by J. Zamudio.

Identification of core and senescence-specific p53 signatures

Of the 4,745 peak-associated transcripts, 1,573 were differentially regulated in response to p53 restoration. 685 genes were upregulated >2-fold while 888 genes were downregulated >2-fold across the three tumor types (Fig. 3A, C). Differentially regulated genes fell into three categories: 1) Tumor type specific (LA, SA, or LY-specific); 2) Outcome-specific (senescence or apoptosis); and 3) Core (common). In order to gain a better understanding of the make-up of the specific p53 transcriptional program(s) enacted in each category, and whether any unique signatures explained the different behaviors of each *KPR* tumor type, we performed gene set enrichment analysis (GSEA) on each category.

Of the transcripts transcriptionally activated by p53, 61 were induced across all three tumor types, including many known p53 targets such as *p21*, *Mdm2,* S*esn2*, and *Bbc3* (*Puma*) (Fig. 3A). Unsurprisingly, GSEA of this core induced set of transcripts revealed significant enrichment for factors previously implicated in the p53 pathway and/or various processes regulated by p53, such as the DNA damage response and apoptosis (Fig. 3B). Moreover, *in silico* motif analysis of the p53 peaks associated with these transcripts performed by J. Zamudio via *p53scan* (Smeenk et al. 2008) revealed an enrichment for the canonical p53 response element (p53RE) (Fig. 3E), further supporting direct regulation by p53. Thus, we were able to identify a core set of genes regulated by p53 in response to oncogenic stress across multiple tumor contexts.

In search for a potential apoptosis signature, we next investigated the transcripts upregulated uniquely in LY cells. However, while we did find enrichment for the p53 pathway itself (Fig. 3A), we found little overlap with

apoptosis gene sets and no enrichment of the p53RE (Fig. 3E), suggesting that other factors beyond transcriptional regulation by p53 may play a more important role in driving apoptosis in LY cells. Similar GSEA analysis of the 51 genes induced in LA and SA but not LY cells did not reveal any significantly enriched datasets, indicating the lack of a senescence-specific p53 activated signature.

In contrast to the well-characterized role of p53 in gene activation, the functional importance of and mechanisms underlying p53-mediated gene repression are less clear (Vousden and Prives 2009; Fischer et al. 2014). By examining the 888 peak-associated transcripts which were downregulated >2-fold, we noticed that a large proportion (35%) of these genes fell within the "senescence-specific" category (*i.e.* repressed only in both LA and SA cells) (Fig. 3C). In contrast, only 3 genes were repressed across all three tumor types, none of which contained p53REs (Fig. 3C, F). These data suggested that p53-mediated transcriptional repression is likely not a dominant feature of the p53 transcriptional network, at least in the context of oncogenic stress, but perhaps is a feature of outcome-specificity, specifically senescence.

Indeed, GSEA of the 312 downregulated senescence-specific genes highlighted the repression of genes involved in the cell cycle, which is consistent with reduced proliferation during senescence (Fig. 3D). In addition, we discovered significant overlaps with E2F and DREAM targets (Fig. 3D), which is in line with a previously proposed model in which repression downstream of p53 is mediated by the p21/E2F/DREAM transcriptional complex (Engeland 2018; Uxa et al. 2019). Only 17% of all p53 repressed genes contained a p53RE, compared to 30% of p53 induced genes (Fig. 3E, F), and this number was not further enriched in

senescence-repressed genes (Fig. 3F). However, as 56 of the 312 senescence-repressed genes (18%) did contain recognizable p53REs, it remains possible that p53 binding may still be important for repression at some loci, whether through contributing to the localization of E2F/DREAM components or through other mechanisms.

In summary, these data indicated that senescence, but not apoptosis or the core p53 response to oncogenic stress, is distinctly characterized by pervasive p53-mediated repression. Moreover, senescence-specific transcriptional repression may possibly be mediated through a repressive partner, such as the p21/E2F/DREAM complex.

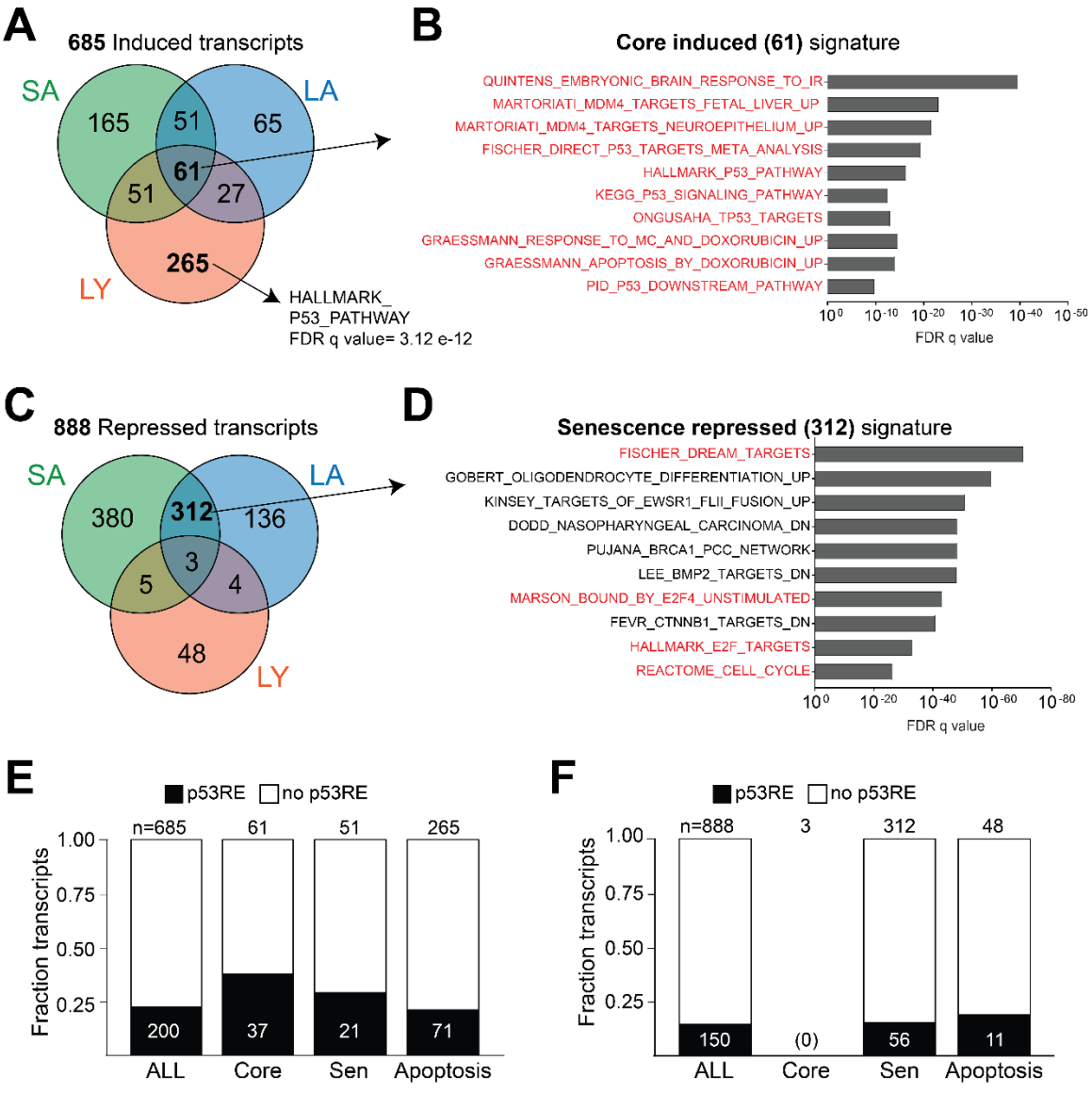


Figure 3: Pathway analysis of p53-responsive transcriptome reveals core and outcome-specific p53 signatures. (A) Venn diagram showing distribution of p53-induced (log2FC>1) transcripts across indicated *KPR* tumor types. Arrows designate results of GSEA of indicated gene category, with FDR q value shown. Highlighted gene categories: Core= LA, SA, and LY. Senescence= LA and SA. Apoptosis= LY. **(B)** GSEA of Core p53-induced category, using the H and C2 collections of MSigDB. Top ten enriched gene sets ranked by q values were plotted. Gene sets related to the p53 pathway are highlighted in red. **(C)** Venn diagram showing distribution of p53-repressed (log2FC<1) transcripts across indicated *KPR* tumor types. **(D)** GSEA of Senescence p53-repressed category, as described in **(B)**. Gene sets related to cell cycle regulation are highlighted in red. **(E-F)** p53-responsive transcript distributions across indicated gene categories in relation to p53RE status as determined by *p53scan* on transcript-associated p53 peaks. **(E)** p53-induced transcripts **(F)** p53-repressed transcripts.

In (E) and (F), *p53scan* motif analysis was performed by J. Zamudio on all Induced and Repressed transcripts.

Many lncRNAs are activated by oncogenic stress following p53 restoration

We next focused on the identification and characterization of lncRNAs activated by p53 in our *KPR* model, wondering whether lncRNAs played an important role in the p53 response to oncogenic stress. Among the genes that were induced by p53 in *KPR* cells more than 2-fold, 21 lncRNAs were identified (described in **Table 3.1**). Interestingly, barring three transcripts which had previously been characterized as p53 targets, [*lincRNA-p21* (Dimitrova et al. 2014; Huarte et al. 2010); *Neat1* (Adriaens et al. 2016; Mello et al. 2017), and *Pvt1* isoform *Pvt1b* (Olivero et al. 2020)], the remaining lncRNAs were either novel or not previously associated with the p53 pathway. These novel lncRNAs were named as follows: antisense (as) according to genes overlapped in the antisense orientation, sense (s) respective to overlaps in the sense orientation, or long intergenic noncoding RNAs (lincRNAs) according to the nearest neighboring protein coding gene. Three lncRNAs, *2310008N11Rik* (Zfp703 >150kb), *Gm10801* (Lrrc4c >1Mb), and *Gm10721* (Alkbh8 >300kb) were located in gene deserts and were thus simply referred to by their MGI identifiers, unless otherwise noted. Of the 21 p53-induced lncRNAs, 13 were further identified by *p53Scan* to contain associated p53REs, providing a basis for direct regulation by p53 (**Table 3.1**).

To confirm that these RNAs were truly noncoding, protein coding potential was assessed using the Coding Potential Assessment Tool (CPAT), which is a commonly employed, alignment-free algorithm for assessing the probability of a given nucleotide sequence containing a translated ORF (Wang et al. 2013). As expected, previously described lncRNAs *lincRNA-p21, Airn, Neat1, Malat1,* and

Pvt1 all had CPAT scores significantly lower than the protein-coding cut-off assigned to the mouse transcriptome (CPAT score >0.440), suggesting a lack of coding potential. Of the novel transcripts, all but one (*Galnt2l-s*) were similarly below the coding threshold, suggesting that these transcripts represent *bona fide* lncRNAs (**Table 3.1**).

Table 3.1: LncRNAs activated by p53 during the oncogenic stress response

#	Name	Class	*p53Scan*	CPAT score	Coding label
1	**lincRNA-p21**	Intergenic	YES	0.246	NO
2	**Zmat3-as**	A.S overlap	YES	0.176	NO
3	**Bahcc1-as**	A.S overlap	YES	0.229	NO
4	**lincRNA-Spag9**	Intergenic	YES	0.046	NO
5	**lincRNA-Gadd45γ**	Intergenic	YES	0.243	NO
6	Airn	A.S overlap		0.243	NO
7	**2310008N11Rik (lncRNA 7)**	Intergenic	YES	0.044	NO
8	lincRNA-Klhdc4	Intergenic	YES	0.995	Yes
9	**Neat1**	Intergenic		0.087	NO
10	**Malat1**	Intergenic		0.056	NO
11	**Pvt1**	Intergenic	YES	0.119	NO
12	Gm10801	Intergenic		0.064	NO
13	lincRNA-Zfp945	Intergenic		0.122	NO
14	Adamts9-as	A.S overlap	YES	0.124	NO
15	**Rhod-as**	A.S overlap	YES	0.304	NO
16	**Sesn2-s**	S. overlap	YES	0.050	NO
17	**Bfsp2-as**	A.S overlap	YES	0.052	NO
18	Anks1-as	A.S overlap		0.213	NO
19	Kdm4c-as	A.S overlap		0.005	NO
20	Gm10721	Intergenic		0.065	NO
21	**Ltc4s-as**	A.S overlap	YES	0.042	NO

Bold= lncRNAs selected for further characterization

Thirteen lncRNAs were selected for initial characterization experiments (bolded in **Table 3.1**). Criteria for selection included likelihood of direct regulation by p53, previous implication in the p53 pathway or other cancer-associated processes, and likely overall abundance as initially determined in our RNA-seq

data. p53-dependent induction of these select lncRNAs across the panel of *KPR* cancer cell lines was validated by qRT-PCR. In agreement with our RNA-seq analysis, 10/13 lncRNAs were induced >2-fold upon 24h Tam treatment in at least three of the six cell lines, barring *Neat1*, *Malat1*, and *Ltc4s-as*. 12/13 lncRNAs met this threshold of induction in at least one *KPR* cell line. Interestingly, we were unable to confirm p53-dependent induction of *Neat1* at this early time point, implying a lack of direct regulation by p53 in contrast to previous studies (Fig. 4A). When a later time point (72h Tam) was examined, all 13 lncRNAs met this >2-fold threshold of induction, suggesting p53-regulated lncRNAs may be induced with different transcriptional dynamics (Fig. 4A). Of note, five lncRNAs (*Pvt1b, Zmat3-as, Bahcc1-as, 2310008N11Rik*, and *Rhod-as*) were induced across all six *KPR* cell lines at both early (24h) and late (72h) time points, thus potentially encompassing a core set of oncogenic stress-specific, p53-activated lncRNAs.

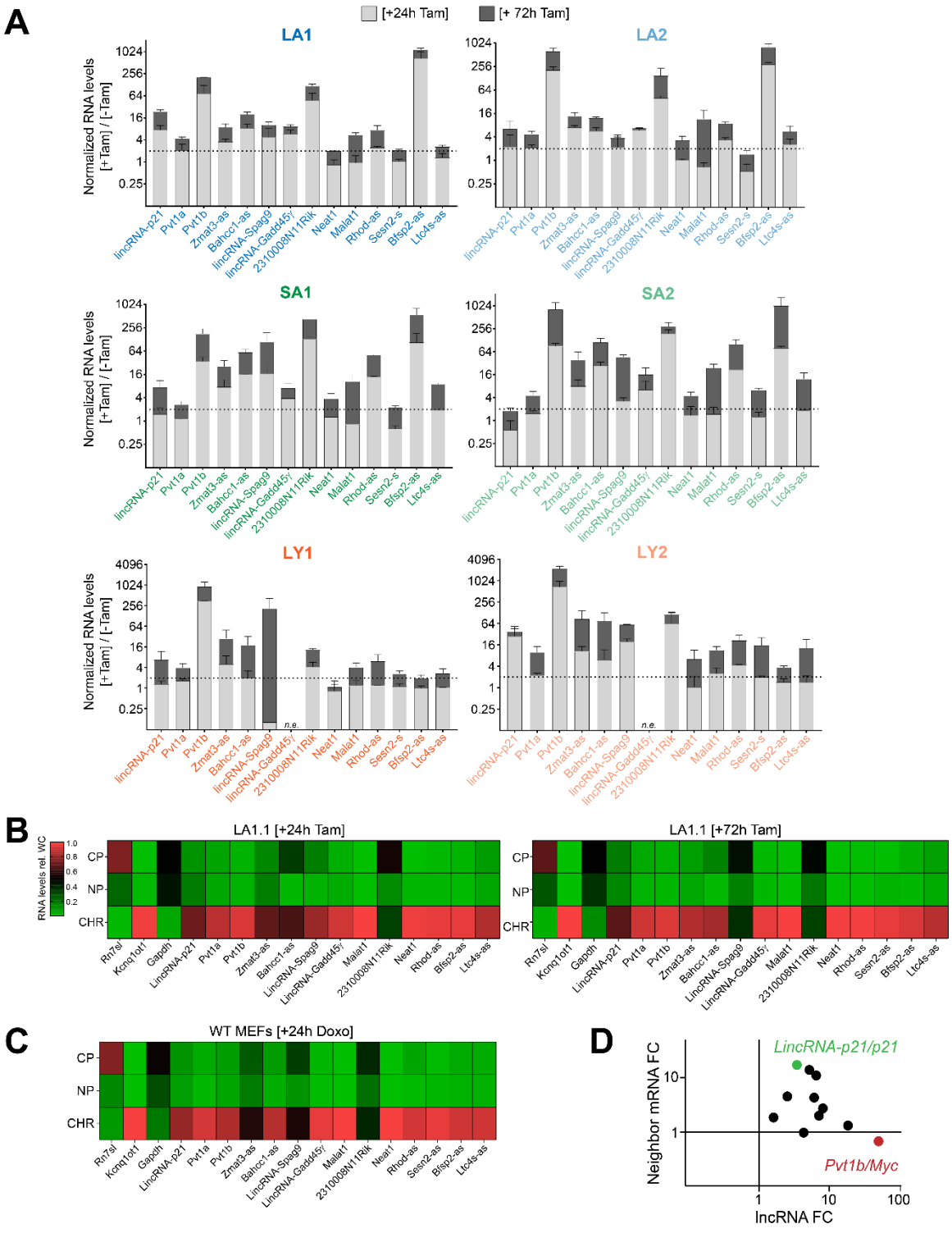
p53-activated lncRNAs are nuclear-enriched and co-regulated with their nearest neighboring mRNAs

We next sought to determine the subcellular localization patterns of the selected lncRNAs using subcellular fractionation followed by qRT-PCR, assessing RNA levels in the cytoplasm (CP), nucleoplasm (NP), and at the chromatin interface (CHR) in an LA1-derived clonal cell line (LA1.1). As anticipated, control transcript *Kcnq1ot1* was enriched in the chromatin fraction, whereas control transcripts *Rn7sl* and *Gapdh* were enriched in the cytoplasm (Fig. 4B). Furthermore, lncRNAs previously reported to be enriched in the nucleus (*lincRNA-p21, Pvt1, Neat1,* and *Malat1*) were similarly distributed in *KPR* cells as

well (Fig. 4B). Intriguingly, we determined that this nuclear-enrichment was common to all tested p53-activated lncRNAs, with most enriched specifically at the chromatin. These general patterns of subcellular localization also held true in wild type MEFs treated with Doxo, demonstrating that these subcellular localization patterns were conserved in the p53 response to genotoxic stress as well (Fig. 4C).

Curious about the implications of this pervasive chromatin-enrichment, we next assessed the p53-responsiveness of each selected lncRNA's overlapping and/or nearest neighboring protein coding genes, wondering whether lncRNA localization reflected lncRNA-mediated local gene regulation. As observed in the parental *KPR* cells, p53 restoration also induced lncRNA expression in LA1.1 cells (Fig. 4D). Strikingly, we found that most lncRNA-associated protein coding genes were also upregulated in response to p53 (Fig. 4D). These data suggested that lncRNAs that are activated by p53 in response to oncogenic stress are frequently transcriptionally co-regulated with nearby protein-coding genes. Notably, this correlation was positive for the majority of lncRNA-mRNA pairs investigated, with the only exception being *Pvt1b*, which showed a negative correlation with its upstream neighbor, *Myc*.

Figure 4: LncRNAs activated by p53 in response to oncogenic stress are chromatin-enriched and positively correlated with neighboring mRNAs. (A) qRT-PCR analysis of lncRNA levels relative to *Gapdh* in indicated *KPR* cell lines treated with 24h or 72h Tam, normalized to untreated cells. n.e.= not expressed. Data show mean ± SD (n=2 biological replicates). **(B-C)** Heat map representation of qRT-PCR analysis after subcellular fractionation. *Rn7sl* and *Kcnq1ot1* served as fractionation controls for cytoplasmic and chromatin-associated fractions, respectively. Also shown is Gapdh mRNA, as an additional control. CP= cytoplasmic. NP= nucleoplasmic. CHR= chromatin-associated. **(B)** LA1.1 cells treated with 24h (left panel) or 72h (right panel) Tam. **(C)** Primary MEFs treated with 24h 0.5 μM Doxo. **(D)** Correlation plots of qRT-PCR analysis of RNA induction upon 24h Tam treatment in LA1.1 cells. Represented are lncRNA and corresponding neighboring and/or overlapping mRNA levels relative to *Gapdh*, normalized to untreated LA1.1 cells. *LincRNA-p21* plotted against *p21* and *Pvt1b* plotted against *Myc* are highlighted in green and red, respectively. Data show mean values (n=3 biological replicates).


A
[+24h Tam]
[+ 72h Tam]
LA1
LA2
SA1
SA2
LY1
LY2
Normalized RNA levels [+Tam] / [-Tam]
lincRNA-p21
Pvt1a
Pvt1b
Zmat3-as
Bahcc1-as
lincRNA-Spag9
lincRNA-Gadd45γ
2310008N11Rik
Neat1
Malat1
Rhod-as
Sesn2-s
Bfsp2-as
Ltc4s-as
n.e.
B
LA1.1 [+24h Tam]
LA1.1 [+72h Tam]
RNA levels rel. WC
CP
NP
CHR
Rn7sl
Kcnq1ot1
Gapdh
C
WT MEFs [+24h Doxo]
D
LincRNA-p21/p21
Pvt1b/Myc
Neighbor mRNA FC
lncRNA FC

To further examine the relationship between p53-activated lncRNAs and their neighboring mRNAs, we next sought to establish a genetic lncRNA LOF strategy. We turned to CRISPR-Cas9 mutagenesis of lncRNA-associated p53REs, reasoning that as p53 is a sequence-specific transcription factor, genetic disruptions of the DNA element recognized by p53 should specifically abrogate p53-dependent lncRNA induction. Of the 13 lncRNAs selected for validation, 11 (excluding *Neat1* and *Malat1*) had p53REs identifiable by *p53scan* (**Tables 3.1, 3.2**). Most lncRNA-associated p53REs were located at or near promoter regions, with 8 out of 11 (73%) p53REs being found either upstream of a lncRNA's TSS or within the lncRNA gene's first exon or first intron, similar to what has been observed for protein coding p53 targets (**Table 3.2**) (Riley et al. 2008).

To test the feasibility of our p53RE mutagenesis approach, we selected 7 lncRNAs for which we were able to design gRNAs that were highly specific (specificity score by CRISPOR (Haeussler et al. 2016) >70/100) and predicted to direct Cas9 cleavage within or adjacent to a p53RE sequence. Introduction of Cas9 and lncRNA p53RE-targeting (ΔRE) gRNAs into a clonal LA2-derived cell line, LA2.5 (cell lines generated by J. Bendor), abrogated the Tam-induced upregulation of 5 of the 7 targeted lncRNAs, confirming direct transcriptional regulation by p53 (Fig. 5A). Two lncRNAs, *2310008N11Rik* and *Rhod-as*, were not affected by this approach. This was likely due to the inefficient disruption of their p53REs by the designed gRNAs, as was revealed by Sanger sequencing individual alleles from populations of cells expressing Cas9 and non-targeting (Con) or ΔRE gRNAs (Fig. 5B).

Table 3.2: Descriptions of lncRNA-associated p53 response elements (p53REs)

#	LncRNA	p53RE Location	p53RE Sequence	Suitable PAM?
1	lincRNA-p21	Exon 1	AGGCAAGCCCGGGCAAGgCC	YES
2	Zmat3-as	Promoter	AGACATGCCTGGGCTTGTTT	YES
3	Bahcc1-as	Down-stream	GAACAAGCaT (GTAGACAGA) GcACATGTTC	No
4	lincRNA-Spag9	Intron 1	GGACTTGTTgGGACATGTCC	YES
5	lincRNA-Gadd45γ	Exon 3	AGGCATGCCT (G) tGACATGCCT	YES
7	2310008N11Rik (lncRNA 7)	Exon 1	AGACATGTCTGAGCAAGCCT	*No
11	Pvt1	Promoter	tGACAAGTTTGGGCTTGTTC	YES
15	Rhod-as	Intron 1	GAGCTAGCTC(TG)GcACATGgCa(GA) tGtCATGaaT	*No
16	Sesn2-s	Promoter	GGAtTTGCCT (GGACTTGAG) AcGCTcGCTT	No
17	Bfsp2-as	Intron 5	GGACATGTAA (CGAAGGGCCTGCCC) GGGCATGTCC	†No
21	Ltc4s-as	Intron 1	GGACTTGCCTGGACTTGCTC	YES

P53RE consensus sequence= RRRCWWGYYY(n)RRRCWWGYY; n=0-15 nt
*Inefficient disruption of p53RE sequence experimentally determined (Fig. 5A)
†Prohibitively low gRNA specificity score

To assess the consequences of this p53RE mutagenesis on local gene expression and broader p53 pathway function, we turned to our LA1.1 cells, which are a better characterized model for LA function within the lab. As a positive control, we added *lincRNA-p21* to our list of targeted p53-regulated lncRNA loci, and focused on lncRNA loci in which we were able to achieve efficient mutagenesis as determined by TIDE (Tracking of Indels by Decomposition) (Brinkman et al. 2014) analysis of sequenced Con and ΔRE LA1.1 populations (Fig. 5C). Similar to what was observed in LA2.5 cells, lncRNA ΔRE-expressing LA1.1 cells exhibited significantly less lncRNA induction upon Tam-treatment compared to cells expressing control gRNAs (Fig. 5C). As expected, the downregulation of *lincRNA-p21* (73% knockdown) in cells expressing gRNA ΔRE-*lincRNA-p21* was accompanied by a corresponding 24% decrease in *p21* levels compared to control

cells, whereas *Pvt1b* downregulation (81%) in Tam-treated ΔRE-*Pvt1b* cells led to a 58% increase in *Myc* expression, confirming previously reported regulatory relationships at these loci (Dimitrova et al. 2014; Olivero et al. 2020).

Intriguingly, we discovered that mutagenesis of the p53REs associated with three previously undescribed lncRNAs, *Zmat3-as*, *lincRNA-Gadd45γ,* and *Ltc4s-as*, also resulted in reduced expression of their respective neighboring and/or overlapping genes, compared to control cells (Fig. 5C). Of note, for *Ltc4s-as*, which is transcribed antisense to two protein-coding genes (*Ltc4s* and *Maml1*), only *Ltc4s* was significantly reduced in ΔRE-*Ltc4s-as*-expressing cells, while *Maml1* expression was not significantly affected, suggesting that coregulation by p53 at this complex locus is tightly regulated. The regulatory relationship between *Ltc4s-as* and its overlapping neighbors is further explored in **Chapter 5**.

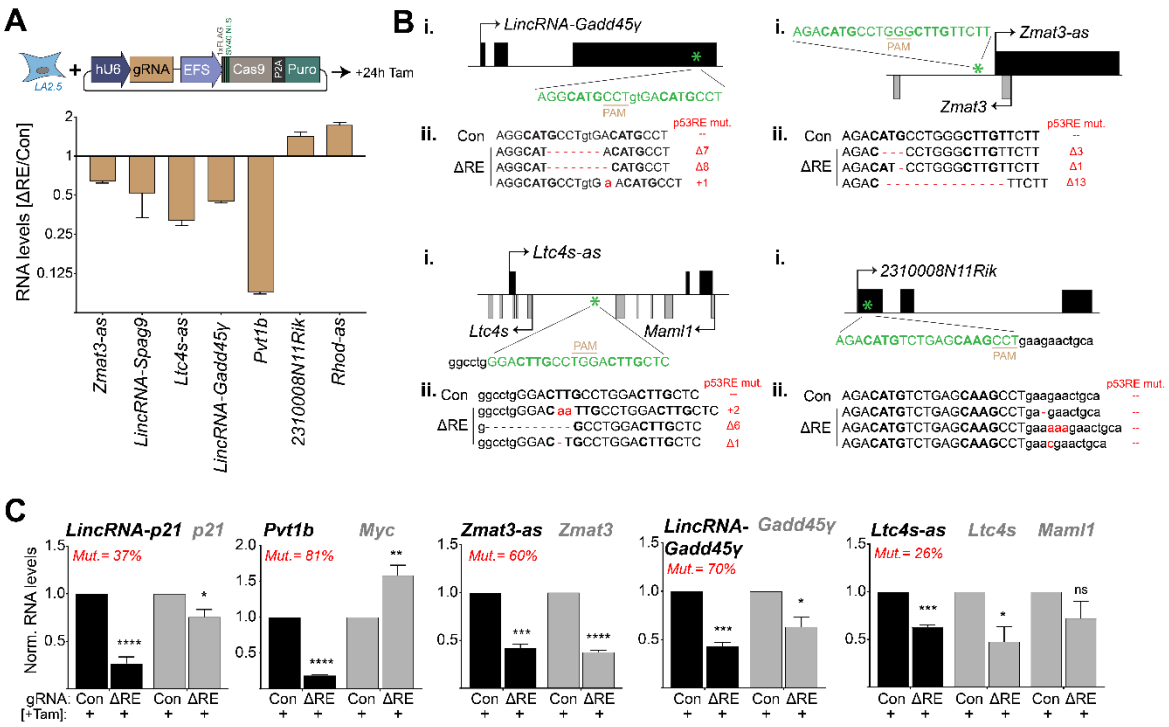


Figure 5: LncRNA p53 response element (p53RE) mutagenesis reveals co-regulatory relationships. (A) Top- schematic of pilot CRISPR-Cas9 p53RE mutagenesis experiment. Vector shown= BRD001 (see supplementary table 1). Bottom- qRT-PCR of indicated lncRNA levels in LA2.5 cells expressing corresponding ΔRE-targeting gRNAs. Data shown as log2FC normalized to LA2.5 cells expressing non-targeting (Con) gRNA ±SD (n=2 biological replicates). **(B) i.** Schematics for indicated lncRNA/mRNA genomic loci and p53RE mutagenesis strategies. Green= p53RE location (asterisks), p53RE sequence (text). Brown= ΔRE gRNA targeted PAM. **ii.** Representative control and ΔRE mutated alleles identified by Sanger sequencing. Red= characterization of p53RE indels. **(C)** qRT-PCR detection of indicated lncRNAs and associated mRNAs in indicated LA1.1 cells and treatments, normalized to cells expressing control gRNAs. TIDE mutagenesis efficiencies (Mut.) are noted in red text. Data show mean ±SEM (n=3-5 biological replicates). *p <0.05, ** p<0.01, ***p<0.001, and **** p<0.0001, ns= not significant, unpaired t test.

Novel p53 target *lincRNA-Gadd45γ* positively regulates *Gadd45γ* in *cis*

Given the role of *lincRNA-p21* in promoting the expression of *p21* and the role of *Pvt1b* in transcriptionally repressing *Myc*, we next wondered whether *Zmat3-as*, *lincRNA-Gadd45γ,* and *Ltc4s-as* might also influence the expression of their neighboring genes. Given the overlapping genomic contexts of *Zmat3-as* and *Ltc4s-as*, making functional dissociation of the lncRNA and mRNA transcripts more challenging, we initially chose to focus on *lincRNA-Gadd45γ,* which represents a discrete transcriptional unit located 125k b downstream of *Gadd45γ (Growth arrest and DNA damage-inducible 45 gamma)*. Furthermore, *Gadd45γ* is a poorly characterized member of a family of proteins that are involved in cell cycle regulation and survival, some through the p53 pathway (E. Tamura et al. 2012), though *Gadd45γ* is not thought to be directly regulated by p53 itself. Thus, multiple features of the *lincRNA-Gadd45γ/Gadd45γ* locus made it an attractive candidate for further study.

We next explored the possibility that *lincRNA-Gadd45γ* might play a functional role in promoting the expression of *Gadd45γ,* analogous to *lincRNA-p21* and *p21*. To determine whether loss of p53 binding at the lncRNA locus or loss of the lncRNA transcript itself contributed to the effects on *Gadd45γ* regulation observed in ΔRE- *lincRNA-Gadd45γ* cells, we turned to CRISPR-based epigenetic modulation of the *lincRNA-Gadd45γ* locus. First, we inhibited *lincRNA-Gadd45γ* expression via transcriptional interference by targeting Cas9 downstream of its TSS using a 12-nt dead RNA (dRNA), which allows the recruitment of catalytically active Cas9 without supporting nuclease activity (Fig. 6A-top) (Dahlman et al. 2015). Importantly, this approach allowed us to abrogate *lincRNA-Gadd45γ*

expression in a manner removed from p53-dependent regulation at this locus. We observed a 50% decrease in *lincRNA-Gadd45γ* levels and a corresponding 30% decrease in *Gadd45γ* expression in Tam-treated LA1.1 cells expressing targeting dRNAs compared to controls (Fig. 6A-bottom). Thus, these data demonstrated that some aspect of *lincRNA-Gadd45γ* biogenesis or RNA positively regulates *lincRNA-Gadd45γ* expression in the p53-dependent response to oncogenic stress.

In the converse experiment, we used the CRISPR-Synergistic Activation Mediator (SAM) system to induce the expression of *lincRNA-Gadd45γ* in either the absence or presence of p53. In this CRISPRa platform, catalytically active Cas9 is recruited to a site upstream of the TSS using a dRNA fused to MS2 repeats, which in turn serve to recruit a complex of transcriptional activators fused to the MS2-binding protein (MBP-HSF1-p65)(Fig. 6B-top) (Dahlman et al. 2015; Olivero et al. 2020). In an experiment performed by E. Martínez-Terroba (who also developed the molecular reagents used in this specific CRISPRa platform), CRISPRa of *lincRNA-Gadd45γ* led to a 28-fold and 26-fold upregulation of *lincRNA-Gadd45γ* over control dRNAs in the absence and presence of p53, respectively. Importantly, p53-independent activation of *lincRNA-Gadd45γ* via CRISPR-SAM was marked by a 9-fold increase in *Gadd45γ* levels as well, further suggesting that transcription from the lncRNA locus is sufficient to promote *Gadd45γ* expression (Fig. 6B-bottom).

As a third orthogonal approach to depleting *lincRNA-Gadd45γ* levels, we employed antisense oligonucleotides (ASOs), which decrease target RNA levels through both RNase-H-dependent and transcription-based mechanisms (Lai et al. 2020; Lee and Mendell 2020). Two independent *lincRNA-Gadd45γ* targeting

ASOs were designed and introduced into LA1.1 cells and RNA levels compared to a non-targeting control ASO (Con) (Fig. 6C-top). Interestingly, while both ASO1 and ASO2 led to a significant decrease in *lincRNA-Gadd45γ* expression in Tam-treated LA1.1 cells (96% and 93% knockdown, respectively), only ASO2 led to a corresponding 47% decrease in *Gadd45γ* levels (Fig. 6C-bottom). Curious as to whether this discrepancy stemmed from each ASO targeting a different complement of *lincRNA-Gadd45γ* isoforms, we designed an additional two qPCR primers against *lincRNA-Gadd45γ*. While primer pairs (1) and (2) revealed comparable levels of *lincRNA-Gadd45γ* knockdown for both ASO1 and ASO2, primer pair (3) showed that solely ASO2 yielded a significant 26% decrease in *lincRNA-Gadd45γ,* paralleling the effect observed on *Gadd45γ* (Fig. 6C-bottom). These data are suggestive of the presence of alternate lncRNA isoforms that differentially contribute to *Gadd45γ* regulation.

Taken together, these data indicate that the production of *lincRNA-Gadd45γ* is both necessary and sufficient to modulate *Gadd45γ* expression, analogous to other *cis*-regulatory lncRNAs, and expands the number of known *cis*-regulatory lncRNAs within the p53 network.

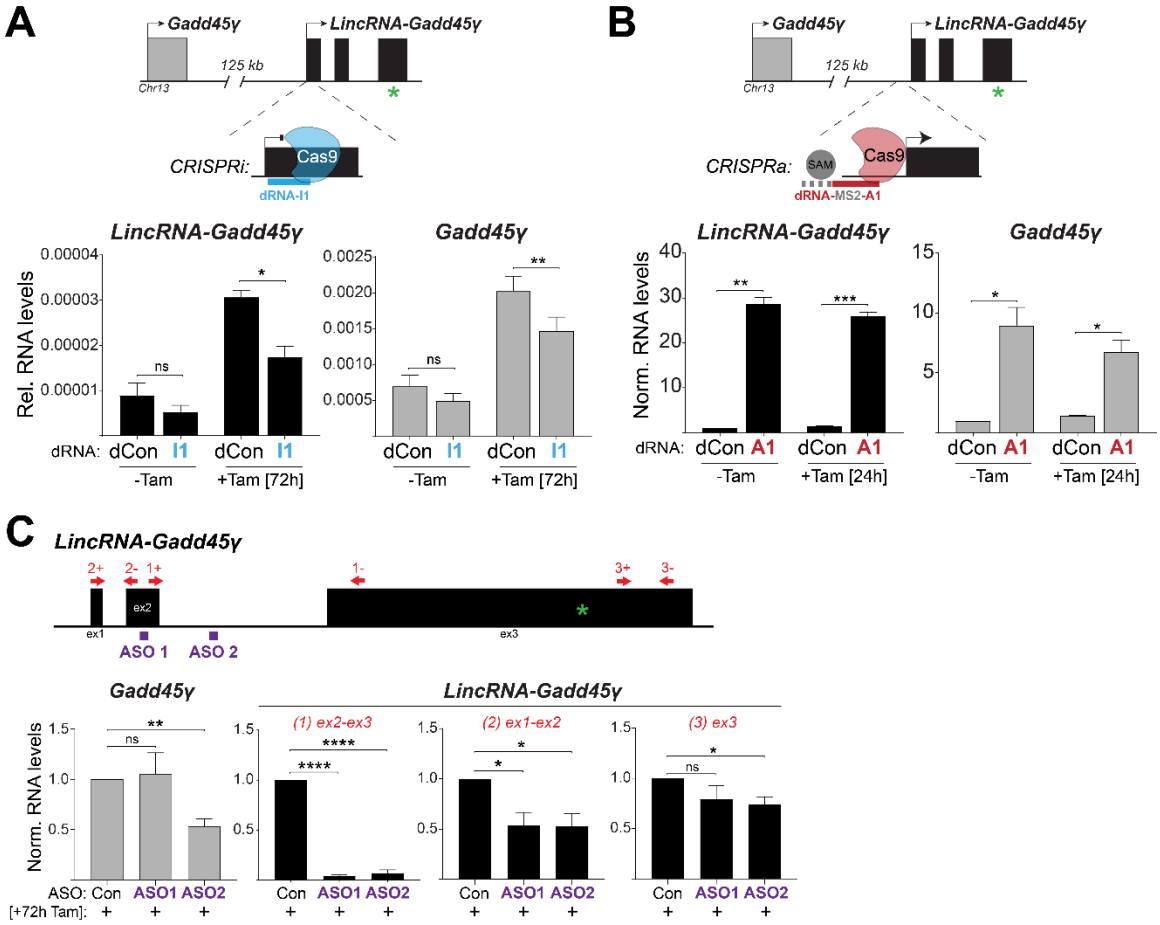


Figure 6: *LincRNA-Gadd45γ* positively regulates *Gadd45γ* in *cis*. (A) Top- Design of *lincRNA-Gadd45γ* CRISPRi experiment. Bottom- qRT-PCR analysis of *lincRNA-Gadd45γ* and *Gadd45γ* levels relative to *Gapdh* in indicated LA1.1 cell lines and treatments. **(B)** Top- Design of *lincRNA-Gadd45γ* CRISPRa experiment. Bottom- qRT-PCR analysis of normalized *lincRNA-Gadd45γ* and *Gadd45γ* levels relative to *Gapdh* in indicated LA1.2 cell lines and treatments. **(C)** Top- Diagram of *lincRNA-Gadd45γ* locus, indicating locations of ASOs (2) and qPCR primer pairs (3). Bottom- qRT-PCR detection of *Gadd45γ* and indicated *lincRNA-Gadd45γ* amplicons in LA1.1 cells treated with indicated ASOs and Tam. Green asterisks indicate p53RE. Data show mean ±SEM (n=3 biological replicates). *p <0.05, ** p<0.01, ***p<0.001, and **** p<0.0001, ns= not significant, unpaired t test.

In (B), CRISPRa was performed by E. Martínez-Terroba.

Pvt1b-Myc co-regulation is necessary for p53's anti-proliferative response to oncogenic stress

Lastly, we examined whether genetic inhibition of the p53-activated lncRNAs *lincRNA-p21*, *Pvt1b*, *Zmat3-as*, *lincRNA-Gadd45γ,* and *Ltc4s-as* affected the antiproliferative outcome observed upon p53 activation in LA cell lines. To assess proliferation, we performed growth curve, BrdU incorporation, and colony formation assays with control, ΔRE-*lincRNA-p21*, ΔRE-*Pvt1b*, ΔRE-*Zmat3-as*, ΔRE-*lincRNA-Gadd45γ,* and ΔRE-*Ltc4s-as* cells. As expected, control cells underwent permanent cell cycle arrest following Tam-mediated p53 restoration and failed to grow or form colonies (Fig. 7A-D). We found that ΔRE-*Pvt1b* cells overcame in part the permanent growth arrest and formed colonies (Fig. 7A-D). In contrast, inhibition of either *Zmat3-as*, *lincRNA-Gadd45γ,* or *Ltc4s-as* did not significantly affect cellular proliferation and/or clonogenicity (Fig. 7A-D). While ΔRE-*lincRNA-p21* cells did exhibit a slight growth advantage at earlier time points (Fig. 7B), likely reflecting the decreased levels of G1/S check point mediator *p21* in these cells, this growth advantage was lost over time and ultimately did not contribute to defects in sustained growth arrest or increased colony formation capability.

We also assessed the effects of lncRNA inhibition on apoptosis, which is not the normal p53 response initiated in LA cells but could theoretically be triggered by the loss of key p53 targets important for driving LA-specific p53 cell fate decisions. As expected, p53 restoration did not promote apoptosis or decrease viability in control cells, in contrast to positive control cells additionally treated with acute DNA damage (Fig. 7E, F). This resistance to cell death in response to

oncogenic stress was unaffected by the inhibition of *lincRNA-p21*, *Pvt1b*, *Zmat3-as*, or *lincRNA-Gadd45γ* (Fig. 7E, F).

Thus, these data demonstrate that, barring *Pvt1b*, loss of no individual p53-activated lncRNA was sufficient to bypass oncogenic stress-mediated arrest, and that furthermore, lncRNAs are unlikely to significantly contribute to p53 cell fate decision making in LA cells.

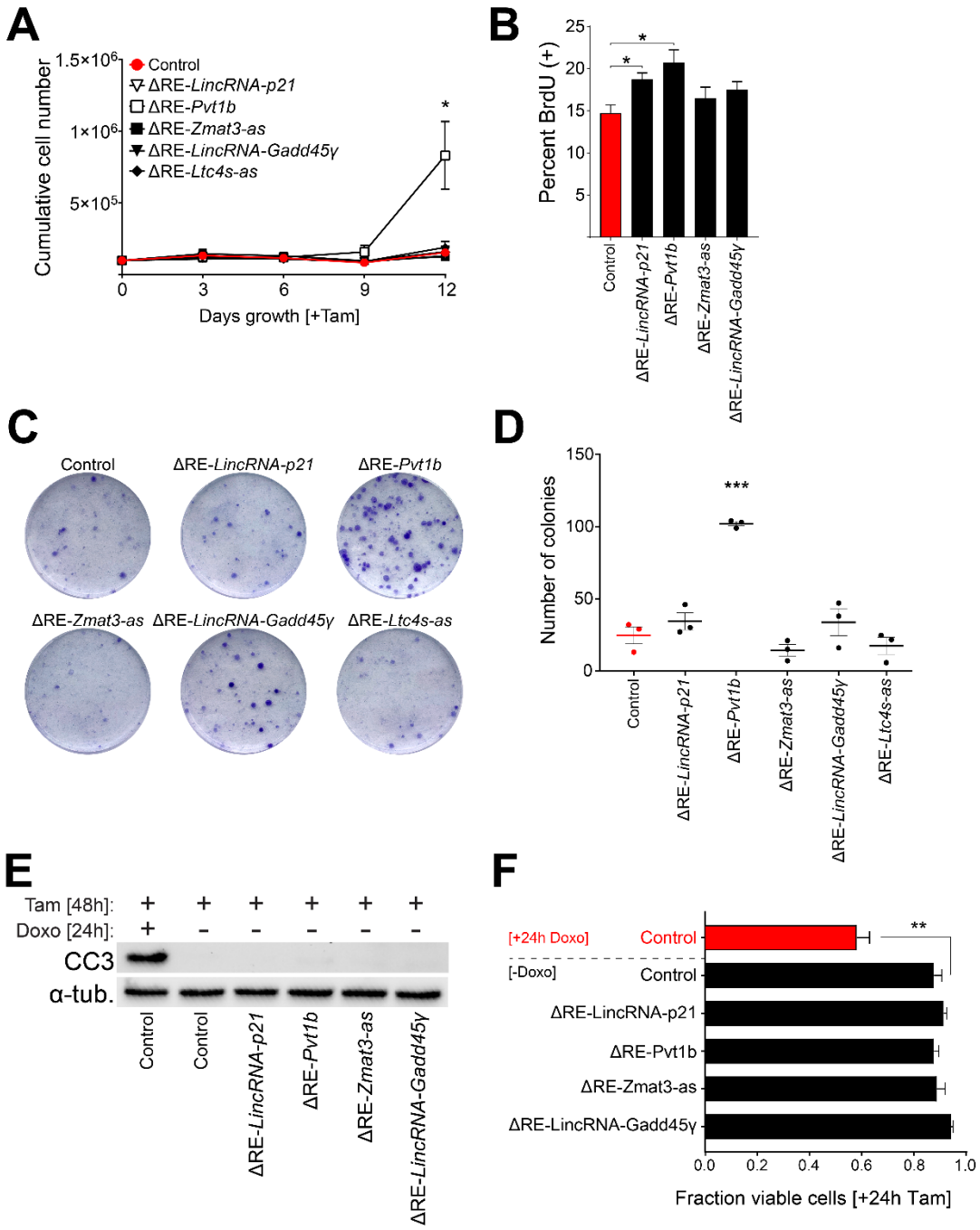


Figure 7: Contributions of lncRNA-mRNA co-regulation to p53's antiproliferative response to oncogenic stress. (A) Cumulative cell numbers of Tam-treated control (indicated in red) or lncRNA ΔRE gRNA-expressing LA1.1 cells over time. **(B)** BrdU incorporation in LA1.1 cells expressing indicated gRNAs following 24h Tam and 2h BrdU treatment. Cells were scored for BrdU (FITC) incorporation by immunofluorescence and total number BrdU positive nuclei plotted over total scored nuclei per cell line. **(C)** Representative images of colony formation assays in Tam-treated LA1.1 cells expressing indicated gRNAs. **(D)** Quantification of **(C)**. **(E)** Immunoblotting for CC3 in LA1.1 cells expressing indicated gRNAs and indicated treatments (Doxo= 1 μM). **(F)** Viability assayed by Trypan blue exclusion in LA1.1 cells expressing indicated gRNAs and treatments (Doxo= 1 μM). Data in **(A-D, F)** represent mean values ±SEM (n=3-4 biological replicates). *p <0.05, ** p<0.01, and ***p<0.001, unpaired t test.

Data summary and conclusions

Collectively, these data represent a comprehensive investigation of the p53 response to oncogenic signaling and the gene networks that underlie common, tumor type-specific, and senescence- or apoptosis- specific p53 responses to this stress. Importantly, we uncovered a small core set of genes, including several novel lncRNAs, that were directly activated by p53 in all *KPR* tumor types, providing an important resource for future studies into the universal effects of p53 re-activation in cancer settings.

Our data refutes the hypothesis that p53 decision-making is driven by the activation of unique, outcome-specific transcriptional networks, as we did not find any obvious senescence-specific of apoptosis-specific programs. These observations were initially surprising, given the striking, apparently stressor-agnostic outcome-specificity of our *KPR* tumor-derived cell lines. However, this conclusion is consistent with an emerging model of p53 pathway function in which p53 is thought to activate a multifunctional and likely redundant core network, regardless of biological context (Andrysik et al. 2017; Verfaillie et al. 2016). In this model, context-specific p53 cellular responses would arise from cell- or stressor-specific "interpretations" of this core p53 network, which could be p53-independent. Interestingly, we did reveal a previously unappreciated connection between p53-mediated gene repression and oncogenic stress-induced senescence, but not apoptosis. The mechanism and importance of this repression remains to be explored further.

This work also greatly expands the known catalogue of p53-activated lncRNAs, linking 16 previously undescribed lncRNAs to the p53 transcriptional

network, and specifically, the p53 response to oncogenic stress. Interestingly, a number of previously described lncRNAs induced by p53 in response to genotoxic stress (see **Table 1.1**), were not found in our work, suggesting significant species-, cell line- or stress-dependent variability in the cohort of lncRNAs activated by p53. We found that most p53-activated lncRNAs remain at the chromatin, presumably at their sites of transcription (Werner and Ruthenburg 2015), and that many are co-regulated with their neighboring genes. These localization and regulatory patterns lead us to suppose that the primary role of p53-activated lncRNAs is *cis*-regulation, as exemplified by *lincRNA-p21*, *Pvt1b*, and two newly characterized lncRNAs: *lincRNA-Gadd45γ* (this chapter) and *Ltc4s-as* (discussed further in **Chapter 5**). Notably, the inhibitory function of *Pvt1b* appears to be unique in the p53 network and not a general mechanism by which p53 enacts gene repression as we did not find other examples of negative lncRNA-mRNA co-regulation. This suggests that lncRNA-mediated *cis* activation, but not *cis* repression is a key feature of the p53 transcriptional network.

Barring Pvt1b, loss of no individual tested p53-activated lncRNA was sufficient to bypass oncogenic stress-induced growth arrest. We interpret this to mean that the highly redundant nature of p53's anti-growth response to oncogenic stress extends beyond just protein coding p53 targets and applies to lncRNA targets of p53 as well. Interestingly, these data also suggest that negative correlations between lncRNAs and neighboring protein coding genes, while uncommon, may be indicative of functionally important lncRNA-mRNA co-regulatory relationships.

Nevertheless, these data demonstrate the potential for p53-activated lncRNAs to contribute to p53 pathway regulation, and in the case of *Pvt1b*, the potential functional importance of *cis*-regulation in mediating p53 growth suppression. This concept of lncRNAs contributing to p53 regulated cellular responses, in possibly, context-dependent ways, is explored further in **Chapter 4** and **Chapter 5**.

Chapter 4:
Characterization of novel p53-activated lncRNA 2310008N11Rik

Introduction

2310008N11Rik, hence-forth referred to as *lncRNA 7*, is one of the several previously undescribed p53-activated lncRNAs we identified in **Chapter 3**, and is an intergenic, spliced and poly-adenylated lncRNA of three exons located in a gene desert on chromosome 8 (Fig. 8A). This chapter is an in-depth characterization of this novel gene, which was chosen as an interesting p53-induced lncRNA for further study for several reasons, most notably its robust induction and expression profile across our *KPR* and genotoxic stress models, unique localization patterns, and possible conservation between mice and humans. Here, we investigate the role of *lncRNA 7* in regulating p53-dependent cell cycle arrest and survival using exogenous overexpression and CRISPR-based epigenetic manipulations for lncRNA LOF and GOF.

Results

2310008N11Rik (*lncRNA 7*) is a previously undescribed lncRNA

To confirm that this unstudied RNA was truly noncoding, protein coding potential was assessed in two ways. First, as discussed in **Chapter 3**, the Coding Potential Assessment Tool (CPAT) was employed. Full length *lncRNA 7* had a CPAT score below the protein-coding threshold assigned to the mouse

transcriptome, suggesting a lack of coding potential (**Table 3.1**). Second, a number of recent publications have highlighted the potential of lncRNA loci to encode expressed and translated micropeptides. LncRNA-encoded micropeptides can be overlooked by both *in silico* coding assessment tools and mass proteomic analyses due to their small size (<100 amino acids) and potential non-canonical start codon usage, but are often identifiable by strong peaks of evolutionary conservation (Lewandowski et al. 2020; Anderson et al. 2015). As *lncRNA 7* is found both in the nucleus and cytoplasm (Fig. 4B, C) and thus feasibly able to associate with the ribosome, manual binformatic analysis was performed to rule out the presence of any small but potentially translated ORFs within *lncRNA 7* that may have been missed by CPAT. Using NCBI ORFinder, two putative small ORFs (sORFs) were identified, predicted to yield micropeptides of 13 (sORF1) and 21 (sORF2) amino acids (Fig. 8B). However, comparing these putative peptides to publicly available protein databases yielded no significant matches within eukaryotes. Additional comparison of the RNA sequences of these putative peptides also revealed a lack of sequence conservation, further suggesting the absence of translated sORFs. In summary, *lncRNA 7* was deemed unlikely to be micropeptide-encoding and thus represent a true novel lncRNA.

LncRNA 7 is induced by oncogenic and genotoxic stress in a p53-dependent manner

In our original RNA-seq experiment in the panel of *KPR* cell lines, *lncRNA 7* was most robustly induced at 24h Tam treatment in the four independent lung adenocarcinoma (LA) and sarcoma (SA) cell lines, which undergo senescence upon

p53 restoration (LA1, LA2, SA1, SA2). In contrast, *lncRNA 7* was less markedly upregulated at this time point in the two lymphoma (LY) cell lines (LY1, LY2), which undergo p53-dependent apoptosis. This observation was confirmed by qRT-PCR in the same panel of six *KPR* cell lines (Fig. 8C), firmly establishing *lncRNA 7* as being induced in a p53-dependent manner under conditions of oncogenic stress and hinting at senescence-specific regulation.

Intriguingly, *lncRNA 7* shares synteny, if not primary sequence similarities, with a lncRNA located in the corresponding region on human chromosome 8, *LINC01605* (Fig. 8A). Looking through publicly available data on p53 binding throughout the human genome (Wei et al. 2006), we noticed that p53 bound near *LINC01605* in human colorectal cancer cells treated with 5-fluorouracil, leading us to wonder whether *LINC01605* represented a putative human orthologue of *lncRNA 7*. Supporting this hypothesis, we saw that in human osteosarcoma (U2OS) cells with in-tact p53, *LINC01605* was upregulated upon Nutlin-3 treatment, which stabilizes p53 protein through antagonizing interactions with MDM2 (Nag et al. 2013), signifying p53-dependent regulation of this human lncRNA (Fig. 8D). Moreover, *LINC01605* has been recently reported to play a role in human colon cancer, indicating another link between this locus and cancer signaling pathways (Forrest et al. 2018). Thus, lncRNA expression from this region under conditions of oncogenic stress may be conserved in mice and humans.

Further characterization revealed that *lncRNA 7* was also induced under conditions of p53 stabilization in other cellular contexts. As discussed in **Chapter 2**, MEFs treated with Doxo represent a well-established *in vitro* system to study p53-dependent processes downstream of genotoxic stress (Attardi et al. 2004).

LncRNA 7 upregulation was observed in Doxo-treated primary MEFs at as early as 8h post-treatment, comparable to direct p53 target *p21*, and reached an average of 5-fold induction at 24h (Fig. 8E). To further establish that this induction was directly through p53, *lncRNA 7* levels were also assessed in *p53$^{LSL/LSL}$; Rosa26-CreER^{T2}* (*p53-restorable*) MEFs. In these MEFs, p53 is constitutively inactivated by the homozygous insertion of a LoxP-flanked STOP cassette into the endogenous p53 locus. Upon treatment with Tam, Cre-mediated excision of the STOP cassette restores p53 expression (Ventura et al. 2007). While treatment with Doxo had negligible effects on *lncRNA 7* and *p21* induction in the absence of Tam, significant induction was observed in *p53-restorable* MEFs upon Tam and Doxo treatment, indicating that induction relied on p53-proficiency (Fig. 8F). Of note, a milder induction of both *lncRNA 7* and *p21* was also observed in Tam only treated cells (Fig. 8F), likely reflecting a milder p53 response triggered by the stress naturally accumulated over time in cells lacking p53 (Ventura et al. 2007).

In further support of direct regulation by p53, *lncRNA 7* was found to harbor a p53 response element (p53RE) within its first exon. The sequence of this p53RE perfectly matches the consensus p53RE motif and contains two directly juxtaposed half sites, both of which are features associated with high affinity p53REs (Riley et al. 2008). Indeed, p53 ChIP-qPCR confirmed p53 binding at this site upon p53 restoration (Fig. 8G).

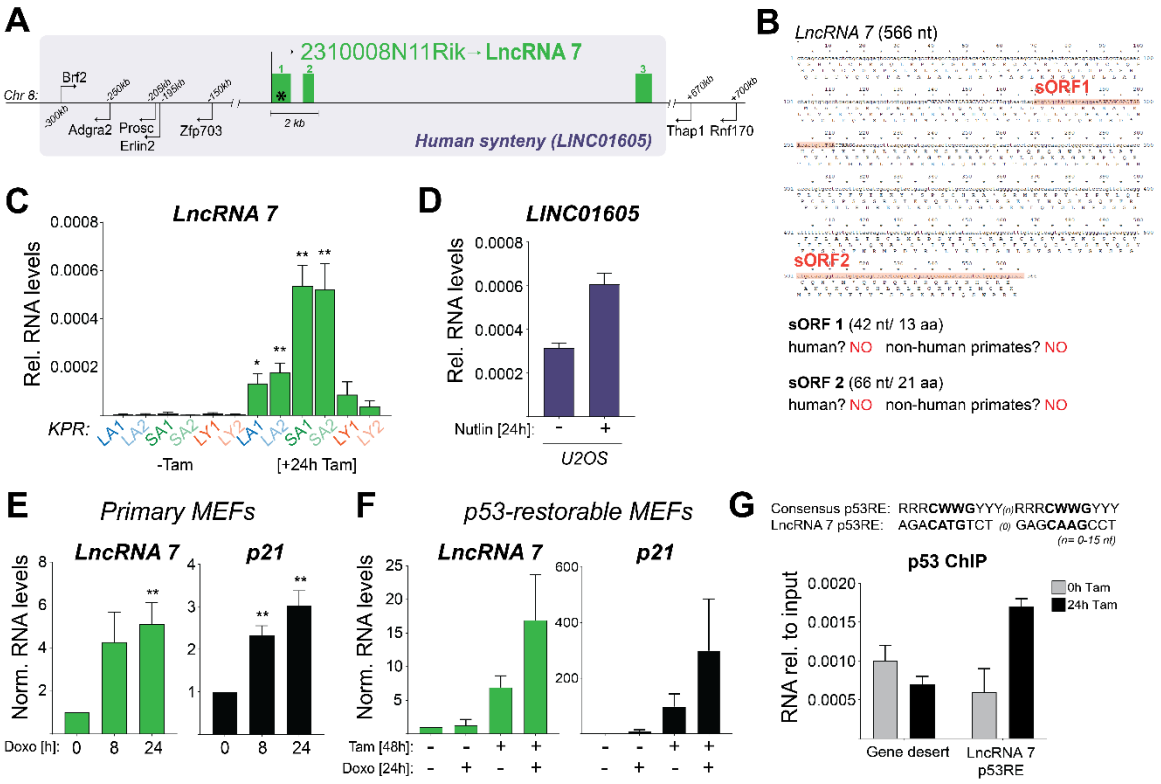


Figure 8: *LncRNA 7* (*2310008N11Rik*) is a novel, p53-regulated lncRNA. (A) Schematic of *lncRNA 7* locus, with nearest neighboring genes indicated in relation to distance from *lncRNA 7*'s TSS. Asterisk indicates p53RE location. Shading marks region of shared genic synteny with human chromosome 8, centered around *LINC01605*. **(B)** Full length *lncRNA 7* sequence and ORF analysis. Two putative sORFs identified by NCBI ORFinder are highlighted in orange. Results of peptide and nucleotide sequence conservation analysis are noted below. **(C)** qRT-PCR analysis of *lncRNA 7* relative to endogenous control RNA *Gapdh* in the indicated *KPR* cell lines and treatments. Data show mean ±SEM (n=3 biological replicates). *p <0.05 and ** p<0.01, unpaired t test. **(D)** qRT-PCR analysis of *LINC01605* relative to *GAPDH* in U2OS cells and indicated treatment (Nutlin= 5 μM). Data show mean ±SD (n=3 technical replicates). **(E)** qRT-PCR analysis of *lncRNA 7* and *p21* relative to *Gapdh* in primary MEFs treated with 0.5 μM Doxo, normalized to untreated control. Data show mean ±SEM (n=3 biological replicates). ** p<0.01, unpaired t test. **(F)** qRT-PCR analysis of *lncRNA 7* and *p21* relative to *Gapdh* in *p53$^{LSL/LSL}$; Rosa-CreER^{T2}(p53-restorable)* MEFs treated as indicated (Doxo= 0.5 μM). Data show mean ±SD (n=2 biological replicates). **(G)** Top= *lncRNA 7* p53RE sequence compared to consensus sequence. Bottom= Enrichment of p53 binding at *lncRNA 7*'s p53RE or a control region (gene desert) by ChIP followed by qRT-qPCR. Data show mean ±SD (n=3 technical replicates).

In (G), p53 ChIP was performed by N. Dimitrova (qRT-PCR was performed by E. Tesfaye).

Taken together, these data established that *lncRNA 7* is a novel lncRNA directly activated by p53 in multiple biological contexts and suggested that *lncRNA 7* may play a significant role in the p53 pathway, either contributing to p53's cell fate decision making in response to oncogenic stress or as a more general regulator of p53 activity. To test these hypotheses, we next set out to establish efficient, locus-appropriate LOF and GOF tools. Subcellular fractionation revealed that although *lncRNA 7* is nuclear-enriched, a sizeable (~40%) fraction is also found in the cytoplasm in both *KPR* cells and primary MEFs (Fig. 4B, C). As discussed in **Chapter 3**, this cytoplasmic localization pattern was revealed to be somewhat unique to *lncRNA 7* amongst the p53-responsive lncRNAs identified in our study. With this localization pattern and *lncRNA 7*'s status as an intergenic lncRNA in mind, the following GOF and LOF approaches were employed in order to interrogate *lncRNA 7* function.

Exogenous overexpression of *lncRNA 7* yields no discernable effects on p53 pathway function

As *lncRNA 7* was initially identified as being preferentially upregulated in cells that undergo senescence, we first tested whether over-expressing *lncRNA 7* might affect cellular proliferation. While transgene overexpression (OE) is unsuitable for studying locally acting lncRNAs, the large fraction of endogenous *lncRNA 7* found in the cytoplasm prompted as to investigate a potential *trans*-acting role by first taking an exogenous lncRNA OE approach. Full-length *lncRNA 7* cDNA was stably introduced into cells using a retroviral vector, with an empty vector (EV) construct serving as a negative control. High levels of *lncRNA 7* OE

were achieved in both *KPR* cells (LA1, LA2, SA1, SA2) and primary MEFs, reaching levels several hundred-fold beyond those achieved via endogenous activation upon either Tam or Doxo treatment, respectively (Fig. 9A).

With these two *in vitro* models established, cellular proliferation was assessed in both the absence and presence of p53, rationalizing that if *lncRNA 7* works as a downstream target of p53, its OE might be able to recapitulate some aspect(s) of p53 activity in lieu of activation of the rest of the p53 network. However, *lncRNA 7* OE had no significant effect on proliferation rates in either normally growing early passage primary MEFs, p53-deficient MEFs (*i.e. p53-restorable* MEFs -Tam), or p53-deficient *KPR* cells. These data suggested that exogenous OE of *lncRNA 7* does not promote senescence or cell cycle arrest in these diverse biological contexts (Fig. 9B). To test whether *lncRNA 7* was perhaps indeed a necessary component of p53's antiproliferative response but not sufficient to drive growth arrest in the absence of a competent p53 pathway, proliferation was also assessed in p53-proficient, *p53-restorable* MEFs. As predicted, Tam-treated *p53-restorable* MEFs grew at a slightly slower rate than un-restored, p53-deficient cells, reflecting a p53-dependent antigrowth response to stress accumulated in these cells prior to p53-restoration. However, this growth rate was comparable between *lncRNA-7-* and EV-expressing control cells, suggesting that exogenous OE of *lncRNA 7* does not promote growth arrest in the presence of a competent p53 network but no additional stress (Fig. 9B).

Primary MEFs exposed to low levels of DNA damage undergo senescence, as detected through morphological changes (enlarged nuclei, enlarged and flattened cellular shape) and positive senescence-associated β-galactosidase (SA-β

gal) staining (Campisi and d'Adda di Fagagna 2007). To test whether exogenous OE of *lncRNA 7* modulated this stress-induced senescence in MEFs, SA-β gal activity was monitored in *lncRNA 7*- and EV-expressing primary MEFs treated with low levels of Doxo for one week. MEFs over-expressing *lncRNA 7* were as proficient at undergoing senescence as EV-expressing cells (Fig. 9C). *LncRNA 7* exogenous OE similarly had no significant effect on growth arrest in Tam-treated LA and SA *KPR* cells (Fig. 9D). Thus, transgene OE of *lncRNA 7* did not appear to have any discernable effects on cellular proliferation in multiple independent cell lines, nor in response to either genotoxicity-induced or oncogene-induced growth arrest.

Next, we tested whether *lncRNA 7* might not promote cellular arrest, but rather prevent the initiation of an alternative cell fate upon p53 activation. Increased resistance to pro-apoptotic signals is a commonly observed feature in senescent cells (Childs et al. 2014; Soto-Gamez et al. 2019). Curious as to whether *lncRNA 7* might play a pro-survival role, we assessed the effects of *lncRNA 7* exogenous OE on apoptosis in our primary MEF and LA and SA *KPR* models.

While primary MEFs are predisposed to undergo p53-dependent cell cycle arrest in response to genotoxic stress, acute exposure to high levels of DNA damage can drive apoptosis (Attardi et al. 2004; Kastan et al. 1992). In the absence of Doxo, exogenous *lncRNA 7* OE had no effect on viability in primary MEFs (Fig. 9E). Under pro-apoptotic conditions (*i.e.* treatment with 1 μM Doxo), apoptosis as measured by cleaved caspase 3 (CC3) was detectable in both *lncRNA 7*- and EV-expressing cells. However, no significant differences were observed between the two conditions, indicating *lncRNA 7* OE did not modulate the apoptotic response

to genotoxic stress in primary MEFs (Fig. 9E). *E1A*-expressing MEFs also undergo apoptosis in response to DNA damage in a specifically p53-dependent manner, and thus represent a widely-employed model to study components of the p53 pathway that contribute to apoptosis (Lowe et al. 1993a). Aiming to refine our investigation of *lncRNA 7*'s role in modulating apoptosis downstream of p53, control- or *E1A*-expresssing *p53-restorable* MEFs were stably infected with EV or *lncRNA 7* and treated with Tam to restore p53 and Doxo to induce apoptosis. Similar to what was observed in primary MEFs, *lncRNA 7* OE did not have any effect on the extent of apoptosis observed in *E1A*-sensitized MEFs exposed to genotoxic stress (Fig. 9F).

Also investigated were the effects of *lncRNA 7* OE on viability in LA and SA *KPR* cells, which preferentially undergo p53-dependent senescence rather than apoptosis. As expected, EV-expressing control LA cells did not undergo appreciable apoptosis, either in the absence (-Tam) or presence (+Tam) of p53, confirming that p53-restoration is insufficient to drive apoptosis in these cells. This phenotype was not altered by lncRNA 7 OE (Fig. 9G). Hypothesizing that *lncRNA 7* OE may confer cytoprotective effects solely under pro-apoptotic conditions, apoptosis was assessed in p53-restored LA and SA *KPR* cells treated with high levels of acute genotoxic stress (1 μM Doxo). While Doxo treatment was indeed able to drive SA cells to undergo apoptosis, *lncRNA 7* OE had no significant effect on the extent of this apoptosis (Fig. 9H). Thus, these data altogether suggest that exogenous *lncRNA 7* OE does not alter p53 outcome-specificity or pathway robustness in primary MEFs or *KPR* cells.

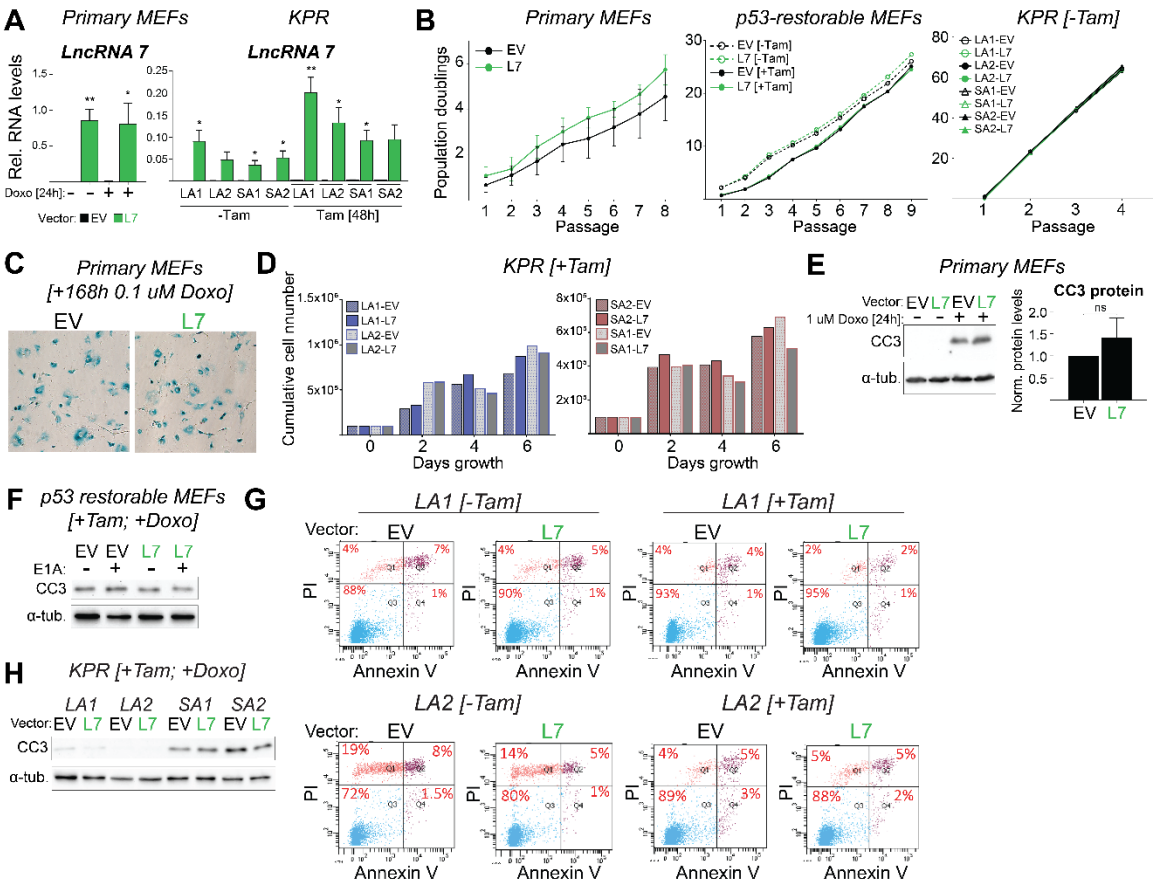


Figure 9: *LncRNA 7* exogenous OE does not affect cellular proliferation or apoptosis under genotoxic or oncogenic stress. (A) qRT-PCR analysis of *lncRNA 7* in primary MEF and *KPR* cell lines expressing either *lncRNA 7* overexpression (OE; L7) or empty vector (EV) control and treatments (Doxo= 0.5 μM). Data show mean ±SEM (n=3 biological replicates). *p <0.05 and ** p<0.01, unpaired t test. **(B)** Growth curves in indicated EV and L7 cell lines and treatments passaged over time. For primary MEF experiments, data represent mean ±SEM (n=3 biological replicates), unpaired t test at each passage. For *p53-restorable* and *KPR* cell lines, data are representative. **(C)** Senescence-associated β-galactosidase staining in indicated cell lines and treatment. **(D)** Cumulative cell numbers over time in indicated Tam-treated EV and L7 *KPR* cell lines. **(E)** Left= Representative immunoblot for CC3 protein in indicated cell lines and treatments. α-tubulin served as a loading control. Right= Densitometry analysis of CC3 levels relative to loading control, normalized to EV. Data show mean ±SEM (n=3 biological replicates). ns=not significant, unpaired t test. **(F)** Immunoblotting for CC3 protein in indicated cell lines treated with 48h Tam and 24h 0.5 μM Doxo. α-tubulin served as a loading control. **(G)** Representative FACS plots of EV and L7 LA *KPR* cells, untreated or treated with 48h Tam prior to Annexin V/PI analysis. **(H)** Immunoblotting for CC3 protein in indicated cell lines treated with 48h Tam and 24h 0.5 μM Doxo. α-tubulin served as a loading control.

Epigenetic downregulation of *lncRNA 7* may affect cellular proliferation during specifically the oncogenic stress response

Next in our efforts to characterize *lncRNA 7* was to develop strategies to manipulate the endogenous expression of *lncRNA 7*, starting with a suitable LOF approach. While CRISPR-Cas9 mutagenesis of lncRNA-associated p53REs proved to be highly effective for the molecular dissection of several other p53-activated lncRNAs (see **Chapter 3**), the lack of compatible PAM sites precluded the use of this genetic strategy for *lncRNA 7* (Fig. 5A, B). Instead, we turned to CRISPR-inhibition (CRISPRi) to epigenetically silence the *lncRNA 7* locus. CRISPRi was achieved by recruiting nuclease-deficient Cas9 fused to the transcriptionally repressive Krüppel- associated box domain of Kox1 (dCas9-KRAB) (Gilbert et al. 2013) to *lncRNA 7*'s TSS. With this approach, we aimed to take advantage of the fact that *lncRNA 7* resides in a gene desert, thus minimizing the likelihood of CRISPR-based modulation of the locus directly interfering with the expression of other nearby genes.

A panel of eight *lncRNA 7*-targeting gRNAs were designed, selected for high specificity (score >70/100 via CRISPOR) and TSS proximity. gRNAs were screened for efficacy in *p53-restorable* MEFs engineered to stably express dCas9-KRAB (Fig. 10A, B). Remarkably, all tested guides were able to knockdown *lncRNA 7* levels by 70-85% compared to a non-targeting control (Con) gRNA (Fig. 10B). Three of the most efficient gRNAs (gRNAs #7, #9, #10) were selected for further functional investigation of the consequences of *lncRNA 7* loss on p53 pathway function in MEFs. Despite achieving high levels of *lncRNA 7* knockdown, no overt effects on cellular proliferation were observed, as assayed through either growth

curve analysis (Fig. 10C-left) or BrdU incorporation (Fig. 10C-right). Similarly, no consistent effects on DNA damage-induced apoptosis were observed (Fig. 10D). Taken together, these data suggested that *lncRNA 7* is dispensable for the p53 response to genotoxic stress in MEFs.

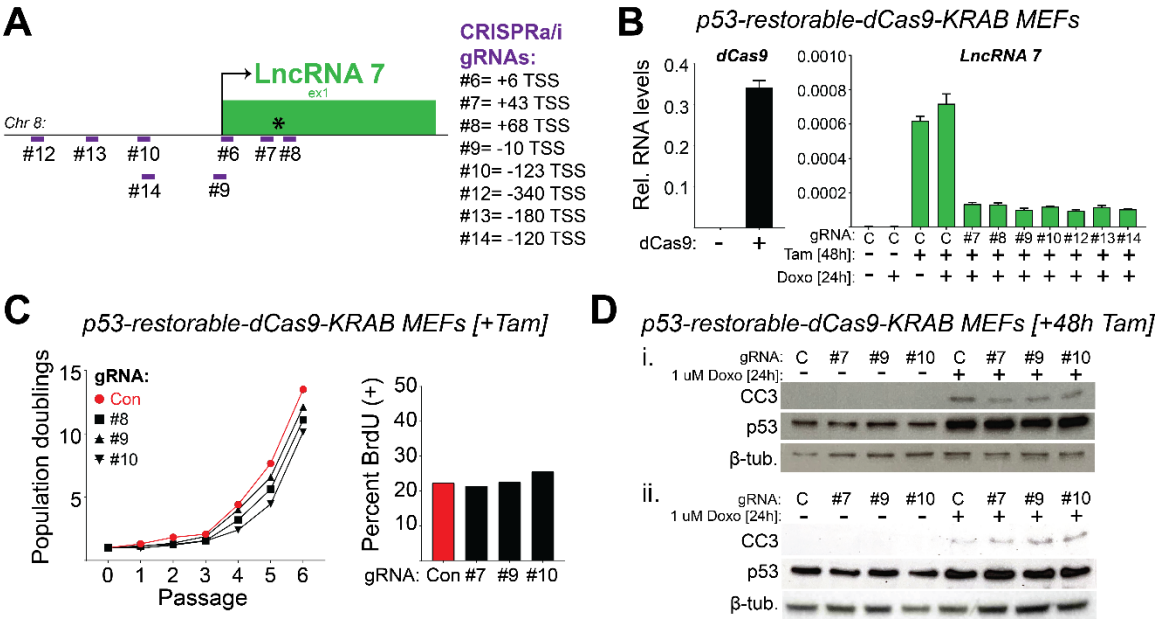


Figure 10: Epigenetic silencing of *lncRNA 7* locus does not affect p53 pathway function in *p53-restorable* MEFs. (A) Diagram of PAM locations of designed *lncRNA 7*-targeting gRNAs. Ex1= exon 1. **(B)** qRT-PCR detection of dCas9-KRAB in engineered p53-restorable-dCas9-KRAB MEFs and *lncRNA 7* levels in indicated gRNA-expressing cells and treatments, relative to *Gapdh* (Doxo= 0.5 μM). C= nontargeting control gRNA. Data show mean ±SD (n=3 technical replicates). **(C)** Left= Growth curves in indicated cell lines and treatments. Right= BrdU incorporation in indicated cell lines following 24h Tam and 4h BrdU treatment. Cells were scored for BrdU (FITC) incorporation by immunofluorescence and total number BrdU positive nuclei plotted over total scored nuclei per cell line. **(D)** Two representative immunoblots for CC3 and p53 protein levels in indicated cell lines and treatments. β-tubulin served as a loading control.

Next we assessed the effects of *lncRNA 7* LOF in our *KPR* oncogenic stress model, hypothesizing that *lncRNA 7* may be induced by p53 across many different contexts but only functionally important in certain cell-type- and/or stressor-specific settings, as has been previously suggested for other p53 targets (Mello et al. 2017; Kastenhuber and Lowe 2017). Surprisingly, screening the same *lncRNA 7*-targeting gRNAs in LA1.1 cells stably expressing dCas9-KRAB revealed that dCas9-KRAB-mediated CRISPRi yielded 40-60% *lncRNA 7* knockdown compared to non-targeting control gRNA-expressing cells, in contrast to the 70-85% knockdown observed in MEFs (Fig. 11A-left). These differences in CRISPRi efficacy may reflect cell-intrinsic differences in chromatin state at this locus. The three top LA1.1 performing gRNAs were subsequently introduced into parental LA1 cells engineered to express a Doxycycline (Doxy)-inducible dCas9-KRAB construct, which would facilitate more precise timing of LOF experiments (Fig. 11A-right). Of the three gRNAs tested, only one guide (gRNA #12) appeared to confer a slight growth defect, despite all three guides yielding comparable levels of *lncRNA 7* knockdown (Fig. 11A, B). This phenotype was initially encouraging and explored further in experiments detailed in **Chapter 5**, in which the role of *lncRNA 7* and several other senescence-associated lncRNAs is more finely examined.

Modest transcriptional activation of endogenous *lncRNA 7* does not affect cellular proliferation in LA *KPR* cells

As discussed in **Chapter 1** and **Chapter 3**, lncRNAs can very broadly be grouped as functioning near (*cis*-acting) or distal to (*trans*-acting) their sites of transcription, with each category having unique constraints on the types of suitable

LOF and GOF approaches. Considering the results of our exogenous *lncRNA 7* OE experiments, we wondered whether the apparent lack of an appreciable phenotype stemmed from *lncRNA 7* indeed playing a local, *cis*-regulatory role. In that scenario, a transgenic OE GOF approach would not be expected to have a phenotype, as transgenic *lncRNA 7* would not be properly localized to its endogenous site of function. To experimentally address this hypothesis, we turned to CRISPRa as an alternative GOF strategy. Specifically, we employed gRNA-mediated recruitment of nuclease-dead Cas9 fused to four copies of the transcription activator VP16 (dCas9-VP64) (Gilbert et al. 2013) to promote the transcriptional upregulation of *lncRNA 7* from its endogenous locus.

Three gRNAs targeting *lncRNA 7*'s TSS (gRNAs #9, #10, #12; annotated in Fig. 10A) were tested in LA1.1 and LA1 cells engineered to constitutively express dCas9-VP64. gRNA #12 did not yield *lncRNA 7* upregulation in either LA1.1 or LA1 cells. In contrast, gRNAs #9 and #10 led to the upregulation of *lncRNA 7* levels to 4x and 8x above a nontargeting control gRNA (Con) in un-restored LA1.1 cells, reaching roughly 22% and 50% of the *lncRNA 7* levels observed upon full p53 restoration. In p53-restored LA1.1 cells, gRNAs #9 and #10 activated *lncRNA 7* to 2x and 4x above control, respectively (Fig. 11C-Left). *LncRNA 7* CRISPRa was slightly more efficient in LA1 cells, with gRNA #9 yielding 5x upregulation (24% full *lncRNA 7* induction) and gRNA #10 yielding 11x upregulation (55% full lncRNA 7 induction) in un-restored cells, and 6x and 5x upregulation, respectively, upon Tam-treatment (Fig. 11C-Right). Thus, recruitment of dCas9-VP64 to *lncRNA-7*'s TSS was able to drive modest induction of *lncRNA 7* for two of the three

gRNAs tested, suggesting that CRISPRa efficacy positively correlates with proximity to TSS, as has been previously reported (Konermann et al. 2014).

Next, the effects of *lncRNA 7* CRISPRa on cellular proliferation were assayed, both in the absence and presence of p53. Upregulation of endogenous *lncRNA 7* had no effect on cellular proliferation in the absence of p53, as measured by growth curve analyses in both LA1 and LA1.1 cells (Fig. 11D). This was despite gRNA #10 driving *lncRNA 7* expression in un-restored cells to levels approaching that seen in control gRNA-expressing cells upon p53 activation (Fig. 11D). Similarly, *lncRNA 7* activation did not significantly affect growth arrest upon p53 restoration in either Tam-treated LA1.1 or LA1 cells (Fig. 11E). Taken together, these CRISPRa data suggested that locally-acting *lncRNA 7* may not contribute to p53's antiproliferative response to oncogenic stress, despite *lncRNA 7* being activated by p53 in such conditions. Alternatively, a threshold of *lncRNA 7* transcription might be required for function, and the levels of activation achieved using solely the recruitment of dCas9-VP64 may be insufficient. The functional consequences of significantly higher endogenous *lncRNA 7* activation are further explored in **Chapter 5**.

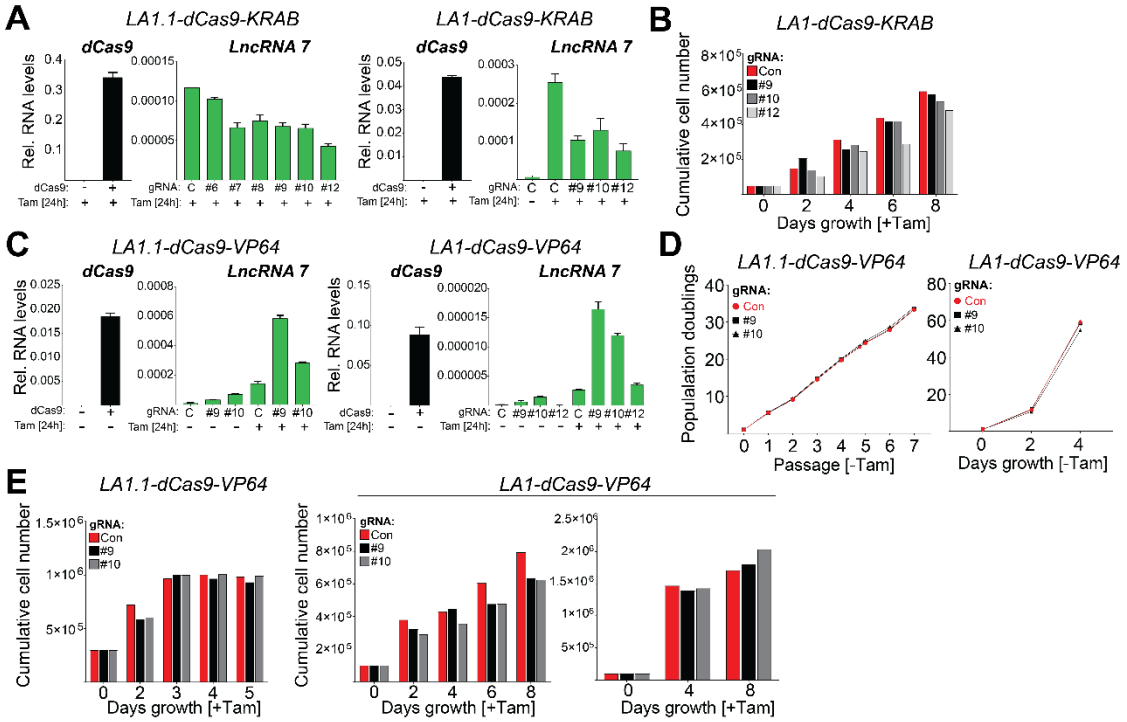


Figure 11: Assessing *lncRNA 7* CRISPRi and CRISPRa in *KPR* LA cells. (A) qRT-PCR detection of dCas9-KRAB in LA1.1 and LA1 cells engineered to stably express dCas9-KRAB and *lncRNA 7* levels in indicated gRNA-expressing cells and treatments, relative to *Gapdh*. Data show mean ±SD (n=3 technical replicates). **(B)** Cumulative cell numbers over time in indicated Tam-treated LA1-dCas9-KRAB cell lines. **(C)** qRT-PCR detection of dCas9-VP64 in LA1.1 and LA1 cells engineered to stably express dCas9-VP64 and *lncRNA 7* levels in indicated gRNA-expressing cells and treatments, relative to *Gapdh*. Data show mean ±SD (n=3 technical replicates). **(D)** Growth curves in indicated cell lines and treatments. **(E)** Cumulative cell numbers over time in indicated Tam-treated cell lines.

Data summary and conclusions

These data firmly establish *lncRNA 7* as a novel lncRNA directly activated by p53 in multiple cellular and stressor contexts. Moreover, we confirm the presence of a syntenic human lincRNA (*LINC01605*) that is also p53-responsive in Nutlin 3-treated human osteosarcoma cells, suggesting conserved p53 regulation, though the importance of this p53 regulation in human cells is an intriguing unknown.

Although we found that *lncRNA 7* was strongly induced by DNA damage in MEFs, we were unable to find any evidence for *lncRNA 7* significantly contributing to cellular proliferation, senescence, or apoptosis in this context. Similarly, exogenous OE of *lncRNA 7* did not influence the p53 outcomes of LA and SA cells, despite the preferential induction of *lncRNA 7* in these four senescing *KPR* cell lines. These data suggest that *lncRNA 7* does not function in *trans* to modulate oncogenic stress-driven cellular senescence. It is also unlikely that *trans*-acting *lncRNA 7* has cytoprotective effects against either genotoxic or oncogenic stress, at least in LA and SA cells and MEFs, which are naturally resistant apoptosis. Thus, we conclude that *lncRNA 7* is unlikely to exert any functions in *trans* downstream of p53 activation, despite its cytoplasmic localization.

The effects of *lncRNA 7* LOF and GOF in *KPR* cells is explored further in **Chapter 5,** building upon the epigenetic tools developed in this chapter.

Chapter 5:

Senescence-associated lncRNAs in p53 pathway function

Introduction

The underlying drivers of p53 cell fate decision making, or why certain cellular conditions lead to one p53 stress response versus another, remain poorly understood (see **Chapters 1** and **2**). Senescence is an irreversible state of proliferative arrest that can be triggered by a number of stress stimuli and is characterized by distinct morphological, epigenetic, molecular, and secretory phenotypes (Campisi and d'Adda di Fagagna 2007). Cellular senescence is thought to act as a safeguard against the persistent growth of cells that have accumulated irreparable DNA damage. As such, senescence has been implicated in numerous homeostatic processes, including tissue repair and immune clearance, as well as several age-associated pathologies, such as neurodegeneration, chronic inflammation, tissue degeneration, and cancer (Muñoz-Espín and Serrano 2014; Campisi 2013). In cancer, senescence can be both tumor suppressive and oncogenic, largely due to the pleiotropic effects of the proinflammatory SASP (senescence-associated secretory phenotype) (Muñoz-Espín and Serrano 2014; Collado and Serrano 2010). Nonetheless, p53 is known to be an important upstream activator of oncogene-induced senescence (Serrano et al. 1997; Campisi and d'Adda di Fagagna 2007) and p53-dependent senescence can be an important barrier to tumor progression *in vivo* (Xue et al. 2007; Ventura et al. 2007; Feldser et al. 2010; Li et al. 2014).

In this chapter, we address the long-standing question of why p53 elicits senescence in certain tumor types while apoptosis is initiated in others (Ventura et al. 2007; Symonds et al. 1994). Specifically, we investigate the importance of p53-activated lncRNAs that are preferentially expressed during oncogene-induced senescence over oncogene-driven apoptosis. While a handful of lncRNAs have previously been associated with senescence (Abdelmohsen et al. 2013; Hu et al. 2014; Ghanam et al. 2017), the broader importance of lncRNAs in establishing or maintaining senescence driven by oncogenic stress is incompletely understood.

Results

Identification of senescence-associated lncRNAs

To identify candidate lncRNA loci that may contribute to p53 outcome-specificity, we leveraged a key feature of the *KPR* mouse model in which cell lines derived from distinct tumor types respond differently to Tam-mediated p53 restoration (Fig. 2). Namely, lung adenocarcinoma cell lines (LA1, LA2) and sarcoma cell lines (SA1, SA2) undergo senescence with no appreciable apoptosis, thus representing an ideal *in vitro* system to identify novel factors contributing to p53-mediated senescence across four independent cell lines representing two independent tissue contexts. As detailed in **Chapter 3**, 21 lncRNAs were found via genome-wide p53 ChIP-seq and RNA-seq analyses to be activated by p53 across various *KPR* cell lines (**Table 3.1**). Amongst these 21 lncRNAs, closer examination of expression patterns across the six *KPR* cell lines revealed that several lncRNAs were preferentially upregulated in LA and SA cells upon p53-restoration, compared to the two independent lymphoma cell lines (LY1, LY2) (Fig. 12A).

Curious as to whether these apparent senescence-specific expression patterns reflected a shared function, four of these oncogenic-stress induced lncRNAs were selected for further study (*2310008N11Rik* [*lncRNA-7*], *Bfsp2-as*, *Ltc4s-as*, and *Neat1*), first confirming the expression patterns observed in our RNA-seq dataset via qRT-PCR across the six *KPR* cell lines (Fig. 12B). All four lncRNAs were also induced in primary MEFs exposed to acute DNA damage, as would be expected of direct targets of p53 (Fig. 12C). Interestingly, *lncRNA-7*, *Bfsp2-as*, *Ltc4s-as*, and *Neat1* levels also accumulated in MEFs under sustained exposure to low levels of DNA damage, which also promotes senescence (Fig. 12D). Indeed, lncRNA expression patterns paralleled those of key mediators of senescence, *p21* and *p16* (Serrano et al. 1997), which also accumulated over exposure time as is characteristic of cells undergoing senescence (Lin et al. 1998; Campisi and d'Adda di Fagagna 2007) (Fig. 12D).

In searching for other p53-regulated lncRNAs that may also have outcome-specific expression patterns, a striking senescence-specificity was revealed for *9230114K14Rik* (henceforth referred to as *lincRNA-RAPT1)*. While *lincRNA-RAPT1* did not meet our stringent filtering criteria to be called as an oncogenic stress-responsive, p53-activated lncRNA in our genome-wide *KPR* dataset, it had previously been implicated as a target of p53 (Guttman et al. 2009) and clearly demonstrated p53-dependent induction in Tam-treated *KPR* cells (Fig. 12B) and DNA damage-treated MEFs (Fig. 12C, D). Thus, *lncRNA-7*, *Bfsp2-as*, *Ltc4s-as*, *Neat1*, and *lincRNA-RAPT1* were determined to be induced by both oncogenic stress and genotoxic stress, with induction patterns suggestive of a role in senescence.

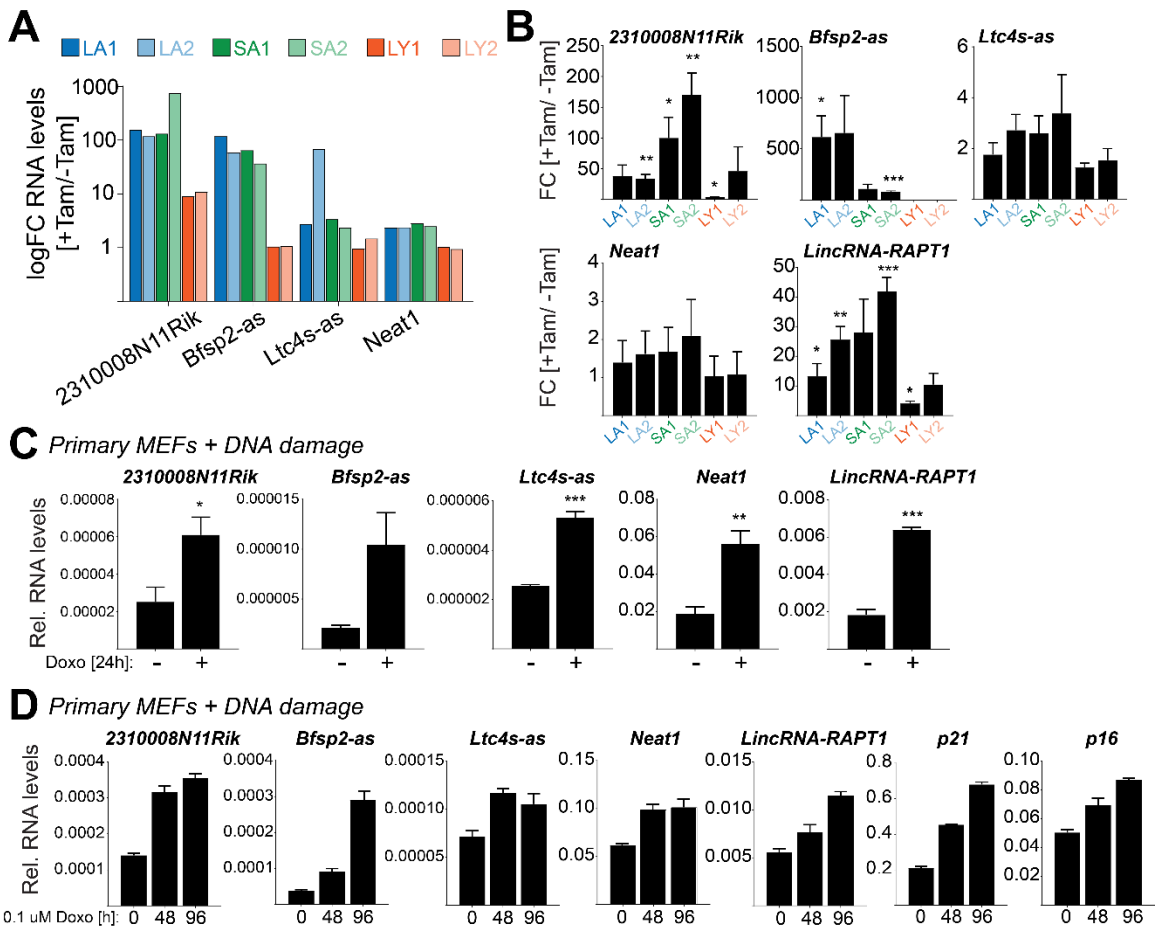


Figure 12: Several p53-activated lncRNAs have senescence-associated expression patterns. (A) RNA-seq values (log2 FC) in indicated *KPR* cell lines treated with 24h Tam normalized to untreated samples. **(B)** qRT-PCR analysis of indicated lncRNAs relative to *Gapdh* in indicated *KPR* cell lines. Fold change were calculated by normalizing to untreated cells. Data show mean ±SEM (n=3 biological replicates). *p <0.05, ** p<0.01, and *** p<0.001, unpaired t test. **(C)** qRT-PCR detection of indicated lncRNAs in primary MEFs treated with 24h mock or 24h 0.5 µM Doxo. Data show mean ±SEM (n=3 biological replicates). *p <0.05, ** p<0.01, and *** p<0.001, unpaired t test. **(D)** qRT-PCR detection of indicated lncRNAs and p21 and p16 mRNA in primary MEFs treated as indicated. Data show mean ±SD (n=3 technical replicates).

In (A), RNA-seq was performed by N. Dimitrova (see Fig. 2).

Characterization of *lincRNA-RAPT*, a novel p53-activated lncRNA

LincRNA-RAPT1 was selected for functional characterization first, due in part to the availability of powerful genetic tools developed and initially characterized in our lab by N. Dimitrova, and several unique features of this lncRNA locus (described shortly).

LincRNA-RAPT1 is a polyadenylated, 9.1 kb-long transcript comprised of four exons. While *lincRNA-RAPT1* had previously come up as a transcript that was induced by DNA damage in a p53-dependent manner in one of the earliest genome-wide lincRNA datasets (Guttman et al. 2009), it had not been functionally followed up on nor established to be a true direct target of p53. Our own investigation of the *lincRNA-RAPT1* locus revealed the presence of multiple p53 response elements (p53REs) within *lincRNA-RAPT1*'s first intron (Fig. 13A), providing evidence for direct regulation by p53 and a basis for the p53-dependent induction patterns observed in our two *in vitro* model systems (Fig. 12B, C). In the UCSC genome browser, *lincRNA-RAPT1* is annotated as RIKEN cDNA *9230114K14Rik*, with several variants reflecting alternative splicing patterns and multiple TSSs and termination sites (Fig. 13A). Deep RNA-sequencing of primary MEFs performed by N. Dimitrova provided further evidence for the presence of multiple transcript variants, while also revealing that full length (FL) *lincRNA-RAPT1* in fact extends 8 kb beyond the last annotated termination site (Fig. 13A).

Intriguingly, early sequence analysis performed by N. Dimitrova also identified a short (15 nt) region of complementarity between *lincRNA-RAPT1* and p53 mRNA's 5' untranslated region (5' UTR) (Fig. 13B). Of note, this region of complementarity, which fell within the extended *lincRNA-RAPT1* exon four,

corresponded to a region of p53 mRNA also predicted to base-pair with its own 3' UTR. Such 5'UTR-3'UTR interactions had previously been shown to be important for promoting the translation of human *p53* under conditions of stress (Chen and Kastan 2010; Chen et al. 2012). Thus, we were intrigued by the possibility that *lincRNA-RAPT1* may contribute to either p53 outcome-specificity or general p53 pathway robustness, potentially though RNA-RNA interactions with *p53* itself.

In order to further interrogate the role of *lincRNA-RAPT1* in the p53 network, two complementary genetic mouse models were developed prior to my taking on the project and will be briefly described here. Care was taken to design a knockout strategy that would abolish p53-dependent *lincRNA-RAPT1* expression without affecting the expression of *Dhx15,* which is a protein coding gene that *lincRNA-RAPT1* shares a promoter with (Fig. 13A). *Dhx15* (also known as *Prp43*) is a DEAD/DEAH box RNA helicase implicated in ribosome biogenesis and spliceosome disassembly (Combs et al. 2006). Unlike *lincRNA-RAPT1*, *Dhx15* did not appear to be significantly regulated by p53 (Fig. 13E). While it is unclear how two genes sharing a promoter can be differentially regulated as such, it is possible that the intronic location of *lincRNA-RAPT1's* p53REs provide an explanation. Importantly, *Dhx15* has not been previously implicated in senescence. Thus, we concluded that *lincRNA-RAPT1* and *Dhx15* are unlikely to be functionally linked.

To avoid disrupting the shared promoter while designing a conditional knockout (KO) allele, *loxP* sites were inserted within the first and second introns of *lincRNA-RAPT1*. This allele will be referred to as the floxed (fl) allele (Fig. 13C-top). The *lincRNA-RAPT1* constitutive KO (null; -) allele was subsequently generated by crossing *lincRNA-RAPT1*$^{fl/fl}$ mice to transgenic mice constitutively

expressing Cre recombinase, leading to the generation of mice in which the region between the two *loxP* sites has been excised. Importantly included in this excised region are *lincRNA-RAPT1*'s p53REs (Fig. 13C-bottom).

The following *lincRNA-RAPT1* characterization experiments were performed in early passage primary MEFs of two complementary *lincRNA-RAPT1* genetic backgrounds: (1) *lincRNA-RAPT1*$^{fl/fl}$*; Rosa26-CreER*^{T2} or (2) *lincRNA-RAPT1*$^{-/-}$ vs *lincRNA-RAPT1*$^{+/+}$. For conditional KO experiments, *lincRNA-RAPT1*$^{fl/fl}$*; Rosa26-CreER*^{T2} MEFs were isolated from homozygous crosses between *lincRNA-RAPT1*$^{fl/fl}$*; Rosa26-CreER*^{T2} mice. In these cells, the addition of Tam facilitates *in vitro* CreER-mediated recombination of the floxed allele and loss of *lincRNA-RAPT1* expression. Mock treatment served as a control, allowing isogenic comparisons of wild type (WT) and KO cells (Fig. 13D- top). Importantly, this strategy led to a 95% decrease in *lincRNA-RAPT1* levels, effectively abolishing p53-dependent induction, whereas *Dhx15* expression was not significantly affected (Fig. 13E). For constitutive KO experiments, littermate *lincRNA-RAPT1*$^{+/+}$ *and lincRNA-RAPT1*$^{-/-}$ MEFs were isolated from crosses of *lincRNA-RAPT1*$^{+/-}$ heterozygotes (Fig. 13D-bottom).

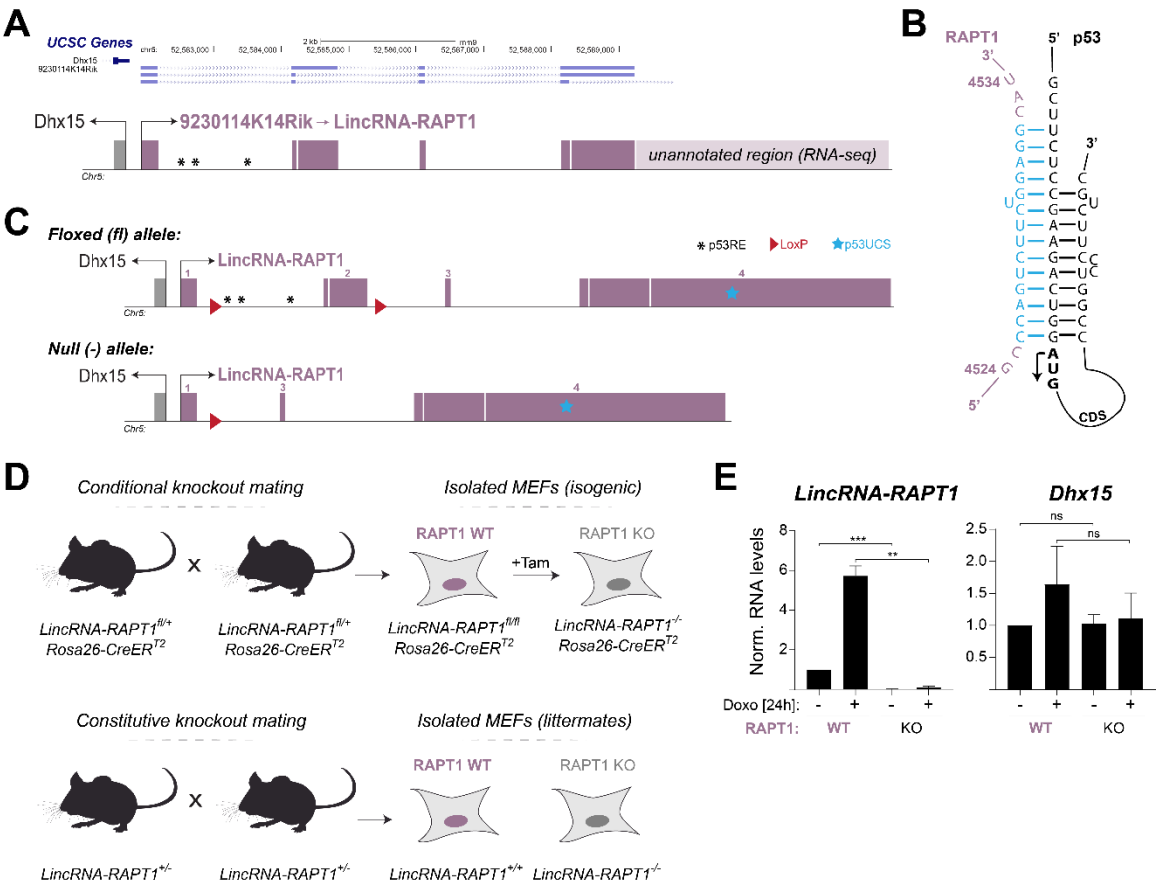


Figure 13: *LincRNA-RAPT1* (*9230114K14Rik*) locus and development of knockout mouse models. (A) Top= UCSC genome browser view (mm10) of Riken cDNA 9230114K14Rik, henceforth referred to as *lincRNA-RAPT1*. Bottom= Schematic of *Dhx15* / *lincRNA-RAPT1* locus, including previously unannotated region of *lincRNA-RAPT1* exon 4 identified by RNA-seq. p53REs are marked by asterisks. **(B)** Schematic of 15 nt region of complementarity between *lincRNA-RAPT1* and p53 mRNA 5' UTR (highlighted in blue). Also illustrated is predicted complementarity between p53 mRNA 5' UTR and 3' UTR. **(C)** Schematic of floxed (fl) and null (-) *lincRNA-RAPT1* alleles, with important features labeled as indicated. p53UCS= p53 UTR complementary sequence. **(D)** Matings and *in vitro* drug treatments for generating primary MEF cell lines of indicated genotypes. **(E)** qRT-PCR detection of *lincRNA-RAPT1* and *Dhx15* in primary MEFs of indicated genotypes and treatments relative to *Gapdh*, normalized to WT untreated cells (Doxo=0.5 μM). Data show mean ±SEM (n=2 biological replicates). ** p<0.01 and *** p<0.001, ns= not significant, unpaired t test.

In (A), *lincRNA-RAPT1* unannotated region indicated was identified by N. Dimitrova via RNA-seq (data not shown). In (B), 15 nt p53UCS was identified by N. Dimitrova. Mouse alleles/genetic models described above and in text were developed by N. Dimitrova, though all characterization experiments shown and discussed in this chapter were performed by E. Tesfaye.

To determine whether loss of *lincRNA-RAPT1* led to any changes in proliferation, growth curve analyses were performed in *lincRNA-RAPT1* WT and KO MEFs. Despite the drastic reduction in *lincRNA-RAPT1* levels achieved, no significant differences in proliferation rate were observed (Fig. 14A). As *lincRNA-RAPT1*-deficient MEFs appeared to grow normally, suggesting *lincRNA-RAPT1* did not regulate growth arrest downstream of p53, we next wondered whether *lincRNA-RAPT1* may regulate other aspects of p53 activity. A few p53-activated lncRNAs have been reported to regulate p53 activity in positive feedback loops (see **Table 1.1**), though none have been reported to interact with p53 mRNA. Given *lincRNA-RAPT1*'s short region of complementarity with p53 mRNA's 5' UTR, we questioned whether the loss of *lincRNA-RAPT1* affected p53 RNA and/or protein levels. In constitutive KO MEFs, p53 mRNA levels were not significantly altered compared to WT MEFs in either the presence or absence of DNA damage (Fig. 43B). This was also true in conditional KO MEFs (Fig. 14D), implying that *lincRNA-RAPT1* did not influence p53 mRNA transcription and/or stability.

Given that cellular stress promotes the translation of p53 mRNA (Takagi et al. 2005) and p53 protein stabilization (Horn and Vousden 2007), we next questioned whether *lincRNA-RAPT1* promoted p53 protein levels during stress. Increases in p53 protein were detectable in both WT and *lincRNA-RAPT1* KO MEFs at as early as 6h Doxo treatment (Fig. 14C). However, in comparing littermate WT and constitutive KO MEFs, significant variability in p53 protein levels was consistently observed, even amongst independent littermate WT controls (Fig. 14C). Considering this apparent variability in p53 protein levels, which could stem from heterogeneity in basal p53 levels or handling stress, we

decided to focus on our conditional *lincRNA-RAPT1* KO model, which allows isogenic comparisons. Similar experiments in *lincRNA-RAPT1* conditional KO (*i.e.* Tam-treated) and WT (*i.e.* mock-treated) MEFs demonstrated no consistent effect on p53 protein levels at either 6h or 24h Doxo treatment (Fig. 14E). Given the accumulation of *lincRNA-RAPT1* under pro-senescent conditions (Fig. 12B, D), we also tested whether *lincRNA-RAPT1* loss affected p53 levels under conditions in which MEFs undergo senescence and did not see a significant difference compared to control MEFs (Fig. 14F), though substantial variability in p53 protein levels across biological replicates was observed.

Curious about potential biological explanations for this persistent phenotypic variability, in which several experiments in multiple independently-derived cell lines suggested some level of positive correlation between *lincRNA-RAPT1* and p53 protein levels, we next performed more experiments further characterizing our *lincRNA-RAPT1* KO allele. Single molecule RNA FISH (smRNA FISH) of WT and KO MEFs treated with DNA damage (Doxo) to induce *lincRNA-RAPT1* expression revealed the persistence of cytoplasmic *lincRNA-RAPT1* signal in KO cells, albeit at drastically reduced levels (Fig. 14G-top). These data were consistent with the endogenous localization pattern of WT *lincRNA-RAPT1* as determined by subcellular fractionation (Fig. 14G-bottom), as well as our qRT-PCR data showing highly efficient (>90%) but not complete *lincRNA-RAPT1* knockdown in KO cells.

In order to better characterize the RNA products of this persistent transcription from the *lincRNA-RAPT1* locus in KO cells, we designed a series of qRT-PCR primers spanning *lincRNA-RAPT1*, aiming to capture transcript variants

utilizing the locus' many alternate start, splice, and termination sites (Fig. 14H-top). Surprisingly, absolute transcript abundance analysis using these primers in WT and KO MEFs exposed the presence of quantifiable amounts of *lincRNA-RAPT1* in KO MEFs (Fig. 14H-bottom), leading us to conclude that the *lincRNA-RAPT1* KO allele is in fact a hypomorph.

In summary, our work establishes *lincRNA-RAPT1* as a direct target of p53, though its contributions to downstream processes regulated by p53 and possibly p53 regulation remain to be fully explored using alternative LOF strategies.

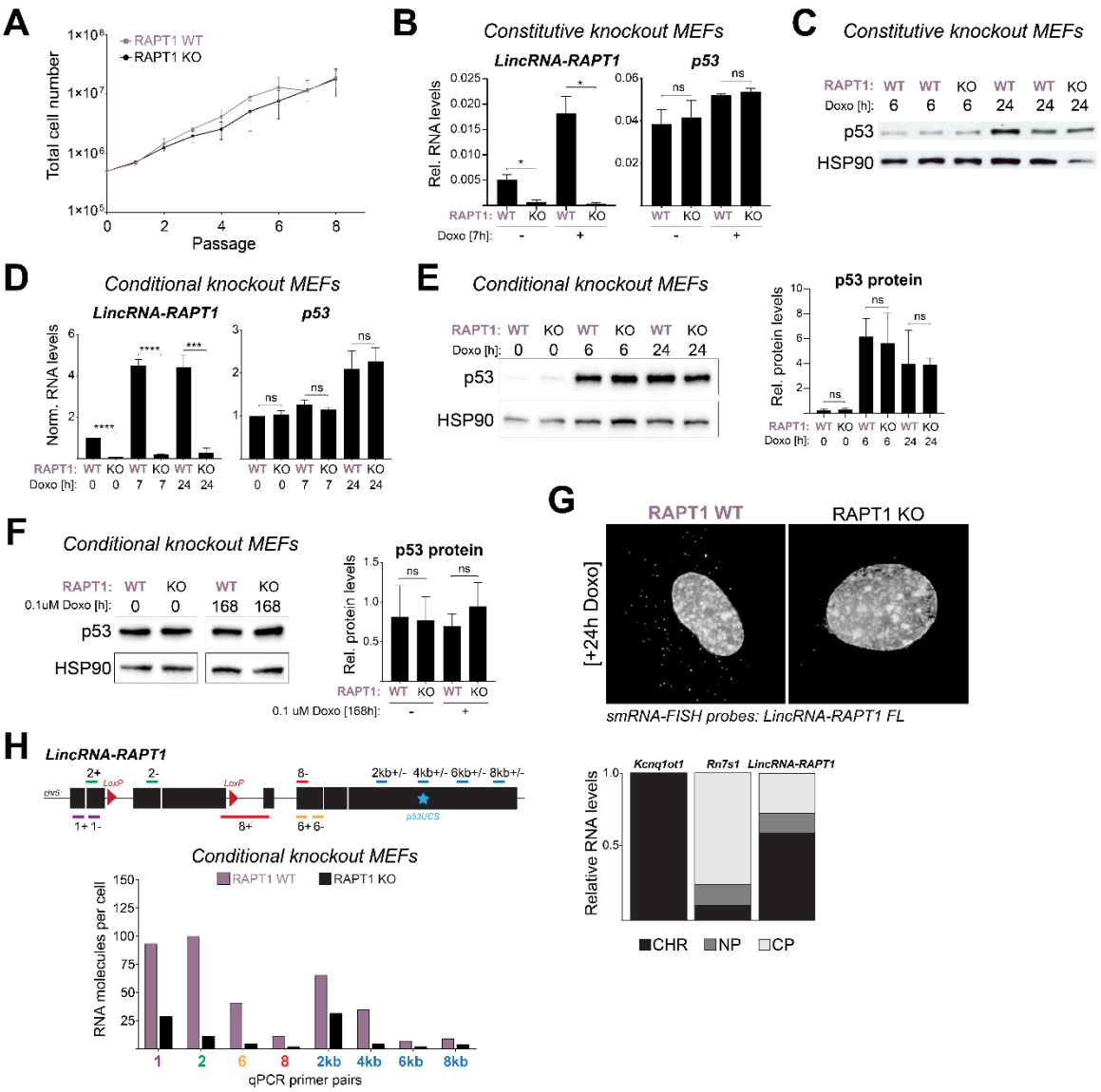


Figure 14: Characterization of *lincRNA-RAPT1*. (A) Growth curves in primary MEFs of indicated genotypes. Data represent mean cell numbers ±SEM (n=4 biological replicates), unpaired t test at each passage. **(B)** qRT-PCR analysis of *lincRNA-RAPT1* and p53 mRNA in primary MEFs of indicated genotypes and treatments relative to *Gapdh* (Doxo=0.5 µM). Data show mean ±SEM (n=3 biological replicates). * p<0.05, ns= not significant, unpaired t test. **(C)** Immunoblotting for p53 protein in indicated cells and treatments. HSP90 served as a loading control. **(D)** qRT-PCR analysis of *lincRNA-RAPT1* and p53 mRNA in primary MEFs of indicated genotypes and treatments relative to *Gapdh*, normalized to untreated WT MEFs (Doxo=0.5 µM). Data show mean ±SEM (n=3 biological replicates). *** p<0.001 and **** p<0.0001, ns= not significant, unpaired t test. **(E, F)** Left= Representative immunoblot for p53 protein in indicated cells and treatments (Doxo in **(E)** =0.5 µM). HSP90 served as a loading control. Right= Densitometry analysis of p53 protein levels relative to loading control. Data show mean ±SEM (n=3 biological replicates). ns=not significant, paired t test. **(G)** Top= smRNA-FISH of *lincRNA-RAPT1* in indicated cells treated with 24h 0.5 µM Doxo. Note- background signal in nuclei from Doxo autofluorescence. Bottom= qRT-PCR detection of controls (*Rn7sl*= cytoplasm; *Kcnq1ot1*= chromatin) and *lincRNA-RAPT1* after subcellular fractionation in WT primary MEFs treated with 24h 0.5 µM Doxo. CP= cytoplasmic. NP= nucleoplasmic. CHR= chromatin-associated. **(H)** Top= Diagram indicating locations of qRT-

PCR primers across *lincRNA-RAPT1* exons. Bottom= Copy number calculations for *lincRNA-RAPT1* as detected by qRT-PCR with indicated primer pairs in WT and KO MEFs

Description of oncogenic stress-induced, senescence-associated lncRNAs

In order to determine whether additional lncRNAs contributed to p53 cellular decision making in our *KPR* model, we selected four candidates from our list of oncogenic stress-induced lncRNAs that were found to have senescence-associated expression patterns (Fig. 12). The lncRNAs selected were: *2310008N11Rik* (*lncRNA 7*), *Bfsp2-as*, *Ltc4s-as*, and *Neat1*. Of these four lncRNAs, only *Neat1* had been previously studied and is a monoexonic intergenic lncRNA located on chromosome 19 (Fig. 15A-iv). Interestingly, *Neat1* has recently been implicated in the p53 response to oncogenic stress (Mello et al. 2017) (discussed further in **Chapter 1**).

The three other senescence-associated lncRNA candidates had not been previously studied, nor linked to the p53 pathway. *LncRNA 7* is an intergenic lncRNA located on chromosome 8 (Fig. 15A-i) and is discussed more in **Chapter 4**, which is an in-depth characterization of this novel lncRNA.

Ltc4s-as (RIKEN cDNA *AK157916*) is a 1.2 kb spliced and polyadenylated transcript of three exons (Fig. 15A-ii). As touched upon in **Chapter 3**, *Ltc4s-as* is transcribed antisense to two protein-coding genes, *Ltc4s* and *Maml1*. *Ltc4s (Leukotriene C4 synthase)* encodes an enzyme involved in the conversion of arachidonic acid to cysteinyl leukotrienes, which are important lipid inflammatory mediators. Although it has not been previously implicated in the p53 pathway, *Ltc4s* has been linked to anaphylaxis and other inflammatory responses in humans

(Funk 2001; Lam and Frank Austen 2002). *Maml1 (Mastermind-like protein 1)* is a transcriptional co-activator in the Notch signaling pathway and, interestingly, has been reported to also interact with p53 protein and promote p53 transactivation in human breast cancer cells (Zhao et al. 2007; Yun et al. 2015). In contrast to *Ltc4s-as* and *Ltc4s*, which were found to be transcriptionally activated in multiple LA and SA *KPR* cells lines upon Tam treatment, *Maml1* expression was not significantly altered by either p53 restoration or mutagenesis of *Ltc4s-as'* p53RE (Fig. 5C), suggesting that *Maml1* is not regulated in a p53-dependent manner.

Bfsp2-as (RIKEN cDNA *5830418P13Rik*) is a polyadenylated transcript 2.2 kb in size, comprised of six exons, and is transcribed antisense to the gene *Bfsp2 (Beaded filament structural protein 2)* (Fig. 15A-iii). *Bfsp2* is an ocular lens-specific intermediate filament important for eye development (Perng et al. 2007). While *Bfsp2* did emerge as a p53-responsive gene in a previously published genome-wide dataset (Tonelli et al. 2017), it has never been studied in the context of the p53 stress response nor implicated in any known p53-regulated cellular processes.

Functional investigation of the role of lncRNAs in mediating p53 outcome-specificity in response to oncogenic stress

Candidate loci determined, we elected to take CRISPR-based approaches to epigenetically down or up-regulate lncRNA expression, which is a strategy that has been employed to functionally interrogate noncoding loci with great success in recent years (Liu et al. 2017; Joung et al. 2017; Fulco et al. 2016). For senescence-

associated lncRNA LOF, we employed CRISPR-based transcriptional interference/inhibition (CRISPRi). In this system, catalytically inactive Cas9 fused to the KRAB (dCas9-KRAB) was recruited to the TSSs of target loci in a gRNA-directed manner (Fig. 15B). We anticipated that both steric transcriptional interference from dCas9 and epigenetic repression from KRAB-mediated recruitment of repressive chromatin modifiers would lead to the knockdown of target lncRNA expression (Gilbert et al. 2014; Thakore et al. 2015). Two TSS-targeting gRNAs were designed for each senescence-associated lncRNA, with care being taken to design guides with high specificity (scores >70/100 via CRISPOR) and suitability for both CRISPRi and CRISPRa manipulations (Fig. 15A- right).

In order to interrogate the effects of lncRNA loss on either the establishment or maintenance of senescence in LA and SA *KPR* cells, a Doxy-inducible dCas9-KRAB system was selected, which would allow more temporal control over lncRNA LOF. LA1 and SA1 cell lines stably expressing (TRE)dCaS9-KRAB were established and confirmed to undergo senescence upon Tam-mediated p53 restoration with dynamics comparable to parental LA1 and SA1 cells (Fig. 16A, B). Individual lncRNA TSS-targeting gRNAs were stably introduced into LA1-dCas9-KRAB or SA1-dCas9-KRAB cells and cells were Doxy-treated for 5-10 days to induce dCas9-KRAB expression and allow sufficient time for KRAB-mediated epigenetic repression (Kearns et al. 2014). Knockdown efficacy was assessed by qRT-PCR, using the average lncRNA expression levels in cell lines expressing two independent, non-targeting gRNAs as controls. Surprisingly, no knockdown was observed across our panel of lncRNA-targeting gRNAs in SA1-dCas9-KRAB cells, even when gRNAs validated to facilitate efficient CRISPRi in other, non-SA cell

lines were assessed. Thus, our data reinforces the notion that both gRNA-specific features and cell-intrinsic factors contribute to CRISPRi efficacy.

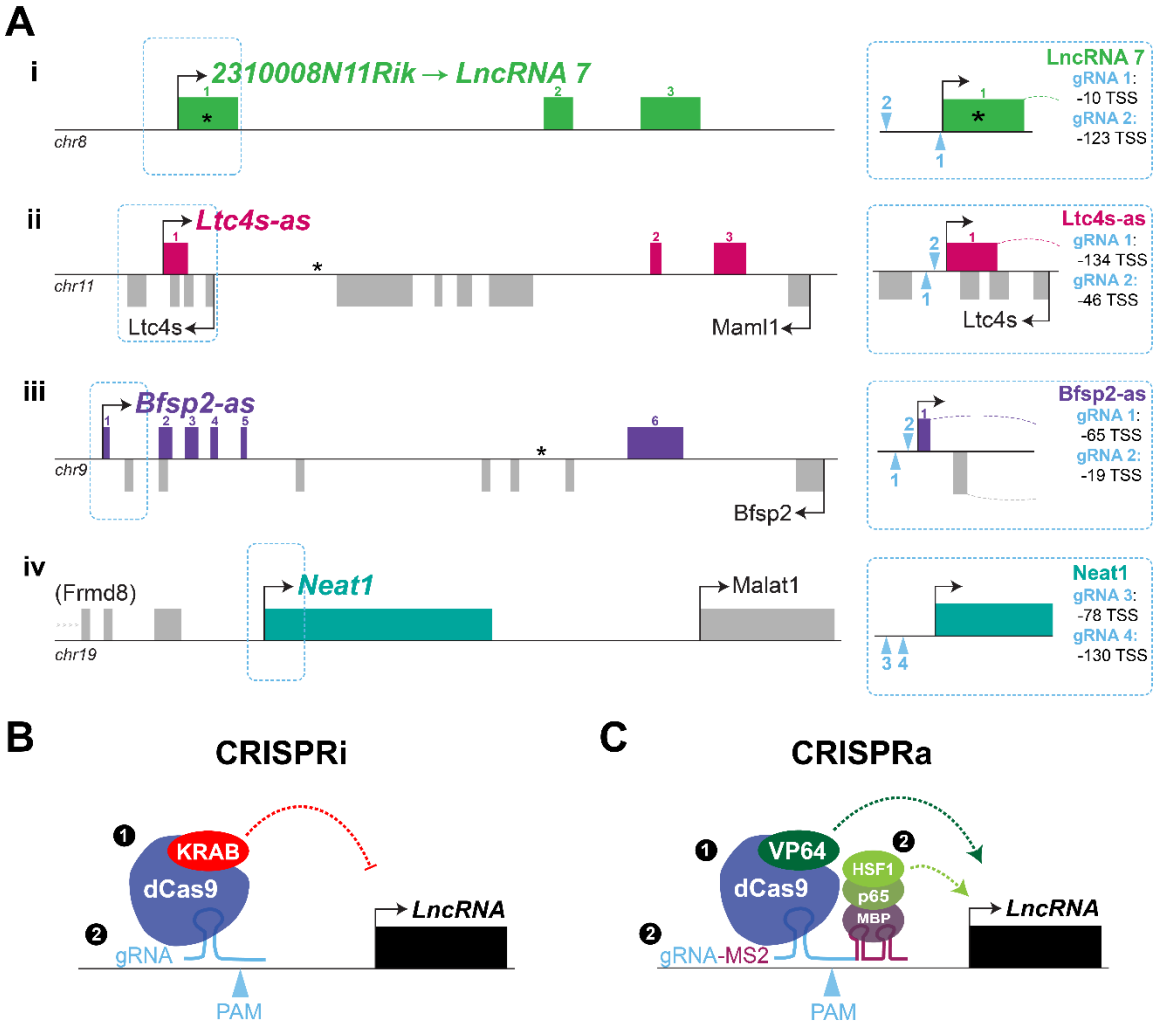


Figure 15: CRISPRi and CRISPRa strategies of senescence-associated lncRNA LOF and GOF. (A) Locus schematics for four (**i-iv**) candidate senescence-associated lncRNAs. Light blue boxes show PAM locations of designed lncRNA TSS-targeting gRNAs. **(B)** Details of CRISPRi strategy and two-vector system for introducing necessary components: (1) dCas9-KRAB; (2) gRNA. **(C)** Details of CRISPRa (VP64 CRISPR-SAM) strategy and two-vector system for introducing necessary components: (1) dCas9-VP64; (2) gRNA-MS2; HSF-p65-MBP.

Of the four senescence-associated lncRNAs targeted in LA1-dCas9-KRAB cells, we were able to robustly knockdown *Ltc4s-as* and *lncRNA 7*. For *Ltc4s-as*, gRNA 1 yielded a 53% knockdown and gRNA 2 yielded a 23% knockdown, compared to the average of two independent control gRNAs (Fig. 16C). For *lncRNA 7*, gRNA 1 led to a 48% decrease in *lncRNA 7* levels, while gRNA 2 led to a 13% knockdown (Fig. 16C), compared to control average levels. One gRNA targeting *Bfsp2-as*, gRNA 2, strangely led to a significant increase in *Bfsp2-as* RNA levels (Fig. 16C). However, a closer examination of cells expressing this gRNA revealed the dysregulation of several other p53-regulated genes, suggesting that this gRNA had off-target effects and was not worth investigating further. *Neat1* levels were not significantly reduced in LA1-dCas9-KRAB cells.

We next asked whether the observed decreases in *Lct4s-as* and *lncRNA 7* levels influenced cellular proliferation or survival in LA1-dCas9-KRAB cells. As both lncRNAs are very lowly expressed in the absence of p53, most of the following experiments were performed in p53-restored cells. However, we did determine that epigenetic repression of either loci had no significant effect on the ability of LA1-dCas9-KRAB cells to form colonies in the absence of p53 (Fig. 16D), suggesting the unlikelihood of these lncRNA loci contributing to p53-independent functions related to proliferation and/or survival.

In p53-proficient cells, knockdown of either *Ltc4s-as* or *lncRNA 7* had no effect on growth rate (Fig. 16E) or the establishment of senescence (Fig. 16F). Moreover, colony formation was similarly unaffected (Fig. 16G), suggesting that lncRNA loss did not significantly disrupt the maintenance of senescence. Lastly, we examined whether lncRNA CRISPRi modulated either resistance or sensitivity

to apoptosis, both of which have been observed in human cells of various backgrounds that have undergone senescence (Marcotte et al. 2004; Childs et al. 2014). As was observed in parental LA and SA cells, LA1-dCas9-KRAB cells did not undergo apoptosis upon p53-restoration, as measured by cleaved caspase 3 (CC3) levels, and this was unaffected by *Ltc4s-as* or *lncRNA 7* reduction (Fig. 16H-i). On average, treatment with 24h 1 μM Doxo drove p53-proficient LA1-dCas9-KRAB to undergo apoptosis to comparable levels in control gRNA-expressing and lncRNA targeting gRNA-expressing cells, though considerable variability in CC3 levels was observed even amongst control cells (Fig. 16H). More tellingly, CC3 levels did not correlate with lncRNA knockdown efficiencies. Thus, we concluded that loss of *Ltc4s-as* and *lncRNA 7* did not alter either growth, senescence or apoptosis in LA1 cells.

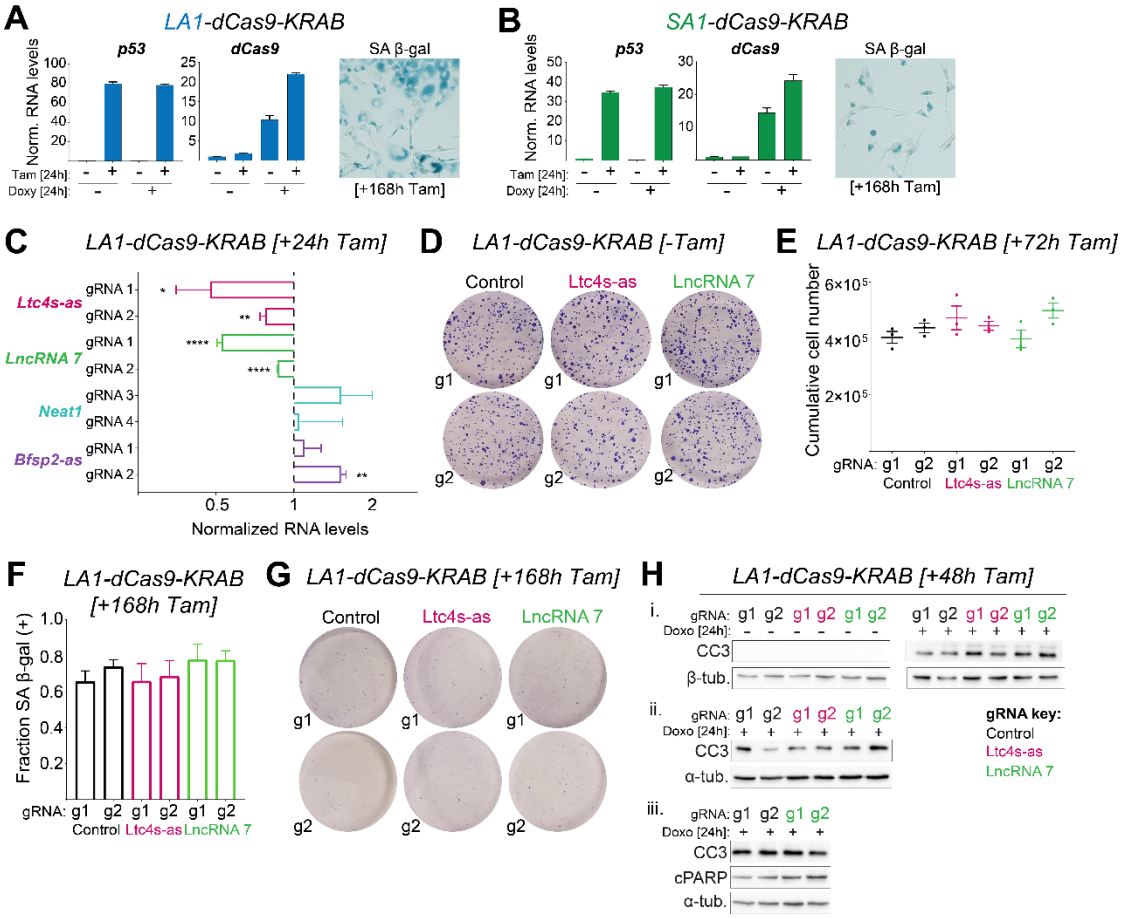


Figure 16: CRISPRi of *lncRNA 7* or *Ltc4s-as* does not affect proliferation or survival in LA *KPR* cells. (A, B) Left= qRT-PCR detection of p53 mRNA and dCas9-KRAB in engineered LA1 **(A)** and SA1 **(B)** cell lines treated as indicated. Data show mean ±SD (n=3 technical replicates). Right= Senescence-associated β-galactosidase staining in indicated cell lines and treatments. **(C)** qRT-PCR analysis of indicated lncRNAs in Tam-treated LA1-dCas9-KRAB cells expressing noted gRNAs. Data show RNA levels relative to *Gapdh*, normalized to the average of two non-targeting control gRNAs. Mean values ±SEM (n=3 biological replicates). * p<0.05, **p<0.01, and ****p<0.0001, unpaired t test. **(D)** Representative images of colony formation assays in untreated LA1-dCas9-KRAB cells expressing indicated gRNAs. **(E)** Cumulative cell numbers in Tam-treated LA1-dCas9-KRAB cells expressing indicated gRNAs after 3d. Data show mean values ±SEM (n=3 biological replicates), one-way ANOVA= no significant differences. **(F)** Quantification of SA β-gal assays in indicated cell lines and treatment. Data show mean ±SD (n=10 technical replicates). **(G)** Representative images of colony formation assays in Tam-treated LA1-dCas9-KRAB cells expressing indicated gRNAs. **(H)** Immunoblots for CC3 (**i, ii**) and cPARP (**iii**) protein levels in indicated cell lines and treatments (Doxo= 1.0 μM). α-tubulin or β-tubulin served as a loading controls.

In **Chapter 4**, we discovered that recruitment of the CRISPR-SAM module to *lncRNA 7*'s TSS in p53-deficient LA1 cells was able to upregulate *lncRNA 7* expression 5-11x fold over a non-targeting control gRNA, reaching 24%-55% of the levels of *lncRNA 7* induction observed upon p53-restoration in these cells (Fig. 11C). While this level of CRISPRa did not yield an observable effect on cellular proliferation, we wondered whether this perhaps stemmed from an insufficient amount of upregulation. Thus, in order to interrogate the functional consequences of *Ltc4s*-as and *lncRNA-7* GOF in our LA1 and SA1 systems, we revisited the CRISPR-SAM system described in **Chapter 4** and made the following modifications to see if we could maximize CRISPRa even further: in place of shortened 'dead' RNAs and nuclease-proficient Cas9, full length gRNAs and nuclease-dead Cas9 fused with four copies of the transcriptional coactivator VP16 (dCas9-VP64) were employed, as the recruitment of this combination of coactivators has been reported to even further improve the synergistic activating effects of CRISPR-SAM (Konermann et al. 2014) (Fig. 15C). dCas9-VP64-expressing LA1 and SA1 cell lines were established, and as expected, p53-restoration in both cell lines led to the acquisition of features of senescence, such as senescence-associated morphological changes and β-Gal staining (Fig. 17A, B).

Using the same two independent gRNAs per lncRNA employed for CRISPRi, we assessed the efficacy of this improved dCas9-VP64 CRISPR-SAM system in transcriptionally activating *Ltc4s-as* and *lncRNA 7*. Two non-targeting gRNAs served as controls. Although we did not observe significant p53-dependent induction of *Ltc4s-as* in control LA1-dCas9-VP64 cells treated with Tam for 24h, we were able to achieve roughly 4x (gRNA 1) and 2x (gRNA 2) upregulation of this

lncRNA in both untreated and Tam treated cells, thus reaching levels of *Ltc4s-as* induction greater than those induced by p53 at this time point (Fig. 17C-left). *Ltc4s-as* was more highly expressed in SA1-dCas9-VP64 cells, induced 3-4x upon 24h p53-restoration in control gRNA-expressing cells (Fig. 17D-left). Interestingly, *Ltc4s-as* CRISPRa was also more effective in this cell line, achieving 9x (gRNA 1) and 3x (gRNA 2) upregulation over controls in untreated cells, or 304% (gRNA 1) and 85% (gRNA 2) of the levels of *Ltc4s-as* seen upon p53-mediated induction in these cells (Fig. 17D-left).

dCas9-VP64 CRISPR-SAM of *lncRNA 7* was also highly efficient. In unrestored LA1-dCas9-VP64 cells, gRNA 1 led to a 16x upregulation of *lncRNA 7*, and gRNA 2 led to a 290x upregulation, compared to nontargeting control gRNAs. Notably, this level of activation in p53-deficient cells represented 65% and 1184% of the lncRNA 7 levels achieved upon p53-restoration in these same cells (Fig. 17C-right). As was seen for *Ltc4s-as, lncRNA 7* CRISPRa was even more effective in SA1-dCas9-VP64 cells, where gRNA 1 promoted 77x upregulation and gRNA 1 433x upregulation of *lncRNA 7*, driving *lncRNA 7* expression to at or 600% p53-driven induction levels (Fig. 17D-right). Thus, dCas9-VP64 CRISPR-SAM of *Ltc4s-as* and *lncRNA 7* was highly efficient in transcriptionally upregulated these two senescence-associated lncRNAs to levels reaching or considerably exceeding endogenous, p53-dependent activation levels.

Given the association between *Ltc4s-as* and *lncRNA 7* induction and senescence-onset, we next asked whether promoting the transcriptional upregulation of these loci affected this process. In the absence of p53, *Ltc4s-as* and *lncRNA-7* upregulation did not have any appreciable effects on LA1-dCas9-VP64

and SA1-dCas9-VP64 proliferation (Fig. 17E), suggesting that expression of these lncRNA loci in themselves was not sufficient to initiate cellular arrest. Colony formation assays in p53-restored LA1-dCas9-VP64 further supported this conclusion, with no differences observed in the number of colonies formed in lncRNA upregulated cells compared to controls, though cells in which *lncRNA 7* had been upregulated appeared to form morphologically distinct colonies (Fig. 17F). Furthermore, neither *Ltc4s-as* nor *lncRNA 7* upregulation affected sensitivity to Doxo-induced apoptosis in either LA1-dCas9-VP64 or SA1-dCas9-VP64 cells (Fig. 17G). Taken altogether, these CRISPRi and CRISPRa data suggested that *Ltc4s-as* and *lncRNA 7* are not integral to either the establishment or maintenance of senescence in response to oncogenic stress and are unlikely to individually contribute to the p53 outcome-specificity observed in LA and SA *KPR* cells.

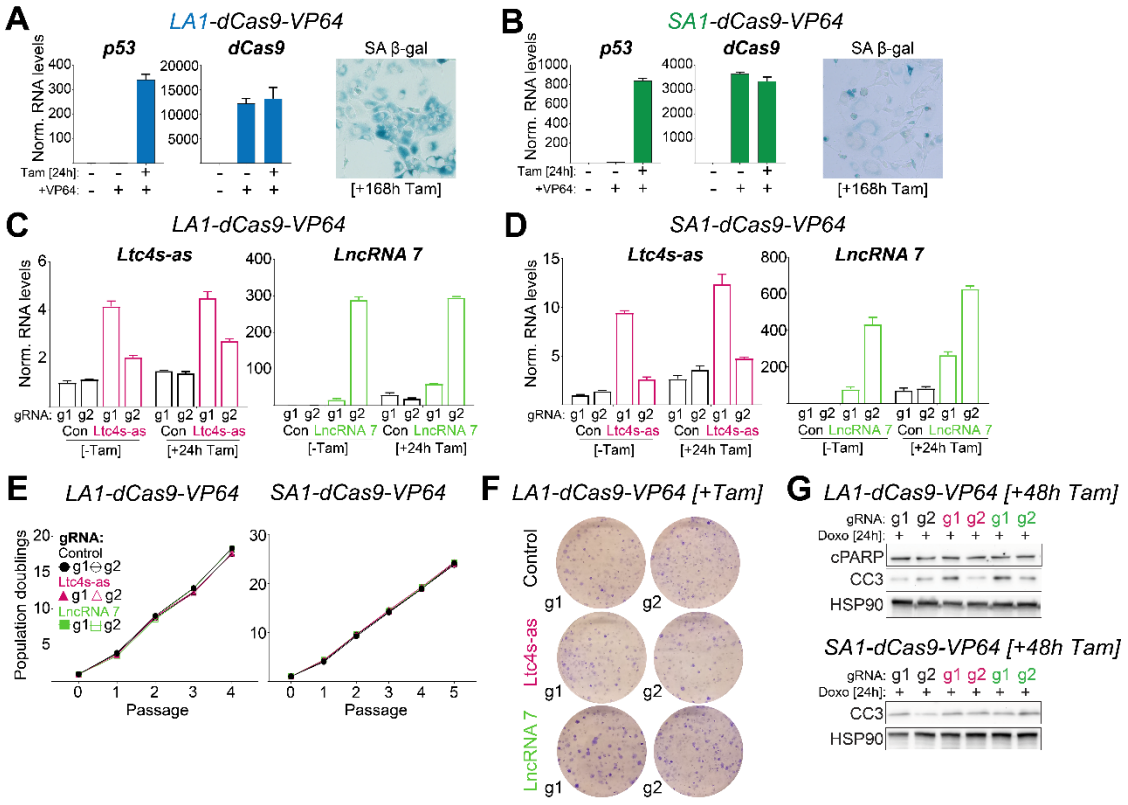


Figure 17: CRISPRa of *lncRNA 7* or *Ltc4s-as* does not affect proliferation or survival in LA and SA *KPR* cells. (A, B) Left= qRT-PCR detection of p53 mRNA and dCas9-VP64 in engineered LA1 **(A)** and SA1 **(B)** cell lines treated as indicated. Data show mean ±SD (n=3 technical replicates). Right= Senescence-associated β-galactosidase staining in indicated cell lines and treatments. **(C, D)** qRT-PCR analysis of indicated lncRNAs in Tam-treated LA1-dCas9-VP64 **(C)** or SA1-dCas9-VP64 **(D)** cells expressing noted gRNAs. Data show RNA levels relative to *Gapdh*, normalized to untreated control gRNA 1. Mean values ±SD (n=3 technical replicates). **(E)** Growth curves of indicated cell lines in the absence of Tam. **(F)** Representative images of colony formation assays in Tam-treated LA1-dCas9-VP64 cells expressing indicated gRNAs. **(G)** Immunoblots for CC3 and cPARP protein levels in indicated cell lines and treatments (Doxo= 1.0 μM). HSP90 served as a loading control.

Given this lack of an obvious senescence phenotype in our CRISPRi and CRISPRa systems and our discovery of a broader *cis*-regulatory lncRNA signature in the p53 transcriptional response to oncogenic stress (**Chapter 3**), we lastly asked whether *Ltc4s-as* and *lncRNA 7* may instead exert more local functions regulating gene expression. In multiple *KPR* cell lines tested, *lncRNA 7* was the only p53-responsive gene within a window encompassing 250 kb upstream of its TSS and 700 kb downstream of its termination site, assessing a generous window given *lncRNA 7* being located in an otherwise gene-depleted region of chromosome 8 (Fig. 8A). As no other genes were either induced or repressed >2-fold upon p53 restoration in this region, we concluded that *lncRNA 7* expression was unlikely to be tied to that of any of its neighbors.

In contrast, *Ltc4s-as* expression positively correlated with that of *Ltc4s*, but not *Maml1*, in multiple *KPR* cell lines. Furthermore, p53RE mutagenesis experiments performed in **Chapter 3** demonstrated that *Ltc4s-as* and *Ltc4s* were co-regulated by p53, independent of *Maml1*. Curious about the effects of transcriptionally silencing or upregulating *Ltc4s-as* on its associated protein coding genes, we assessed *Ltc4s* and *Maml1* levels in our LA1 CRISPRa and CRISPRi cell lines. Consistent with our p53RE mutagenesis data, we saw a strong positive correlation (R=0.85) between *Ltc4s-as* and *Ltc4s* levels and no correlation between *Ltc4s-as* and *Maml1* (R=0.00) (Fig. 18A).

Given the proximity and potential overlap of *Ltc4s-as'* and *Ltc4s'* promoters, raising the possibility that CRISPR-based epigenetic manipulations of one could directly affect the other, we decided to take an alternative approach to reduce *Ltc4s-as* levels by designing an antisense oligonucleotide (ASO) targeting

its third exon. Introducing this ASO into LA1.1 cells led to a roughly 30% decrease in *Ltc4s-as* RNA, both in the presence (+Tam) and absence (-Tam) of p53, compared to a non-targeting control ASO (Con) (Fig. 18B). *Ltc4s* levels were also reduced in these cells, by 27% and 41% in unrestored and p53 restored cells, respectively. As this ASO targeted a unique (*i.e.* non-overlapping) region of *Ltc4s-as* more than 50 kb downstream of either its or *Ltc4s'* TSS, it is unlikely that the observed *Ltc4s* knockdown stemmed from direct ASO-mediated interference of the protein coding gene's transcription. Notably, ASO depletion of *Ltc4s-as* did not significantly affect *Maml1* (Fig. 18B), consistent with the results of our other *Ltc4s-as* LOF and GOF experiments. Taken altogether, these data led us to conclude that either *Ltc4s-as'* transcription or RNA is important for optimal expression of *Ltc4s*, but not *Maml1*, firmly establishing the *Ltc4s-as* locus as an additional, previously undescribed *cis*-regulatory lncRNA locus within the p53 network.

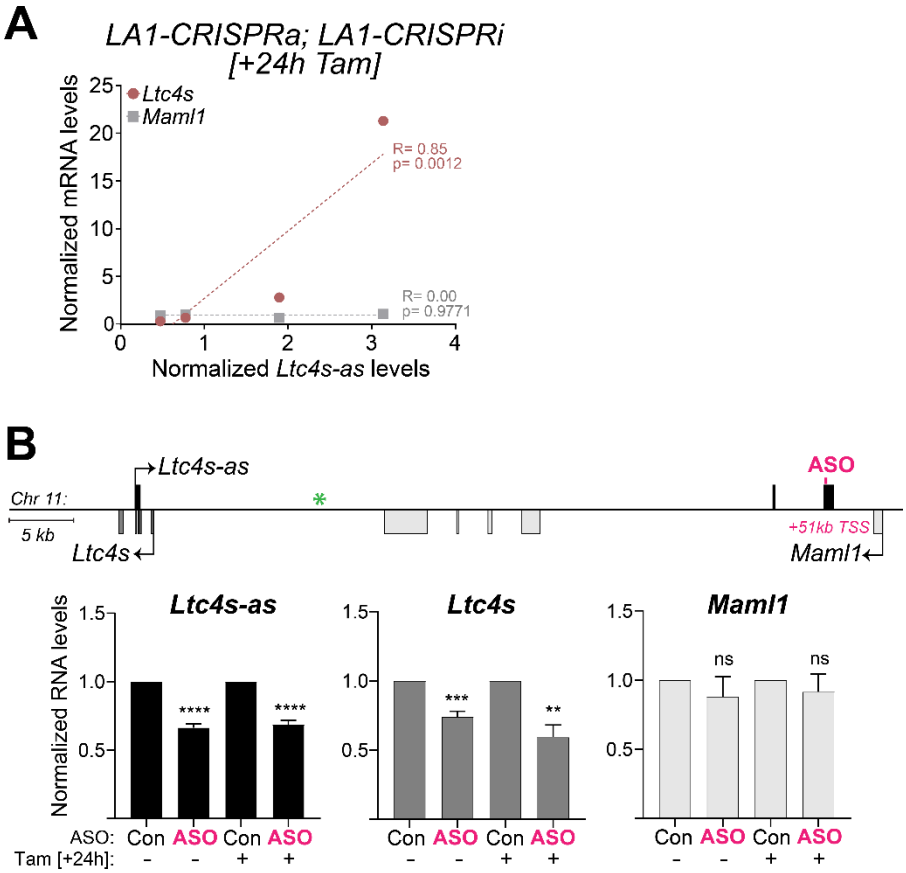


Figure 18: *Ltc4s-as* positively regulates *Ltc4s*, but not *Maml1*, in *cis*. (A) Correlation plot of *Ltc4s-as* levels against *Ltc4s* and *Maml1* levels, as detected by qRT-PCR upon 24h Tam treatment in CRISPRa and CRISPRi experiments performed in LA1 cells. RNA values relative to *Gapdh* were calculated and normalized to control gRNAs within each respective experiment before plotting normalized values. Simple linear regression analyses were performed on *Ltc4s-as* vs *Ltc4s* and *Ltc4s-as* vs *Maml1* levels. Lines of best-fit, goodness of fit values, and significance of slopes are indicated on graph. **(B)** qRT-PCR analysis of *Ltc4s-as, Ltc4s,* and *Maml1* in mock or Tam-treated LA1.1 cells transfected with indicated ASOs. RNA values relative to *Gapdh* were normalized to control (Con) ASO-treated cells in each Tam condition. Data show mean ±SEM (n=4 biological replicates). ** p<0.01, ***p<0.001, and **** p<0.0001, ns= not significant, unpaired t test.

Data summary and conclusions

Broadly, the research detailed in this chapter explores the hypothesis that lncRNA expression patterns can be reflective of functional significance. In the case of p53-dependent senescence driven by oncogenic stress (or in the case of *lincRNA-RAPT1*, genotoxic stress), we were unable to find evidence for lncRNAs significantly contributing to cellular senescence, despite these lncRNAs being transcriptionally activated in a senescence-specific manner. For *lincRNA-RAPT1*, further work (utilizing alternative LOF approaches) will be necessary to determine whether this senescence-associated lncRNA contributes to p53 pathway function in other ways. Of the two lncRNAs we were able to efficiently knockdown in our *KPR* model systems, *lncRNA 7* and *Ltc4s-as*, we did not observe any phenotypes related two key aspects of the senescent state, proliferation and survival, suggesting that these lncRNAs are not necessary for the establishment or maintenance of oncogene-induced senescence. Furthermore, transcriptional upregulation of these lncRNAs did not alter the cell fates of LA and SA cells, demonstrating that induction of these lncRNAs is not sufficient to initiate or accelerate senescence.

One interpretation of these data is that outcome-specific lncRNA expression patterns are the consequence and not the cause of specific cell fates. It is possible that the same yet-to-be determined factors that determine the outcome-specificity of *KPR* cells also influence the outcome-specific expression patterns of lncRNAs, which are as a class known to be more cell- or tissue-specific than mRNAs (Cabili et al. 2011). This is a meaningful conclusion, as questions regarding the biological significance of these oft-observed associations between lncRNA expression

patterns and cellular states are longstanding in the field. Here, we have tested one such correlation in a biological system perfectly set-up to do so.

It is also possible that senescence-specific lncRNAs do functionally contribute to senescence but do so in incremental and/or redundant ways that would not be uncovered by individual lncRNA LOF experiments. Importantly, it is exceedingly rare for the loss of individual protein coding targets of p53 to have dramatic effects on pathway function or tumorigenesis (discussed further in **Chapter 6**). Thus, it remains possible that a network of senescence-associated p53-activated lncRNAs work in concert to ensure a robust, sustained antiproliferative response to oncogenic stress.

Cis-acting lncRNAs, such as the ones we describe in **Chapter 3** and here, would be well-suited to finely regulate a broad, flexible network of genes not directly regulated by p53 themselves towards this end. The *Ltc4s-as* locus illustrates how gene-specific this lncRNA-mediated *cis*-regulation can be, in which we see that *Ltc4s* but not *Maml1* expression is modulated by *Ltc4s-as* expression, despite the lncRNA overlapping both protein coding genes. *Ltc4s'* role in proinflammatory signaling, which is a phenotype associated with senescence *in vivo*, also raises the possibility of a model in which the functional importance of lncRNA-mediated *cis*-regulation could be highly context-specific.

Chapter 6: Summary and Perspectives

Our research expands our understanding of the ways in which lncRNAs contribute to the regulation of gene networks, as well as provides new insights into gene regulation by the central tumor suppressor p53. LncRNAs have garnered a significant amount of attention over the past decade or so as a largely untapped pool of potentially important biomolecules, including within the p53 pathway where many have been implicated as important effectors of p53 function (see **Chapter 1**). But the extent to which lncRNAs contribute to the p53 pathway remains ambiguous. This is in part due to cross-study comparisons of individual lncRNA studies often being complicated by variability in approaches taken or biological conditions under which the p53 pathway was studied. For example, whether lncRNAs contribute to the p53 response in a stress-specific manner is largely unknown. Here, we have employed a powerful *in vitro* model of p53-driven tumorigenesis and multiple state-of-the art lncRNA LOF and GOF tools to address several important questions about the role of p53-activated lncRNAs within the p53 response to oncogenic stress.

As sequencing technologies have become more sensitive and robust, so too has the catalogue of known lncRNA genes and associated transcripts grown (Fang et al. 2018; Uszczynska-Ratajczak et al. 2018). Understandably, determining what fraction of these many thousands of uncharacterized transcripts represent biologically important RNAs has been a pressing challenge in the study of lncRNAs. The operational definition of lncRNA and an incomplete understanding of the relationship between lncRNA primary sequence and lncRNA function have

made *a priori* predictions of importance impractical. Instead, various approaches to further subdivide lncRNAs based on observed expression patterns have often served as starting points for functional studies. These expression pattern-based associations have been especially provocative in the context of cancer, where lncRNAs have been noted to be transcriptionally regulated in ways that exquisitely parallel various aspects of cancer pathophysiology [*e.g.* tissue of origin or metastatic vs nonmetastatic disease (Iyer et al. 2015; Ji et al. 2003; Gupta et al. 2010)]. Our work adds to this wealth of cancer-associated lncRNA data by greatly expanding the number of known lncRNAs regulated by p53. Importantly, our work provides evidence for direct regulation by p53 for many of these previously undescribed lncRNAs, thus giving a more complete picture of the total p53-regulated lncRNA transcriptome.

An additionally important facet of our work is that these lncRNAs are induced by p53 in response to oncogenic stress and may thus represent a stressor-specific cohort of lncRNAs. This is supported by the observation that many lncRNAs that have previously been shown to be activated by p53 in the context of genotoxic stress (**Table 1.1**) did not come up in our list of lncRNAs. While it is possible that this is a reflection of cell type-specific differences rather than stress stimuli, it is worth noting that our *KPR* model allowed us to assess the p53 response to oncogenic stress across six unique cell lines and three distinct tumors/tissue types of origin, thus permitting us to sample a breadth of cellular contexts. Importantly, we identified five lncRNAs (*Pvt1b, Zmat3-as, Bahcc1-as, 2310008N11Rik*, and *Rhod-as)* which were significantly upregulated by p53 across all cell lines and tumor types. Prior to this study, none of these five Core-induced

lncRNAs, nor the majority of the other lncRNAs we identified as p53-activated in our *KPR* model had been previously linked to the p53 pathway. Thus, these data suggest that our work has in fact identified a set of lncRNAs linked to specifically p53 regulation in the context of oncogenic stress and support a previously proposed model in which the transcriptional networks enacted by p53 in response to acute genotoxic stress and oncogenic stress are at least partially distinct from each other (Brady et al. 2011; Kastenhuber and Lowe 2017).

Surprisingly, we found an extremely limited overlap between the lncRNAs identified in our work and previously published p53-activated lncRNAs, restricted to only *lincRNA-p21* and *Neat1*, which are established direct targets of p53 (Huarte et al. 2010; Mello et al. 2017), and *Malat1*, which has been reported to be regulated by a distal p53 bound enhancer (Melo et al. 2016). Thus, even lncRNAs that have been demonstrated to be p53-responsive in other *in vitro* models of oncogenic stress were not identified as such in our *KPR* model. For some lncRNAs, such as the human-specific lncRNA *PANDA*, our data simply confirm previously noted absences of orthologous mouse genes. For others (*e.g. GUARDIN, LED, Linc-Pint*), it is possible that our results reflect species-specific differences in p53 network activation. However, it is important to note that most studies of human lncRNAs in the context of the p53 response to oncogenic stress have been performed in a limited number of human cancer cell lines, thus making cell line-specific differences in p53 regulation an equally likely possibility. Notably, our identification of a p53-responsive human lncRNA at the genomic location syntenic to *2310008N11Rik (lncRNA 7)* in this work and our lab's previous description of an orthologous human *PVT1B* isoform (Olivero et al. 2020) demonstrate that at

least two of the five lncRNAs we found to be activated by p53 across all *KPR* tumor types are also regulated by p53 in human cells, under at least some stress conditions. The full extent of human conservation of our newly identified catalogue of p53-regulated lncRNAs remains to be explored in future studies.

Our work also considerably increases our understanding of the broader p53 transcriptional response to oncogenic stress. It is clear that transcriptional regulation is a key aspect of p53-mediated tumor suppression (Brady et al. 2011; Jiang et al. 2011), but the full set of specific p53 targets that underlie said tumor suppression are elusive. Moreover, p53 suppresses tumorigenesis through a variety of cellular processes, two important examples being apoptosis and cellular senescence, though by what mechanisms these different cellular processes are initiated in different tumor types is less clear. By characterizing the p53 response across multiple tumor types facing the same oncogenic stressor, we shed new light onto these fundamental questions of p53 regulation in several ways. First, we identified a set of target genes activated by p53 in LA, SA, and LY *KPR* cells, suggesting that this small but robustly-activated group of genes may be critical for p53 sensing and/or responding to oncogenic signaling. These Core genes included important mediators of central p53 processes, such as cell cycle regulation and apoptosis, as well as genes involved in autophagy, metabolism, and tumor microenvironment signaling, which are more recently appreciated p53-regulated processes that have been associated with tumor suppression (Kaiser and Attardi 2018). As expected, many of these genes represented known p53 targets that are activated by p53 in other stress conditions as well. This was in contrast to the panel of lncRNAs we uniquely identified as p53 targets for the first time in this work.

Thus, it may be the case that p53-activated lncRNAs are more stressor-specific than protein coding p53 target genes.

Additionally, our data strongly suggest that tumor type-specific differences in the cellular consequences of p53 stabilization do not stem from significant differences in p53 transcriptional activation. This is in contrast to some postulated models of p53 decision-making, in which the expression of clearly delineated outcome-specific gene networks is proposed to underlie outcome-specificity (Weinberg et al. 2005; Schlereth et al. 2010). Instead, our data is in line with more recent reports where genomic p53 binding patterns (Nikulenkov et al. 2012; Botcheva and McCorkle 2014) and p53 network activation correlated more with cell identity than stress stimuli or ultimate cellular outcome enacted in a number of human and mouse cell lines (Tonelli et al. 2017; Catizone et al. 2019; Zhang et al. 2013b). These cell identity-specific factors may be related to p53-dependent functions but have fate-determining patterns of regulation that are either partially p53-independent or pre-exist p53 pathway activation. “Mitochondrial priming” is one example of this, in which a given cell type’s characteristic ratio of mitochondrially-localized pro-apoptotic versus anti-apoptotic BCL-2 family member proteins can determine whether said cell type is inherently apoptosis-resistant or apoptosis-prone (Chonghaile et al. 2011; Liu et al. 2013a). However, it is indisputable that the p53 network is regulated at multiple, intricately-woven levels. We acknowledge that stimuli-specific inputs such as the nature of the stress encountered can also influence p53 pathway activation in some settings, for example by dictating where in the genome p53 binds (Menendez et al. 2013) or by altering the dynamics of p53 protein stabilization (Purvis et al. 2012). Our work

joins this dialogue to provide additional, novel perspective on this fundamental question regarding how inputs into the p53 network modulate network activation.

Interestingly, we uncovered an unexpected association between p53-mediated gene repression and senescence. In contrast, gene repression downstream of p53 was not a feature of the core transcriptional response to oncogenic stress, nor apoptosis. How p53 represses this senescence-specific gene network remains to be determined, as experimental evidence for direct gene repression by p53 is very limited (Vousden and Prives 2009). P53's role in indirect repression is clearer, as p21 activation downstream of p53 is important for gene repression by the E2F/DREAM complex (Engeland 2018). Intriguingly, a recent cross-species metanalysis of orthologous human and mouse genes regulated by p53 found that p53-repressed genes were more likely to be similarly regulated across the two species than p53-activated genes (Fischer 2019), raising the fascinating question of whether a link between p53-dependent gene repression and senescence is also conserved. The functional importance of this p53 gene repression in senescence is largely unknown, though our work does provide one specific example by demonstrating that *Pvt1b/Myc* negative co-regulation is necessary for the maintenance of growth arrest in LA *KPR* cells. However, our lncRNA data suggest that most p53-dependent gene repression is not accomplished through lncRNA *cis*-regulation. Further exploring the mechanisms by which p53 gene repression is established and the biological importance of repressive p53 regulation are important avenues of inquiry for future work.

Similar to other genome-wide profiles of the p53 network, we found that key protein coding p53 targets involved in cell cycle arrest and apoptosis were activated

by oncogenic stress in an outcome-agnostic manner. This in part led us to explore whether lncRNAs, which did exhibit outcome-specific activation in our data, could underlie outcome-specificity. LncRNAs have previously been hypothesized to be drivers of cell fate due to their cell identity- or state-specific expression patterns (Cabili et al. 2011; Derrien et al. 2012). However, our lncRNA GOF and LOF studies did not yield any evidence for any individual lncRNA contributing to the establishment of cellular senescence in LA and SA *KPR* cells, even when lncRNA expression patterns were highly correlated with senescence and anticorrelated with apoptosis in our *KPR* model. Thus, while examples of lncRNAs demonstrably regulating cell fate decisions do exist (Kretz et al. 2013; Grote et al. 2013; Lewandowski et al. 2019), our research joins an growing body of literature (Goudarzi et al. 2019) encouraging caution when interpreting correlative lncRNA associations as functional in the absence of experimental validation. It is possible that our panel of senescence-associated lncRNAs are not drivers of senescence but are rather transcriptional read-outs of specific cellular characteristics that are unique to cells primed to undergo senescence in response to p53 activation. Alternatively, it is also possible that these lncRNAs function at a longer time-scale than investigated in this work and contribute to the long-term maintenance of senescence, which is a uniquely stable biological state.

However, our single lncRNA LOF data are also consistent with the functional redundancy of the p53 network hinted at in our *KPR* transcriptome profiling data and other studies. In this paradigm of p53 network function, the loss of expression of one or even several p53 target genes can be compensated for by others to maintain a robust p53 response. For example, in one recent shRNA

screen for p53 targets important for mediating growth arrest in human osteosarcoma cells, the only significant hit identified was p53 itself (Andrysik et al. 2017), suggesting that even the loss of key protein coding mediators of growth arrest can be insufficient to bypass p53-directed growth arrest. Importantly, there has not been a single p53 target gene to date in which genetic knockout *in vivo* is able to recapitulate the dramatic spontaneous tumorigenesis observed in p53 knockout mice, including mice lacking key drivers of p53-dependent cell cycle arrest [*p21* (Brugarolas et al. 1995); *Gadd45α* (Hollander et al. 1999); *PML* (Gang Wang 1998)], apoptosis [*Puma*; *Noxa* (Villunger 2003); *Perp* (Ihrie et al. 2006); *Bax* (Knudson et al. 2001)], or combinations of target genes important for these processes (Michalak et al. 2008; Valente et al. 2013). Nevertheless, these protein coding genes demonstrably contribute to p53-dependent anti-growth responses in various *in vitro* settings (perhaps reflecting tissue- or cell type-specific functions) and/or in the context of specific *in vivo* cancer models. Thus, many of the lncRNAs we have found to be activated by p53 in response to oncogenic stress may also play subtle, redundant, or context-specific roles in the p53 pathway that collectively support the overall robustness of this tumor suppressive stress response. This functional subtlety and/or redundancy seems to be likely true for many lncRNAs in other tightly regulated biological processes as well, such as development (Goudarzi et al. 2019).

Our research strongly suggests that *cis*-regulatory lncRNAs predominate within the p53 network. While we cannot formally exclude *trans* activities for all identified lncRNAs, the vast majority of lncRNAs found in this study were enriched at the chromatin, relatively low in abundance, and transcriptionally linked to the

expression of their neighboring genes, all of which are features associated with *cis*-regulation. Moreover, our thorough locus investigation of one of the more abundant, partially cytoplasmically-localized lncRNAs, *2310008N11Rik (lncRNA 7)*, did not reveal a *trans* phenotype. Thus, our research adds to a growing body of work suggesting that most p53-activated lncRNAs function in the nucleus to regulate the transcriptional output of the p53 network (reviewed in **Chapter 1**).

We demonstrate that lncRNA locus-mediated *cis*-regulation is a much more common mechanism by which lncRNAs achieve this than previously appreciated. As our characterization of the *lincRNA-Gadd45γ* locus demonstrates, this *cis*-regulation can tie-in protein coding genes not directly regulated by p53 into the broader p53 transcriptional network. Our LOF characterization of several other lncRNA loci suggests that this positive *cis*-regulation is not unique to this locus. Conversely, our work also demonstrates that *Pvt1b*'s regulation of *Myc* represents an exceptional example of *cis*-repression within the p53 network, suggesting that lncRNAs do not significantly contribute to p53-mediated gene repression. Thus, we show that lncRNAs can fine-tune p53's transcriptional response on a locus-by-locus basis, with likely locus-specific RNA-dependent and RNA-independent mechanisms.

Furthermore, our discovery of *Pvt1b*'s importance in mediating growth arrest in LA *KPR* cells demonstrates that lncRNA-mediated *cis*-regulation can be important for p53 pathway function downstream of oncogenic stress. Our data suggest that this phenotype is not driven through changing the cell fate of LA cells, which remained resistant to apoptosis, but rather through *Pvt1b*'s effect on *Myc* levels. Indeed, other work from our lab further supports the conclusion that the

cellular consequences of *Pvt1b* loss are solely mediated through *Myc* derepression (Olivero et al. 2020). *Myc* is a universal regulator of cellular growth, thus it is not surprising that dysregulating *Myc* levels would have profound effects on cellular proliferation. The functional consequences of disrupted *cis*-regulation at other p53-activated lncRNA loci may be more context-specific in a manner related to the normal functions of the protein coding genes under regulation.

The senescence-associated *Ltc4s-as* locus and its positive reinforcement of *Ltc4s* expression may represent an example of this scenario. *Ltc4s* is an enzyme involved in the catalysis of leukotrienes, which are important lipid proinflammatory molecules (Funk 2001). One hallmark of senescence is the secretion of a variable suite of proinflammatory and ECM remodeling molecules that drive senescence-associated autocrine and paracrine signaling (*i.e.* senescence-associated secretory phenotype; SASP), which can be oncogenic or tumor suppressive (Campisi 2013; Collado and Serrano 2010). Leukotriene signaling has recently been shown to be a part of the SASP of several senescent human cell lines and linked to senescence-associated lung fibrosis (Wiley et al. 2019). Thus, we imagine that *Ltc4s-as*-dependent *cis*-regulation of *Ltc4s* could be important for phenotypes associated with cellular senescence or inflammation more broadly in contexts beyond the *in vitro* stress models we assessed in this work.

Lastly, we do not claim that all lncRNA-mRNA co-regulatory relationships are indicative of direct *cis* regulation. Rather, they are demonstrative of linked gene regulation within the p53 network and provide a conceptual platform for potential secondary, fine-tuning transcriptional inputs from lncRNA loci downstream of the

primary input from p53 at these lncRNA-mRNA transcriptional units. Prior to our work, direct lncRNA-mediated *cis*-activation within the p53 network had only been demonstrated for *lincRNA-p21*; we expand this to two novel lncRNAs chosen from our identified catalogue of co-regulated loci. Support for *Ltc4s-as* and *lincRNA-Gadd45γ* being representative examples of a wider *cis*-activating cohort of p53-induced lncRNAs includes the fact that co-regulation in addition to chromatin enrichment has been shown to be predictive of *cis*-regulation in other cellular systems (Werner et al. 2017). Whether true, lncRNA locus-driven *cis*-activation contributes to the positive co-regulation observed at the other loci we uncovered will be work for future investigation.

As for *cis*-repression, we speculate that the presence of a negative co-regulatory relationship is likely to be predictive of true lncRNA mediated *cis*-regulation, especially when negative co-regulation involving intergenic (*i.e.* non-overlapping) lncRNA loci is observed. This is based on the rationalization that lncRNA *cis*-activation may more readily evolve through either the simple act of a lncRNA locus being transcribed or through the modulation of pre-existing transcription-activating signals at a given locus. For example, the transcription of a lncRNA locus may be sufficient to promote the recruitment of positive transcriptional regulators or RNA pol II itself to another genomic region as well, if said region is in close proximity to the lncRNA locus in either linear or three dimensional genomic space, in much the same way enhancers are thought to function. In contrast, *cis*-repression is likely to necessitate additional molecular inputs that are able to overcome and reverse these activating inputs (*e.g.* the recruitment of repressive epigenetic machinery by *XIST*). Furthermore, the rarity

of such negative co-regulatory relationships compared to positive ones in our data and in other genome-wide surveys of lncRNA regulatory patterns (Basu and Larsson 2018) further suggests that sites of negative lncRNA-mRNA co-regulation arise from an evolutionary trajectory distinct from those of positive co-regulation. *Pvt1b*'s activation by p53 and *cis*-repression of *Myc* (a transcription factor in its own right) suggest that such negative *cis*-regulatory relationships may be most likely to emerge at key nodes in transcriptional networks, functioning to adjust critical biological outputs.

Final remarks

Mammalian genomes are complex, both in organization and in regulation. It's long been appreciated that lncRNAs can significantly contribute to both of these features, but our understanding of the importance of lncRNAs in complex regulatory networks remains incomplete. One emerging regulatory theme our work expands upon is that of lncRNA-mediated *cis*-regulation [*e.g.* (Dimitrova et al. 2014; Olivero et al. 2020; Joung et al. 2017; Werner et al. 2017; Engreitz et al. 2016)], namely its previously underappreciated prevalence in the centrally important tumor suppressive p53 pathway. Unlike transcription factors or other nuclear-acting proteins, which are invariably translated distally from their sites of action, lncRNA loci are uniquely positioned to rapidly respond to stimuli by engaging with and modulating the expression of other nearby genes. Within the p53 transcriptional network, we find at least one example of lncRNA-mediated *cis*-regulation being important for tumor suppression in the context of oncogenic stress and speculate that lncRNA local regulation may contribute to the highly tissue- and stimuli-specific nature of the p53 stress response in other settings as well. The ability of *cis*-acting lncRNA loci to dynamically and flexibly fine-tune transcriptional outputs may be especially important in stress responses in general, warranting closer examination of this mode of lncRNA-mediated gene regulation in other tumor suppressive or oncogenic transcriptional networks.

Chapter 7: Materials and Methods

Cell lines and treatments: *KPR* cell lines were previously established from spontaneously arising primary lung adenocarcinoma (LA1, LA2), sarcoma (SA1, SA2), or lymphoma (LY1, LY2) tumors isolated from *Kras$^{LA2\text{-}G12D/+}$; p53$^{LSL/LSL}$; Rosa-CreER^{T2}* mice (Feldser et al. 2010). Puromycin-sensitive LA1 cells (LA1.1) were previously described (Olivero et al. 2020). In brief, LA1 cells were transiently transfected with a guide RNA targeting the ORF of puromycin to inactivate the puromycin-resistance gene expressed from the *Rosa-CreER^{T2}* allele, followed by clonal expansion. gRNAs were introduced cloned downstream of a U6 promoter in a lentiviral vector co-expressing SpCas9 and GFP (BRD004; gift from the Broad Institute, MIT). Puromycin-sensitive LA2 cells (LA2.5) were similarly generated. Primary WT mouse embryonic fibroblasts (MEFs) were isolated from embryos at E13.5 from timed matings of WT C57BL/6J mice. *LincRNA-RAPT1$^{fl/fl}$; Rosa26-CreER^{T2}* MEFs were isolated at E13.5 from timed homozygote matings, while littermate *lincRNA-RAPT1$^{+/+}$* and *lincRNA-RAPT1$^{-/-}$* MEFs were isolated from *lincRNA-RAPT1$^{+/-}$* (heterozygote) matings. All primary MEF experiments were performed at passages 2-10. *p53$^{LSL/LSL}$; Rosa-CreER^{T2} (p53-restorable)* MEFs were previously described (Ventura et al. 2007).

LA and SA cells were maintained in DMEM (GIBCO) supplemented with 10% FBS, 50 U/mL pen/strep, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 0.055 mM β-mercapatoethanol. LY cells were cultured in IMDM (GIBCO) supplemented with 10% FBS, 50 U/mL pen/strep, and 0.055 mM β-mercapatoethanol. MEFs were cultured in DMEM supplemented with 15% FBS,

50 U/mL pen/strep, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 0.055 mM β-mercapatoethanol. All cells were grown at 37°C in a humidified incubator with 5% CO2.

To excise the LSL cassette and restore p53 expression, *KPR* cells and *p53-restorable* MEFs were treated with 0.5 μM 4-hydroxytamoxifen (Tam, Cayman Chemical Company) at time points indicated in text and figure legends. To ablate *lincRNA-RAPT1* expression in the conditional knockout system, *lincRNA-RAPT1$^{fl/fl}$; Rosa26-CreER^{T2}* MEFs were treated with 0.5 μM Tam for 72-96h. To induce DNA damage, cells were treated with Doxorubicin (Doxo, Sigma-Aldrich), at dosage concentrations and lengths indicated in text and figure legends. To activate Tetracycline-dependent gene expression in LA1-TRE-dCas9-KRAB and SA1-TRE-dCas9-KRAB cell lines, cells were treated with 2 μg/mL Doxycycline (Doxy, Sigma-Aldrich) for lengths of time indicated in text and figure legends.

Viral transductions: Lentivirus was produced in 293 cells by co-transfecting lentiviral constructs with pCMV-Δ8.2 (Addgene plasmid #8455) and pCMV-VSV-G (Addgene plasmid #8454) viral packaging constructs. Fresh virus-containing supernatants supplemented with 4 μg/mL polybrene (Millipore Sigma) were used to infect cells in 2-3 consecutive lentiviral infections, collected and delivered at 24h intervals. For retroviral transduction experiments, virus was produced in Phoenix cells transfected with retroviral constructs. Viral supernatant was collected at four consecutive 12h intervals, supplemented with 4 μg/mL polybrene, and immediately used to infect cells. Following infections, cells were selected with Puromycin (2 μg/mL for MEFs, 5 μg/mL for *KPR* cell lines), hygromycin (400

μg/mL for MEFs, 800 μg/mL for *KPR* cell lines), neomycin (400-600 μg/mL), or blasticidin (5-10 μg/mL), as appropriate. All viral constructs and gRNA/dRNA sequences used in this work can be found in Supplementary Tables 1 and 2.

LncRNA p53RE mutagenesis: CRISPR-Cas9 mutagenesis was performed by infecting LA1.1 or LA2.5 cells with site-specific gRNAs cloned downstream of an hU6 promoter in BRD001, a lentiviral vector co-expressing SpCas9 and an IRES-driven puromycin-resistance gene (gift from the Broad Institute, MIT). dTomato-targeting gRNA served as a negative control. Mutagenesis efficiency was assessed by Sanger sequencing using PCR and sequencing primers in Supplementary Table 2 and quantified using the TIDE (Tracking of Indels by Decomposition) webtool (Brinkman et al. 2014). For sequencing of individual mutated alleles, PCR products were subsequently cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and individual clones submitted for Sanger sequencing.

LincRNA-Gadd45γ CRISPRi and CRISPRa: CRISPRi was performed by expressing 15-mer 'dead RNAs' (dRNAs) (Dahlman et al. 2015) from BRD001, targeting a region downstream of the transcription start site of *lincRNA-Gadd45γ* or a non-targeting control region (Con). For CRISPRa experiments, an SpCas9-IRES-GFP-expressing stable cell line was first generated by introducing lentiviral vector BRD004 (gift from the Broad Institute, MIT) into LA1 cells and selecting for GFP expression by FACS. CRISPRa of *lincRNA-Gadd45γ* was subsequently achieved by the CRISPR-SAM system employed in (Olivero et al. 2020). In brief, MS2-tagged gRNAs and HSF-p64-MBP (SAM module) were stably introduced into LA1-SpCas9-GFP cells via a single vector co-expressing both, lenti SAM Hygro (generated by E. Martínez-Terroba), followed by hygromycin selection.

LncRNA 7 exogenous overexpression and E1A MEF sensitization: Full-length lncRNA 7 cDNA was synthesized as a gene block (IDT) and cloned into MSCVpuro and pWZL Hygro (Addgene# #18750) retroviral vectors. Empty vectors (EV) served as controls. MSCVpuro was used to deliver *lncRNA 7* into primary MEFs, while pWZL Hygro was used for LA and SA *KPR* cells, followed by puromycin or hygromycin selection, respectively. For E1A sensitization experiments, pLPC 12S E1A puro (Addgene plasmid #18740) or EV control retroviral constructs were introduced into control or *lncRNA 7*-overexpressing *p53-restorable* MEFs and puromycin selection applied.

LncRNA 7 and senescence-associated lncRNAs CRISPRi and CRISPRa*:* For *lncRNA 7* CRISPRi experiments in *p53-restorable* MEFs and LA1.1 cells, stable dCas9-KRAB cell lines were first generated by introducing pHR-SFFV-dCas9-BFP-KRAB (Addgene plasmid #46911) into cells via lentiviral transduction and selecting for BFP expression by FACS. gRNAs targeting *lncRNA 7*'s TSS were subsequently delivered to cells by BRD003, a lentiviral vector for expressing hU6-driven SpCas9 gRNAs (gift from the Broad Institute, MIT), and cells selected for puromycin resistance. CRISPRi efficacy was assessed in comparison to dTomato-targeting Con gRNA. For *lncRNA 7* CRISPRa experiments performed in **Chapter 4**, dCas9-VP64-expressing cell lines were generated by introducing Lenti dCas-VP64_Blast (Addgene plasmid #61425) into LA1.1 and LA1 cells and selecting for blasticidin resistance. gRNAs were lentivirally delivered by either BRD003 for LA1.1 cells or pLenti BsmBI sgRNA hygro (Addgene plasmid #62205) for LA1 cells, due to the latter's puromycin resistance. After the appropriate selection regimes,

lncRNA 7 CRISPRa was assessed compared to the same dTomato-targeting Con gRNA.

For CRISPRi experiments performed in **Chapter 5**, Doxy-inducible dCas9-KRAB cell lines were generated by introducing Phage TRE-dCas9-KRAB (Addgene plasmid #50917) into LA1 and SA1 cells and selecting for neomycin/G418 resistance. gRNAs were introduced into cells by lentiviral delivery in pLenti BsmBI sgRNA hygro, followed by hygromycin selection. For CRISPRa experiments performed in **Chapter 5** (VP64-SAM), dCas9-VP64 cell lines were generated by introducing Lenti dCas-VP64_Blast into LA1 and SA1 cell lines. MS2-tagged gRNAs and HSF-p64-MBP were subsequently introduced into cells by lenti SAM Hygro, followed by hygromycin selection. For calculations of CRISPRi and CRISPRa efficiencies, RNA values relative to an endogenous control gene (*Gapdh*) were individually calculated for two independent non-targeting control gRNAs and averaged, yielding a control average relative expression value. LncRNA TSS-targeting gRNA relative expression values were then normalized to this control average relative expression value.

RNA isolation and qRT-PCR: For RNA analysis of MEF samples, RNA was isolated with the RNeasy Mini Kit (Qiagen). For RNA analysis of *KPR* samples, RNA was isolated using TRIzol reagent (Invitrogen). 1.0 μg total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). SYBR Green PCR master mix (Kapa Biosystems) was used for quantitative PCR in triplicate reactions. Primers sequences are listed in Supplementary Table 3. RNA expression levels relative to the housekeeping gene

Gapdh were calculated using the ddCt method and normalized to control samples when noted.

Genotyping: *KPR* cells were lysed in 1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0, 20% SDS, 5 M NaCl supplemented with 1 mg/mL Proteinase K at 55 °C. Total genomic DNA was extracted by isopropanol precipitation, and genotyping was performed by PCR with primers listed in Supplementary Table 3.

Immunoblotting: Cells were lysed in 2x Laemmli buffer (100 mM Tris-HCl pH6.8, 200 mM DTT, 3% SDS, 20% glycerol) at $1x10^4$ cells/µL. Samples were heated at 95°C for 7 minutes and passed through an insulin syringe. Protein from $1x10^5$ cells was separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). After blocking (5% milk-PBST), membranes were incubated overnight at 4°C in primary antibody, then 1hr at RT in secondary antibody. The following antibodies were used: anti-p53 (1C12) (1:1,000, 2524S, Cell Signaling Technology), anti-p21(F-5) (1:250, sc-6246, Santa Cruz Biotechnology), anti-cleaved caspase 3(Asp175) (1:250, 9661, Cell Signaling Technology), anti-cleaved PARP (Asp214)(7C9) (1:500, 9548S, Cell Signaling Technology), anti-α tubulin (DM1A) (1:1000, 3873S, Cell Signaling Technology), anti-β tubulin (9F3) (1:1000, 2128S, Cell Signaling Technology), anti-Hsp90 (C45G5) (1:1,000, 4877S, Cell Signaling Technology), goat anti-mouse secondary antibody (1:50000,1706516, Bio-Rad), and donkey anti-rabbit secondary antibody (1:50000, 711-035-152, Jackson ImmunoResearch). Protein bands were visualized using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Quantitative densitometry analysis was performed using background adjusted volume intensities normalized to loading control on Image Lab software (Bio-Rad).

Gene set enrichment analysis (GSEA): GSEA was performed on p53-induced and p53-repressed gene sets identified by RNA-seq analysis using the Molecular Signature Database (MSigDB) (Subramanian et al. 2005).

Antisense oligonucleotide (ASO) knockdown: For *lincRNA-Gadd45γ* knockdown, 1 μM lncRNA-targeting (ASO1, ASO2) or control (CON) antisense LNA Gapmers (Exiqon, QIAGEN) were transfected into $1x10^6$ LA1.1 cells treated with Tam for 48h using the Amaxa Primary Mammalian Epithelial Cells Nucleofector Kit (Lonza, VPI-1005) and the Nucleofector 2b Device (Lonza). Knockdown of *lincRNA-Gadd45γ* and subsequent effects on *Gadd45 γ* expression were assayed by qRT-PCR at 24h post-transfection (72h Tam treatment). For *Ltc4s-as* knockdown, 1 μM lncRNA-targeting (ASO) or control (Con) antisense LNA Gapmers were transfected into $3x10^5$ LA1.1 cells using Attractene transfection reagent (Qiagen). *Ltc4s-as*, *Ltc4s*, and *Maml1* expression was assessed by qRT-PCR at 24h post-transfection, in mock- or Tam-treated cells. ASO sequences are listed in Supplementary Table 2.

Subcellular fractionation: Subcellular fractionation was performed as previously described (Conrad and Ørom 2017) with some modifications. Briefly, cells were harvested by trypsinization, rinsed once in PBS, and re-suspended in

PBS-1 mM EDTA. Half of this cell suspension was set aside for whole cell (WC) RNA isolation using TRIzol reagent. The remaining cells were lysed in 0.4 mL cell lysis buffer for 5 min on ice. Lysis buffer composition for primary MEFs= 10 mM TrisHCl pH 7.5, 0.15% NP-40, 150 mM NaCl, 100 U/mL RNase-IN (Promega); for LA1.1 cells= 10 mM TrisHCl pH 7.5, 0.20% NP-40, 150 mM NaCl, 100 U/mL RNase-IN. Lysate was layered on a sucrose cushion (24% w/v sucrose, 150 mM NaCl, 10 mM TrisHCl pH 7.5,100 U/mL RNase-IN) and centrifuged for 10 min at 3500g for primary MEFs and at 3000g for LA1.1 cells, yielding the cleared cytoplasmic fraction (supernatant) and pelleted nuclei. Nuclear pellets were washed once in PBS-1 mM EDTA, re-suspended in 0.25 mL glycerol buffer (50% glycerol, 20 mM Tris-HCl pH 7.5, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 100 U/mL RNase-IN), and lysed by the immediate addition of an equal volume nuclear lysis buffer (10 mM HEPES pH 7.6, 7.5 mM MgCl2, 0.2 mM EDTA, 300 mM NaCl, 1% NP-40, 1 mM DTT, 1M Urea, 100 U/mL RNase-IN) with 2 min incubation on ice. Centrifugation for 2 min at 18800g yielded the nucleoplasmic and chromatin-associated fractions in the supernatant and pellet, respectively. Chromatin pellets were washed once in PBS-1 mM EDTA and solubilized in 1 mL TRIzol reagent by syringing. RNA was extracted from the cytoplasmic and nucleoplasmic fractions using TRIzol-LS (Invitrogen) and from the chromatin-associated fraction using TRIzol, following the manufacturer's protocols. Subcellular RNA enrichment was determined by qRT-PCR, normalizing fraction Ct values to WC Ct values. Cytoplasmically-enriched RNA *Rn7s1* and chromatin-enriched RNA *Kcnq1ot1* served as fractionation quality controls. Primers sequences can be found in Supplementary Table 3.

Single-molecule RNA FISH (smRNA-FISH): smRNA-FISH was performed as previously described (Olivero et al. 2020). In brief, cells were grown on coverslips and fixed for 10 min at RT in 4% methanol-free formaldehyde (Thermo Scientific). Cells were permeabilized overnight at 4°C in 70% EtOH-DEPC H_2O. Coverslips were then transferred to a hybridization chamber, equilibrated in Wash Buffer A (Stellaris, LGC Biosciences) supplemented with formamide (Millipore Sigma), and incubated overnight at 30°C with probes diluted 1:50 in Hybridization Buffer (Stellaris, LGC Biosciences) with formamide. The following day, cells were washed twice for 30 min at 30°C in Wash Buffer A, incubated for 5 min at RT in Wash Buffer B (Stellaris, LGC Biosciences), and mounted in Vectashield Mounting medium with DAPI (Vector Laboratories). *LincRNA-RAPT1* spanning probes were labeled with Quasar® 570 (LGC Biosearch Technologies). Images were captured using an Axio Imager 2 microscope with a PlanApo 63x 1.4 oil DIC objective lens (Zeiss) and edited using Adobe Photoshop.

Senescence-associated β-gal assay: Primary MEFs were cultured in 0.1 μM Doxo for 168 hours to induce senescence. LA and SA *KPR* cells were grown in normal media or media supplemented with Tam for 168 hours. Cells were fixed for 15 min at RT in 0.5% glutaraldehyde diluted in PBS, washed 3 times in 1 mM $MgCl_2$ in PBS (not pH adjusted) and once in 1 mM $MgCl_2$ in PBS (pH 5.5 for MEFs and LA cells; pH 5.0 for SA cells). Cells were then stained in β-gal staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mg/mL X-gal, 1 mM $MgCl_2$ in PBS, pH 5.0 or 5.5) for 8-12h at 37°C. Cells were rinsed once in PBS before imaging.

Proliferation and colony formation assays: Growth curves were generated for various transgenic and genetically engineered primary MEF lines, *p53-restorable* MEFs, and mock-treated (no Tam) *KPR* cell lines by passaging cells, counting cumulative cell numbers, and calculating population doublings over time.

p53-proficient growth or growth arrest bypass was assessed by plating cells in Tam-supplemented media and counting cells at intervals of indicated lengths, changing media every three days. Cumulative cell numbers over time were plotted as the average of three independent experiments, when appropriate. For p53-proficient colony formation assays, $4x10^5$ cells/6 cm dish were grown in Tam-supplemented media and monitored for colony growth. For p53-deficient (-Tam) colony formation assays, $1x10^5$ cells/6 cm dish were plated and grown in normal media. After 7-10 days, dishes were washed with PBS, fixed in 0.5% Crystal Violet; 25% methanol for 10 minutes, and thoroughly washed in ddH_2O prior to colony counting. Whenever shown, averages represent three independent experiments.

BrdU incorporation: Cells were grown on coverslips to roughly 50% confluency in Tam-supplemented media. At 22h post-Tam treatment, cells were labeled with 10 μM 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) for 2h for *KPR* cells or 4h for *p53-restorable* MEFs, washed once in PBS, and fixed overnight at -20 °C in Fixation Buffer (75% ethanol, 25% 0.05 M glycine pH 2.2). Fixed cells were then washed in PBS and incubated for 10 min at RT in 4N HCL, followed by three PBS washes. In a covered humidified chamber, coverslips were blocked in PBG (0.5% w/v BSA [Sigma A-2153], 0.2% w/v cold water fish gelatin [Sigma G-7765] in PBS)

and stained with FITC-conjugated anti-BrdU antibody diluted 1:100 in PBG for 45 min at RT. Coverslips were washed three times in PBG and mounted in Vectashield Mounting medium with DAPI before imaging. Nuclei were scored for BrdU positivity and data presented as fraction BrdU positive over negative.

Apoptosis assays: Cleaved caspase 3 (CC3) levels, as assessed by immunoblotting, were used as a read out for apoptosis induced by oncogenic and genotoxic stressors, with the appropriate Tam and/or Doxo treatments indicated in text and figure legends. Pro-apoptotic genotoxicity was induced in MEFs and *KPR* cells by treating cells with 1 μM Doxo for 24h. Apoptosis was also monitored in LA1 and LA2 cells using the Alexa Fluro 488® Annexin V/Dead Cell Apoptosis kit (Invitrogen), following the manufacturer's protocol, and analyzed on a FACS Aria III sorter equipped with FACS DIVA software (BD Biosciences).

Statistical Analysis: Statistical tests and sample sizes used for each analysis are indicated in figure legends. Analyses were performed using GraphPad Prism 8. $p<0.05$ was considered statistically significant, with the following significance thresholds used: * $p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Supplementary Tables

Supplementary Table 1: Key plasmids used in this work

Name	Details	Source
pCMV-dR8.2	lentiviral packaging	Addgene plasmid #8455
pCMV-VSV-G	lentiviral packaging	Addgene plasmid #8454
BRD001	lentiviral; SpCas9; hU6-gRNA; Puro	Broad Institute gift
BRD003	lentiviral; hU6-gRNA; Puro	Broad Institute gift
BRD004	lentiviral; SpCas9; hU6-gRNA; GFP	Broad Institute gift
lenti SAM Hygro	lentiviral; p65-HSF1-MBP; hU6-gRNA-MS2; Hygro	Generated by E. Martínez-Terroba
MSCV Puro	retroviral mammalian expression; Puro	
pWZL Hygro	retroviral mammalian expression; Hygro	Addgene plasmid #18750
pLPC 12S E1A puro	retroviral; E1A; Puro	Addgene plasmid #18740
pHR-SFFV-dCas9-BFP-KRAB	lentiviral; constitutive dCas9-BFP-KRAB; BFP	Addgene plasmid #46911
Lenti dCas-VP64_Blast	lentiviral; constitutive dCas9-VP64; Blast	Addgene plasmid #61425
Phage TRE-dCas9-KRAB	lentiviral; Doxy-inducible dCas9-KRAB; Neo/G418	Addgene plasmid #50917
pLenti BsmBI sgRNA hygro	lentiviral; hU6-gRNA; Hygro	Addgene plasmid #62205
pCR-Blunt II-TOPO vector	Blunt DNA cloning vector	Invitrogen (K280002)

Supplementary Table 2: gRNA, dRNA, and ASO information

SpCas9 gRNAs			
Name	**Target**	**Sequence**	***Score**
gRNA-Control 1 (Con)	dTomato	GGCCACGAGTTCGAGATCGA	--
gRNA-Control 2	mCherry	GTATTACTGATATTGGTGGG	--
ΔRE-Zmat3-as	lncRNA p53RE	CATGCTCCCAAGACATGCCT	88
ΔRE-lincRNA-Spag9	lncRNA p53RE	CTGCTGTGCTTGGACTTGTT	84
ΔRE-lincRNA-Gadd45γ	lncRNA p53RE	GTGACATGCCTGTCCCCGAC	91
ΔRE-Ltc4s-as	lncRNA p53RE	TCTGAGGCCTGGGACTTGCC	70
ΔRE-2310008N11Rik	lncRNA p53RE	GGTCCACATTGCAGTTCTTC	90
ΔRE-Pvt1b	lncRNA p53RE	ATATGGGCAGTGACAAGTTT	87
ΔRE-lincRNA-p21	lncRNA p53RE	GATGCAACCGAAGGCAAGCC	90
ΔRE-Rhod-as	lncRNA p53RE	TCAGAGCTAGCTCTGGCACA	81
gRNA #7	lncRNA 7 TSS (+43)	TGCTCAGACATGTCTAAGCC	91
gRNA #8	lncRNA 7 TSS (+68)	GGTCCACATTGCAGTTCTTC	90
gRNA #9; lncRNA 7 gRNA 1	lncRNA 7 TSS (-10)	AAACACAGTGCCTTCGCTCT	94
gRNA #10; lncRNA 7 gRNA 2	lncRNA 7 TSS (-123)	AGCTATAGATCTGTCAGTAC	91
gRNA #12	lncRNA 7 TSS (-340)	AGTCACCAGCTTTGGCGCCC	92
gRNA #13	lncRNA 7 TSS (-180)	TACATGGTACTCATGACCCT	90
gRNA #14	lncRNA 7 TSS (-120)	GCTTTCTGGCTTAACATGTA	90
Ltc4s-as gRNA 1	TSS (-134)	CGGGCTACACCGGCTCGCTA	99
Ltc4s-as gRNA 2	TSS (-46)	CCCACCCTTTGAGCGGCGTT	98
Bfsp2-as gRNA 1	TSS (-65)	AGCTATAGATCTGTCAGTAC	90
Bfsp2-as gRNA 2	TSS (-19)	TGCAGCTGTCTCAGCGACAT	78
Neat1 gRNA 3	TSS (-78)	TCCTGATGGCTTTTGCACCC	92
Neat1 gRNA 4	TSS (-130)	GGCCATGACCCGCCCCTCGT	86
SpCas9 dRNAs			
Name	**Target**	**Sequence**	--
dRNA-Control (dCon)	dTomato	gCGAGTTCGAGATCGA	--
dRNA-A1	lincRNA-Gadd45γ TSS (-10)	GACCTACCGCGGCTC	--
dRNA-I1	lincRNA-Gadd45γ TSS (+3)	CTCAGAAGAGTGACT	--
ASOs			
Name	**Target**	**Sequence**	--
Con	N/A	GCTCCCTTCAATCCAA	--
ASO 1	lincRNA-Gadd45γ	GGTCAAATCGGTGCAG	--
ASO 2	lincRNA-Gadd45γ	ACCATTACTCCTCAAT	--
ASO (Ltc4s-as)	Ltc4s-as	TGTCGGTTAGTAGATG	

*CFD specificity score determined by CRISPOR (Haeussler et al. 2016); scores range from 0-100.

Supplementary Table 3: qRT-PCR, PCR, and sequencing primer information

qRT-PCR primers (mouse); 5'→ 3'		
Gene	**Forward sequence**	**Reverse sequence**
Gapdh	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG
p53	ACGCTTCTCCGAAGACTGG	AGGGAGCTCGAGGCTGATA
p21	TCCACAGCGATATCCAGACA	GGACATCACCAGGATTGGAC
p16	CATCTGGAGCAGCATGGAGTC	TACGACCGAAAGAGTTCGGGG
Rn7s1	CTGTAGTGCGCTATGCCGA	GTTCACCCCTCCTTAGGCAA
Kcnq1ot1	GGCCAGAAGCAGAGGTGATT	CCGAGCCGTAACTGCAAAAC
Zmat3-as	AGCCTACCCTGAAAGCGATG	TTCCAATGTCTTTGGGCGGA
Bahcc1-as	AAGGCCGCTTGATGAAAGGA	TGGCCTGATTCAATCGCGTA
lincRNA-Spag9	GCCTCATGAGACCTGCTGAA	GCCCCTGTGTAGCAATCACT
lincRNA-Gadd45γ	GCTCCGTTCCACGAAGTTTT	TGGGCTAGGCAGGTTAGGTA
lincRNA-Gadd45γ (2)	CTCAGAAGAGTGACTCGGCG	AGGCAATAACTCCAGCTCTTTGA
lincRNA-Gadd45γ (3)	ACTCTTATCAGCACACTCCAGC	CAGGCATGAGGGTCTGTGTC
Ltc4s-as	CCTTGGCTGACACCCGAACC	CCGAGTTCGAGCGCGTCTTC
2310008N11Rik (lncRNA 7)	AGGAAAGAAGCGACTGAACACT	GATGACGAAGGTGAGGCACA
Neat1	GGTTGACGCCTACACAGTGA	CTGCTGCCATTCATGCATCC
Malat1	AACCAGTTTCCCCAGCTTTT	CTACATTCCCACCCAGCACT
Pvt1a	ACTTAGCATTCCCAGAGCCC	TGGAGGGCATCTTCTTACCG
Pvt1b	CCATGACTGGGAAAAACCTCG	TGGAGGGCATCTTCTTACCG
Rhod-as	GGCAAGTTCTGAAGCCCACA	CCTGGGGGATTTCTCATCAGC
Sesn2-as	GGTCCGAGTGCTTTATGGGT	TTGCTCTGTTCCCACACCAC
Bfsp2-as	TTGGCTATGGGTGCTTCGGC	AGCCGGGACTCTATCCATCC
lincRNA-p21	CCTGTCCCACTCGCTTTC	GGAACTGGAGACGGAATGTCTC
Zmat3	TGTCACCTTGAACTCCGCTC	GCACAGCTTACAGTAGTCATTCT
Bahcc1	GGGATTCATGGGATTGCCCT	GGCCAGCACATCCTCATCTT
Spag9	GAATGGGTCGTGAGGTGGAG	TCTGCACGAGCTTTCCTGAG
Gadd45γ	GTGGCCAGGATACAGTTCCG	CAGGGTCCACATTCAGGACTT
Ltc4s	CCCTCGTGGGAGTTCTGTTG	GCGTATAGGGGAGTCAGCCT
Maml1	ACACAAAGCACCTTCCCACA	GAGGACAGCTGGAGTTGGAC
Myc	TTCATCTGCGATCCTGACGAC	CACTGAGGGGTCAATGCACTC
Sytl12	ACAGCAATGAGGGCAAAGGA	GCTGGATCCACCAAACTGGA
Rhod	GCTCCTCTGCTTCGATGTGAC	TGAGAGCCACTTCTGCTGCTT
Sestrin2	CACTGCGTCTTTGGCATCAG	GGTCGTCTTCTCAGGGTAGC
Bfsp2	GGAAAACAGCTTGCACGACG	TGAGGTTCTTCAGACGTCACC
Thap1	CCAGAAGGATGGTGCAGTCC	GGGCGAGTAAGAGGAAACTTGT
Zfp703	CCAATTGAGCTGGACGCCAA	CGGAGTTGAGCTTGGATGAG
Erlin2	CTGGAGGGACAGCCTGGATA	ACAGCTGAGAAGAGAGATGCA
Rnf170	AGCTAGGTAGTCCCCGGAAA	CTTAGACGCCCTAAGCTCG
LincRNA-RAPT1 [aka (2)]	ACAGCTGGCAAGTGGCAAAGCA	CCACCCACCTGCTTCTGTGTCC
Dhx15	CAGCCCAGGAGAGTGGCTGC	ACGCTCCAGAAGGGGATCGTTCA
LincRNA-RAPT1 (1)	GCCGCCTGGAGTTCGTTGGG	CCTGCTTTGCCACTTGCCAGC
LincRNA-RAPT1 (6)	TGTTAGCACGTGGTTTGTCTAAG GTG	GCCTCTCGTCCATCTCTGGGGA
LincRNA-RAPT1 (8)	AGCTTCTCAGGACCTCTCTGCA	AGCTATACTTTCCTGAACCCAGAC GA
LincRNA-RAPT1 (2kb)	GCCTGATAGACTGAGAGGCAC	CTAGGCAAGTGTGAGTGGTGAA
LincRNA-RAPT1 (4kb)	TGACCCCATCCACATCAGTC	GTATGGAGCCCCGATTGAGA
LincRNA-RAPT1 (6kb)	TACCAAAACTCAGGGTAAGGGAA	AAGGCATCCGTGAGGTTGATGG
LincRNA-RAPT1 (8kb)	TATGGCTGAATAGTGTCTCTGCT	CCCTTCTCACTCTACGCTCGTG
qRT-PCR primers (human); 5'→ 3'		
Gene	**Forward sequence**	**Reverse sequence**
LINC01605	AGGCTGGAGAGAGGGAACATG	CCAAGTTCGATGGAGAGCCTT

Genotyping PCR primers; 5'→ 3'		
Name	**Sequence**	
p53 LSL-1	CTT GGA GAC ATA GCC ACA CTG	
p53 LSL-2	CAA CTG TTC TAC CTC AAG AGC C	
SD5	AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC A	
Mutagenesis efficiency PCR primers; 5'→ 3'		
Name	**Forward sequence**	**Reverse sequence**
Zmat3-as PCR	GAGCTTCCCTGTCTGTGATGA	ATCCTTCGTGTCTGCCATCG
lincRNA-Gadd45γ PCR	CTTGCAGACCCGTCTTGAGT	CAGGCATGAGGGTCTGTGTC
lincRNA-p21 PCR	ATTGGCTAACCCCATGAGCAC	CTCTTCTTGCAGAAAGCCGCA
Pvt1b PCR	ATTGCGTTCCTGTGTTCGTG	AGGCAAAACAACAAGGCAGTC
Ltc4s-as PCR	GCAGCTGGACAGATAGATACC	GCTAACAACCACAGTGCAGG
TIDE sequencing primers; 5'→ 3'		
Name	**Sequence**	
Zmat3-as seq	CAACTACCCGTATCTCTGGAAAC	
lincRNA-Gadd45γ seq	CTGACTCCAGTTCTACCACAAG	
lincRNA-p21 seq	GCCCTCTCTCCATCCTCAATG	
Pvt1b seq	CTGCTCTTGTGTTTTTAAGGGG	
Ltc4s-as seq	GGACCCTTAGAGTGTTGTTGTTG	

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