2015

Investigation of Gain-of-Function Induced by Mutant p53

Catherine Vaughan
Virginia Commonwealth University

INVESTIGATION OF GAIN-OF-FUNCTION INDUCED BY MUTANT P53

A Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

Catherine A. Vaughan
B.S. Virginia Commonwealth University 2008

Director: Sumitra Deb, Ph.D.
Professor, Department of Biochemistry and Molecular Biology

Advisor:	Sumitra Deb, Ph.D.
Advisory Committee:	Swati P. Deb, Ph.D.
	Steven Grossman, M.D., Ph.D.
	Kristoffer Valerie, Ph.D.
	Brad Windle, Ph.D.
	Andrew Yeudall, BDS, Ph.D.

Virginia Commonwealth University
Richmond, Virginia
July, 2015

Acknowledgement

I would like to thank my advisor, Sumitra Deb, for his guidance and support throughout the seven years I have been a member of his laboratory. I would also like to thank my committee members for all of their advice along the way. Thanks to past lab members for teaching me how to perform the experiments used, as well as past and current lab members for help with completion of many of the assays included. I would like to thank the Integrative Life Sciences Department for their support as well as several graduate student assistantships that I have been awarded over the past few years from the VCU Graduate School. This work was supported by grants from the National Institute of Health to Sumitra Deb (CA70712 and CA121144) and Swati Palit Deb (CA74172), Pilot Project Awards from the VCU Massey Cancer Center to Sumitra Deb, Swati Palit Deb, Andrew Yeudall, and Brad Windle as well as a grant from the Jeffress Memorial Trust to Brad Windle and in part with funding from NIH-NCI P30CA016059 to the VCU Massey Cancer Center. Thanks also to Arnold Levine and Bert Vogelstein for providing us with cells, plasmids, and advice as well as past technicians for providing support.

Last, but certainly not least, I would like to thank my family and friends for their constant support and encouragement throughout my graduate school career.

Table of Contents

List of Tables

Page

List of Figures

List of Abbreviations

Aa	Amino acid
Ab	Antibody
AcH3	Acetylated histone H3
AcH4	Acetylated histone H4
BSA	Bovine serum albumin
CBP	CREB Binding Protein
cDNA	Complimentary DNA
ChIP	Chromatin Immunoprecipitation
CREB	cAMP Responsive Element Binding Protein
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECL	Electrochemiluminescence
EGFR	Epidermal Growth Factor Receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescent Protein
GOF	Gain-of-Function
HAT	Histone Acetyltransferase
IgG	Immunoglobulin
MDM2	Mouse Double Minute 2
mRNA	Messenger RNA
NFκB2	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells 2

NSCLC	Non-Small-Cell Lung Carcinoma
PAGE	Polyacrylamide Gel Electrophoresis
PARP	Poly (ADP-Ribose) Polymerase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
QPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNAi	RNA Interference
RPMI	Roswell Park Memorial Institute Medium
RTK	Receptor Tyrosine Kinase
SCC	Squamous Cell Carcinoma
SCLC	Small-Cell Lung Carcinoma
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
STAT2	Signal Transducer and Activator of Transcription 2
TAD	Transactivation Domain
TF	Transcription Factor
TSS	Transcription Start Site
WT	Wild-type

Abstract

INVESTIGATION OF GAIN-OF-FUNCTION INDUCED BY MUTANT P53

By

Catherine A. Vaughan, B.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University 2015

Major Director: Sumitra Deb, Ph.D.
Professor, Department of Biochemistry and Molecular Biology

p53 is mutated in 50% of all human cancers, and up to 70% of lung cancer. Mutant p53 is usually expressed at elevated levels in cancer cells and has been correlated with a poor prognosis. Cancer cells that express mutant p53 show an increase in oncogenic phenotypes including an increase in growth rate, resistance to chemotherapeutic drugs, and an increase in motility and tumorigenicity to name a few. We have identified several genes involved in cell growth and survival that are upregulated by expression of common p53 mutants: NFκB2, Axl, and epidermal growth factor receptor (EGFR). The aim of this study was to determine the role NFκB2, Axl, and EGFR play in mutant p53's gain of function (GOF) phenotype and to determine a mechanism for upregulation of mutant p53 target gene upregulation.

Inhibition of mutant p53 in various cancer cell lines using RNAi in the form of transient siRNA transfection or stable shRNA cell line generation caused a decrease in the gain of

function ability of those cells in the form of reduced chemoresistance, reduced cell growth and motility, and a reduction in tumor formation. Additionally, inhibition of NFκB2, Axl, and EGFR also showed similar effects. Promoter deletion analysis of the NFκB2 promoter did not show a specific mutant p53 response element needed for mutant p53 mediated transactivation. Similarly, deletion of the p53/p63 binding site on the Axl promoter did not inhibit mutant p53 transactivation. Sequence analysis of the NFκB2, Axl, and EGFR promoters revealed several transcription factor binding sites located throughout the promoters. ChIP analysis of mutant p53 and the promoter-specific transcription factor binding revealed that in the presence of mutant p53, individual transcription factor binding is increased to the NFκB2, Axl, and EGFR promoters as well as an increase in acetylated histone binding. This data suggests that mutant p53 promotes an increase in transcription by inducing acetylation of histones via recruitment of transcription factors to the promoters of mutant p53 target genes.

Introduction

Discovery of p53. p53 was discovered in 1979 by five independent groups when it coprecipitated with the large T antigen in simian virus 40 (SV40) transformed hamster cells (1-6). It was later found in high concentrations in various cancerous and transformed cells (2, 5, 7-9). p53 was originally thought to behave as an oncogene when co-transfection of mouse p53 cDNA with activated Ras transformed rat embryonic fibroblasts (REF), however it was later learned that the p53 cDNA clone used contained a mutation (10-12). Afterwards, co-transfection of wild-type (WT) p53 cDNA with activated Ras reduced the transformation potential of REFs and even inhibited the transformation potential when a known oncogene was added as well, leading to the belief that p53 acts as a repressor of cellular transformation (13, 14). The role of p53 in tumor formation was later investigated through the creation of a mouse model system where one or both alleles of p53 were deleted, or left as wild-type. p53 was shown to behave as a tumor suppressor when the majority of p53 null mice formed tumors by six months of age while WT mice showed very little tumor onset when aged three times as long (15).

Structure and function of p53. The p53 gene is located on the short arm of chromosome 17 and is a phosphoprotein that is localized mainly in the nucleus (16, 17). It exists as an oligomer but mainly as a tetramer in the cell and binds to DNA sequence-specifically (18-21). Not only is p53 a transcriptional activator, it can also repress transcription (22-27). The p53 consensus sequence is defined as two half-sites of 5'-PuPuPuCWWGPyPyPy-3' (Pu indicates a

purine base, Py indicates a pyrimidine base, and W indicates either adenine or thymine) that are separated by 0-13 bases (28).

p53 is a 393 amino acid long protein with a well-established structure-function relationship. The protein contains five functional domains: the transactivation domain (TAD, amino acids 1-92) contains both TADI (aa1-42) and TADII (aa43-92), the proline rich domain (aa64-92), the DNA binding domain (aa101-300), an oligomerization domain (aa307-355), and a C-terminal regulatory domain (aa356-393) (29-39) (Figure 1). There are five highly conserved domains among p53 genes from evolutionarily distant species, the first is located within the first transactivation domain, and the remaining four are found within the DNA binding domain (40, 41).

WT p53 is activated in response to a variety of stressors including DNA damage, heat shock, and hypoxia to name a few. Upon activation, p53 stabilizes and either causes transient or permanent (senescence) cell cycle arrest, activates DNA repair, or initiates apoptosis (Figure 2) (42-47). WT p53 is a transcription factor that activates a large group of genes whose regulatory sequences have p53 consensus binding sites. (48, 49). Although WT p53 has biological activities that are transcription independent, the majority of its functions are dependent on its transcriptional ability (50). WT p53 has been shown to be involved in many biological activities including aging (51), DNA damage response (52), autophagy (53), maintenance of genetic stability (54), metabolism (55), miRNA transcription (56), and stem cell fate determination (57), amongst others.

p53 regulation. The tumor suppressor p53 is highly unstable due to its short half-life (21). Under normal circumstances WT p53 is regulated via interaction with mouse double

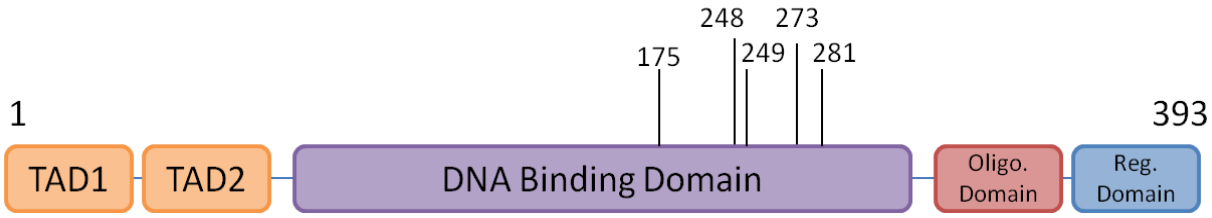

Figure 1. Schematic of p53 protein indicating several mutations within the DNA binding domain.

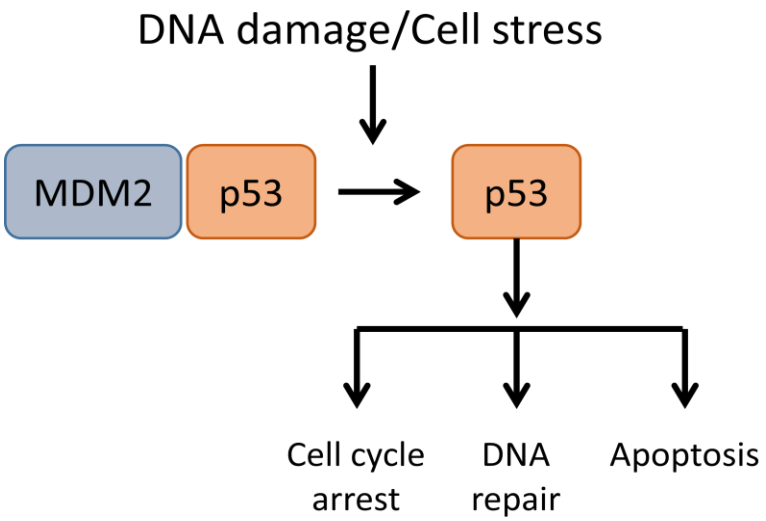

Figure 2. Simplified cartoon for the activation of WT p53 and its major functions.

minute 2 (MDM2) (58, 59). MDM2 binds to p53 at the N-terminus, blocking transcriptional activity, and promotes p53 degradation by transporting it from the nucleus to the cytosol where it is marked for ubiquitination (58-65). When cell stress or DNA damage activates p53 it is phosphorylated at several sites within the N-terminus which free it from MDM2, abrogating ubiquitination, leading to p53 stabilization and accumulation (66, 67).

Another pathway that regulates p53 is the PI3K/Akt pathway. Akt has been shown to phosphorylate MDM2 which translocates MDM2 to the nucleus where it targets p53 for ubiqutination (68-73). p53 acetylation has also been shown to increase the stability of the protein, leading to increased transcriptional activity due to an open conformation. This can be accomplished through histone acetyltransferases (HATs) such as p300/CBP/PCAF or alternately, a decrease in histone deacetylases such as HDAC1 OR SIRT1 (74-78).

Transcription by WT p53. WT p53 mediated transactivation occurs when p53 binds to its consensus site on the DNA and assembles HATs to contact the RNA polymerase II complex at the site of transcription initiation via TBP associated factors (79, 80). WT p53 was also shown to function as a transcriptional repressor in the early 90's when it was shown to inhibit transcription from a number of cellular and viral promoters (27, 81). Repression of transcription by p53 can be either direct or indirect. Direct transcriptional repression could happen by p53 binding to its consensus site within the regulatory region of the genes it regulates which competitively inhibiting other activating transcription factors (82). p53 has also been shown to bind to its site on the regulatory sequences of genes and recruit co-repressors such as mSin3A, which inhibits HATs and thereby represses transcription (83).

p53 mutations. p53 is the most frequently mutated gene in human cancer with approximately 50% of all human cancers carrying a mutation in the p53 protein (84-87). The

majority (73%) of mutations that have been discovered within p53 are considered missense mutations that are located within the DNA binding domain (http://p53.iarc.fr/) (88, 89). In most human tumors only the mutant protein is expressed; usually one allele is point mutated and the other allele is deleted (85-87, 90, 91). The IARC database defines "hot spot" mutations as mutations that represent at least 20% of all reported p53 mutations, which include mutations at codons 175, 245, 248, 273, and 282 (89, 92, 93). Mutations of p53 are classified as those that either affect protein-DNA contact (Class I, i.e. R273H), or alter the protein conformation (Class II, i.e. R175H) (94-96).

Types of p53 mutations. p53 mutations found in human cancer can be divided into three categories: (1) Loss of function mutations. These mutations are responsible for the loss of the tumor suppressor function of p53. Almost all of the p53 mutants that have been identified are categorized as loss of function mutations. In general, they are defective in sequence-specific transactivation of genes containing WT p53 consensus binding sites. (2) Dominant negative mutations. Hetero-oligomeric complex formation between mutant p53 and WT p53 causes changes in the properties of WT p53, with the mutant becoming dominant over the WT protein's properties, therefore inactivating its tumor suppressor functions. An example of this is the immortalization and transformation of rodent embryo fibroblasts by mutant p53 (9, 97). This is thought to cause the increased cancer susceptibility of patients with Li-Fraumeni syndrome as well as p53 +/- mice (98). (3) Gain of function (GOF) mutations. In this case, mutant p53 acquires a dominant oncogenic role that does not depend on complex formation with WT p53. An example of this would be expression of mutant p53 in cells where WT p53 is absent which show enhanced oncogenic properties compared to p53-null cells.

Gain-of-Function mutant p53. Mutations that are characterized as GOF mutations not only lose the WT tumor suppressor function, but also gain new oncogenic functions. Overexpression of mutant p53 in a large number of human cancers not only distinguishes it from other tumor suppressors, but also suggests that there is a selective pressure behind its accumulation. Expression of mutant p53 in cells lacking p53 altogether has been shown to stimulate growth and carries a worse prognosis in patients (99-103). This also suggests that tumors containing mutant p53 may be more aggressive than p53-null tumors.

Gain of function mutant p53 properties. Biological gain of function activity was confirmed when expression of human mutant p53 in the 10(3) murine fibroblast cell line that is endogenously p53-null, caused tumor formation in nude mice even though 10(3) cells alone or 10(3) (vector transfected) cells do not (100). Expression of mutant p53 has been shown to result in oncogenic and proliferative processes such as increased tumorigenicity (100, 104, 105), increased growth in soft agar (106), decreased sensitivity to chemotherapeutic drugs (107-110), increased DNA synthesis and increased growth rate (111-116), induction of gene amplification (117-119), induction of cellular motility, invasive capability and metastasis (120-125), increased tumor angiogenesis (126), and promotion of chronic inflammation and associated cancer (127). These phenotypes show that mutant p53 has a physiological role that leads to the aggressive tumor development and poor prognosis.

Mouse models of mutant p53. Many groups have tried to use mouse models to demonstrate the gain of function ability of mutant p53. An increased metastatic potential was shown in mice inheriting knock-in p53-R172H (homologous to human p53-R175H) (128), and evidence of gain-of-function associated with genomic instability was explored using transgenic

p53-R172H mice (129). The necessity of stabilization for mutant p53 gain of function activity in a mouse system by MDM2 or p16 knock out has also been demonstrated (130).

Mouse models of pancreatic cancer were used to show increased tumor formation and metastases in the presence of mutant p53 versus loss of WT p53 (131, 132). The degree of GOF between mutations was studied using knock-in mice expressing two different hot spot p53 mutations to investigate the difference in tumor formation and survival. A marked increase in tumor formation and decrease in survival in mice expressing R248Q versus G245S was ultimately found (133).

Transcription by mutant p53. p53 mutants have been shown by several groups to upregulate genes involved in cell growth and oncogenesis through the use of microarray analysis in cells overexpressing mutant p53 in a p53-null background (110, 134, 135). Mutant p53 has also been demonstrated to repress expression of a number of genes including inhibitor of differentiation (Id2) (136, 137) p21, gadd45, PERP and PTEN (137), among others. Some of these genes are involved in pro-apoptotic activities, including CD95 (Fas/ApoI) (138, 139), caspase-3 (140) and others (141, 142).

Mechanism of mutant p53 transactivation. There are two possible mechanisms to explain transactivation by GOF mutant p53: one that involves its direct binding to DNA and transcriptional modulation of gene expression, and one in which mutant p53 does not directly come near regulatory sequences on the chromosome. This second category can be subdivided further: (1) protein-protein interactions between mutant p53 and other cellular protein(s), such as the p53 family members, p63 and p73, DNA machinery proteins, and/or proteins of the apoptotic pathway (143, 144); (2) modulation of target genes by mutant p53 (Figure 2), such as activating

growth promoting genes, disrupting DNA repair or apoptotic activities or inhibiting growth suppressive genes (138, 145-148).

As far as the first possible mechanism goes, there is evidence to show that GOF p53 does in fact bind to DNA on the chromosome, particularly in G/C rich areas around transcription start sites of some genes that are characterized by active chromatin marks (149-153).

As mentioned above, regulation of transcription by protein-protein interactions can be either via interaction of mutant p53 with its family members p63 and/or p73, or through interactions with transcription factors. The fact that p53 family members p63 and p73 retain the capability of interacting with tumor-derived p53 mutants led to the possibility that such interactions may inhibit p63/p73 function (147, 154-158). In this model, it is thought that mutant p53 may inhibit p63/p73's DNA binding ability by interacting with the DNA binding domain of p63/p73, as model systems predict (155). Some of the GOF activities of mutant p53 assume that mutant p53 would block the inhibitory effects of p63/p73 (mostly p63). However, transactivation observed by GOF p53 cannot be explained by this model alone, since there are genes that are transactivated by p63 as well as GOF p53 (159, 160). There is another model that is evolving that involves mostly p63 (and perhaps p73), in which it is assumed that mutant p53 can anchor to p63 and become nucleated on promoters with p63/p73 sites. There is evidence for this possibility also (161).

It was proposed about 20 years ago that mutant p53 may transactivate genes it up-regulates indirectly through interactions with transcription factors that normally bind to the regulatory regions of those target genes (162-164). Various groups have shown interactions of p53 (both WT and mutant) with several transcription factors including Sp1, E2F1, Ets1, CREB, p300/CREBP, NFY-A (74, 113, 157, 164-168) and others. Although in some cases evidence has

been presented that indicates nucleation of mutant p53 on DNA by anchoring onto another transcription factor, in most cases there is a lack of clear-cut evidence. Overall it is assumed that mutant p53 becomes situated on the promoter-regulatory sequence of a target gene by its interaction with a transcription factor, and then through its intact transactivation domain it is able to interact with the transcription initiation complex presumably near the transcription start site. It is most likely that the transactivation domain interacts with the component of the "mediator" complex (79, 169, 170). Also, it is generally assumed that mutant p53-CREBP/p300 interaction would facilitate nucleation of these histone acetylases on the chromatin. This results in an opening up of the chromatin by acetylation of histones (171, 172).

Addiction to GOF mutant p53. Oncogene addiction describes the situation where cancer cells cannot function without the many chromosomal abnormalities that have accrued within those cells (173). Knock-down of a particular protein that a cancer cell is addicted to can cause the cell to cease functioning and apoptose, enter a slower growth phase, or growth arrest (173). Many lung cancer cell lines with endogenous expression of different p53 mutants have been tested for their addiction to mutant p53 expression; interestingly, the addiction seems to be allele specific in some gain of function activities, although all the cell lines characteristically lose their enhanced growth rate on elimination of GOF p53 (174). Understanding the mechanism behind the addiction to mutant p53 and its GOF activity is of the utmost importance toward improving cancer's survival rate.

Future therapeutic potential. Reducing mutant p53 expression in various cell lines has been shown to be an effective way to diminish cell growth, migration, and tumorigenicity. Unfortunately, there is no way at present to inhibit the mutant form of p53 in a patient without also targeting WT p53 in healthy cells. Several therapies have been proposed within the last few

years that might prove to be useful. One such therapy would be through the use of peptides to change the conformation of the mutant protein to that of WT (175). Another would be to restore degradation of mutant p53 through the use of HDAC inhibitors (176, 177). Small molecule inhibitors such as siRNA could be also be used to preferentially target the mutant protein while leaving WT p53 intact. Since the goal of cancer research aims to discover what causes a cell to become oncogenic in the hope of developing a way to cure the disease, understanding the mechanism of gain of function is essential to be able to effectively target mutant p53 for cancer therapy. It may be possible that due to the activation of different pathways a combination treatment strategy will have to be used.

Hypothesis

Major hypothesis. The main goal of pursuing cancer research is to eventually discover a cure for the disease. Unfortunately, there is no method for targeting mutant p53 without also causing harm to WT p53 in patient's cells. The next best approach would be to develop therapies against mutant p53 target genes or to inhibit mutant p53-mediated upregulation of those genes. Mutant p53 utilizes several target genes to accomplish its gain of function phenotypes of increased cell growth, chemoresistance, and survival, but the molecular events that lead to cancer with GOF p53's participation, and the mechanisms of how GOF p53's transactivation is involved in oncogenesis are unknown. Previous research has shown that a group of genes involved in oncogenesis are upregulated by mutant p53 with differential requirements of the transactivation domain. Knock-down of mutant p53 in several breast and lung cancer cell lines abolishes GOF activity of mutant p53 indicating the potential of targeting mutant p53 for cancer therapy. ChIP sequencing has shown that mutant p53 interacts with promoters of its target genes. Studies of histone acetylation by chromatin immunoprecipitation-sequencing and gene expression show that histones at mutant p53 target promoters are acetylated in the presence of mutant p53. The major hypothesis is that targeting GOF p53 in cancer would substantially inhibit tumor growth and that the gain-of-function phenotype exhibited by various cancer cells containing mutant p53 requires genes such as NFκB2, Axl, and epidermal growth factor receptor (EGFR), which are required for cancer progression. The specific aims are designed to better understand the

relationship between mutant p53 and these three target genes and to determine a mechanism for mutant p53-mediated transactivation.

Specific aims.

The following are the specific aims: (1) Determine the role of gain-of-function mutant p53 in NFkB2 transactivation, (2) Determine how mutant p53 utilizes receptor tyrosine kinase Axl to accomplish its gain-of-function activity, and (3) Determine how up-regulation of epidermal growth factor receptor (EGFR) affects addiction of lung cancer cells to gain-of-function mutant p53.

Chapter 1

DETERMINE THE ROLE OF GAIN-OF-FUNCTION MUTANT P53 IN NFKB2 TRANSACTIVATION

The work presented in this chapter has been published in the Archives of Biochemistry and Biophysics research journal (Arch Biochem Biophys. 2012 Feb 1;518(1):79-88.). Some chemoresistance assasys were performed by Amber Heck and Amanda High and the motility assays were performed by Andrew Yeudall.

Introduction.

WT p53 induces apoptosis after DNA damage caused by cytotoxic drugs, and it appears that a cell's p53 mutational status may determine the efficacy of many of these drugs (107, 108, 178). It has been shown that mutant p53 expression (in cells devoid of WT p53) can lead to decreased sensitivity to drugs such as doxorubicin, etoposide, cisplatin and others (107, 108). This can be partially explained by an effect of mutant p53 on p73 and p63 (179, 180). In a previous study, evidence was presented to show that part of this chemoresistance may also arise as a result of up-regulation of p52/p100 NF-κB2 by mutant p53 (110).

The NF-κB family of transcription factors regulates expression of many genes involved in growth, differentiation, survival, development and inflammation (181, 182). In mammals this group has five members: Rel A (p65), Rel B, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2) (183); the factors function primarily as p50/Rel or p52/Rel heterodimers, although they may also function as different homo- and heterodimers. The NF-κB2 protein is synthesized as a p100 precursor that gets processed to p52 for more functional activities upon activation of the pathway. p52 over-expression can lead to lymphocyte hyperplasia and transformation (184).

Constitutive nuclear NF-κB activity has emerged as a hallmark for many human leukemias, lymphomas, and several other cancers (185).

Inhibition of NF-κB sensitizes many tumor cells to death-inducing stimuli, including chemotherapeutic agents (185-189). Thus, transactivation of NF-κB could be a crucial step by which mutant p53 induces oncogenic progression. Activation of NF-κB appears to protect tumor cells from apoptosis, through induction of anti-apoptotic genes (190, 191), while p52 over-expression has been shown to inhibit pro-apoptotic genes as well (192).

In this study, using a number of cancer cell lines expressing mutant p53, we show that GOF activities are dependent on the p53 level. We also demonstrate that up-regulation of NF-κB2 in H1299 lung cancer cells expressing mutant p53 is caused by changes in chromatin structure on the NF-κB2 promoter, and increasing interaction of crucial transcription factors with the promoter.

Materials and Methods.

Cell lines. Five human lung cancer cells lines: H23 (p53-M246I, NSCLC), H1048 (p53-R273C, SCLC), H1437 (p53-R267P, NSCLC), KNS-62 (p53-R249S, NSCLC), H1299 (p53-null, NSCLC); two human breast cancer cell lines: MDA-MB-468 (p53-R273H) and SK-BR-3 (p53-R175H); and a human melanoma cell line MDA-MB-435 (p53-G266E) were used in these studies, and grown in media prescribed by ATCC.

Generation of stable cell lines. Stable cell lines were generated after transfection of p53-null H1299 lung carcinoma cells with mutant p53 expression plasmids (or expression vector alone), which contain a neomycin resistance gene as described (104). Mutant p53 knock down cell lines were generated by using lentivirus expressing short hairpin RNA (shRNA) against p53

utilizing lentivirus systems (Open Biosystems) following the manufacturer protocol. Clones were isolated using puromycin selection at 1-3 µg/ml.

Drug sensitivity assay. Drug sensitivity assays were carried out as described by us earlier (112). In general, cancer cells expressing mutant p53 were plated at 10,000 cells/10cm dish and treated with a final concentration of 1-6 uM etoposide (Sigma; St. Louis, MO); concentration of the drug used depended on the cell lines used and preliminary earlier experiments. In our hands different cell lines respond differently towards the same concentration of etoposide as far as cell death is concerned as measured by colony formation assays. We have done preliminary experiments where we used different concentrations of the drug to determine the sensitivity for individual cell lines. These experiments dictated what concentration we ultimately chose for the final experiment the data of which we have presented. For control, in order to avoid over-crowding, we plated 1000 cells/10cm dish treated with DMSO vehicle. The number of cells used per assay varies depending on the cell line, its plating efficiency of the cells and sensitivity towards the chemotherapeutic drug under consideration; but for the same cell line and experiment we used identical number of cells.

After drug/DMSO treatment, plates were washed and the media replaced. The surviving cells were allowed to form colonies for 2-3 weeks with periodic changes of media. Colonies were fixed with methanol, stained with methylene blue and counted as described earlier (193). The percent survival was calculated by dividing the average number of drug-treated colonies by the average number of DMSO-treated colonies and multiplying by 100. This was done for each siRNA treatment, either si-control or si-p53. All experiments were done in triplicate, and repeated multiple times. The error bars represent standard deviation from the average number of colonies counted.

Growth assay. Growth assays were carried out as described by us earlier with slight modifications (110, 135). Cells were plated at 50,000 cells/6cm dish in triplicate for five time points and harvested after incubation with trypsin and counted using a Coulter Counter (Beckman). Multiple cell clones were used for each assay. All experiments were done in triplicate, and repeated multiple times. The error bars represent standard deviation from the triplicates.

PARP cleavage assay. To determine if p53 knock down results in apoptotic cell death, we performed a transient infection with lentivirus expressing p53 shRNA (or GFP shRNA) to knock down mutant p53 in H1048 lung cancer cells expressing mutant p53 and assess apoptosis without selection. For the PARP Western blot experiments we used H1048 cells treated with etoposide (9 uM) for 48 hours as a positive control for apoptosis. The assay was carried out using an antibody from Cell Signalling.

Xenograft assay. Nu/nu mice were used for the tumorigenicity studies. For all injections, $1x10^7$ cells/250µl media were used. Mice were injected subcutaneously on the flanks and tumors allowed to grow to a maximum size of 1cm, measuring periodically as described before (17, 18). Three different clones of H1048 cells with mutant p53 levels reduced by shRNA were used in comparison to two GFP shRNA control cell lines to rule out clonal variations.

siRNA transfection. Breast or lung cancer cells were nucleofected with siRNA directed against a specific or non-specific gene (luciferase) using a nucleofector and a nucleofector kit following the manufacturer's protocol (Amaxa Inc.; Gaithersburg, MD). Sequences used were: Control (C): 5'- CAU GUC AUG UGU CAC AUC ACT T -3' and 5'-GAG AUG UGA CAC AUG ACA UGT T -3', p53: 5'-GCA UGA ACC GGA GGC CCA UTT-3' and 5'-AUG GGC CUC CGG UUC AUG CTT-3' (162) and NF-κB2: 5'-GCC CUG AGU GCC UGG AUC UTT-

3’ and 5’-CGG GAC UCA CGG ACC UAG ATT-3’. Twenty-four hours after nucleofection, cells were trypsinized, counted, plated and then exposed to etoposide or DMSO as a control for 48h and colony formation assays performed as described above.

Chromatin immunoprecipitation. Chromatin immunoprecipitations (ChIP) were performed as described (135, 194). To crosslink protein and DNA, cell cultures were incubated in 2% formaldehyde for 10min at ambient temperature and then 200mM glycine was added for a further 10min. Cells were washed in cold PBS, scraped and centrifuged. Pellets were resuspended in lysis buffer containing 1% protease inhibitors and then sheared by multiple passages through a 27.5 gauge needle followed by 25min of sonication on ice such that the chromatin was fragmented to 500–2000 bp length. Following centrifugation, the protein content of the supernatants was determined and equal amounts used for immunoprecipitation overnight at 4ºC with gentle tilting with anti-acetylated histone H3 antibody or IgG as a control. Immune complexes were captured using Protein A-agarose, then washed sequentially once in RIPA buffer (150mM NaCl, 50mM Tris pH8, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40), once in high salt buffer (500mM NaCl, 50mM Tris pH8, 0.1% SDS, 1% NP-40), twice in LiCl buffer (250mM LiCl, 50mM Tris pH8, 0.5% sodium deoxycholate, 1% NP-40) then twice in TE buffer. Protein-DNA complexes were eluted from beads in fresh elution buffer (20% SDS, 10mM DTT, 100mM $NaHCO_3$), crosslinking was reversed overnight at 65ºC in the presence of NaCl, and then samples were ethanol-precipitated. Following centrifugation, pellets were resuspended in TE buffer and incubated sequentially with 10mg/ml RNase A (30min) and 20mg/ml proteinase K (1h). Samples were phenol/cholorform-extracted, ethanol-precipitated, and the pellets washed in 70% ethanol, dried and resuspended in sterile water. Acetyl histone H3 Ab and normal rabbit IgG (17-615) were from Millipore; CREB (sc-186), c-Rel (sc-272), NFkB p65 (sc-372), STAT2

(sc-476), p53 DO1 (sc-126) and AcH4 K16 (sc-8662) were from Santa Cruz Biotechnology, Santa Cruz, CA. Quantitative PCR (QPCR) was used to quantify precipitated NF-κB2 promoter-specific DNA segments. We have performed two sets of QPCR: one with NF-κB2 specific primers and another with GAPDH specific primers. The second set of QPCR (with GAPDH) has been done to normalize the NF-κB2 values as GAPDH expression remains unchanged by p53. Primers used to analyze ChIP samples were: GAPDH F: 5'-GTC AAC GGA TTT GGT CGT ATT-3' and R: 5'-GAT CTC GCT CCT GGA AGA TGG-3' and NF-κB2 F: 5'-GAG GGA GGA GGG GGC TTA ACC C-3' and R: 5'-CGG GAG GCC CTC GAC AGT CTA C-3'.

Luciferase reporter assays. H1299 cells were transfected in triplicate with 200ng of the promoter-luciferase reporter constructs and 1µg of vector only (pCMV Bam) or p53 expression plasmid for 48 hr (112). After transfection, cells were harvested and luciferase activity measured using the Promega luciferase assay kit (#E1500, Promega, Madison, WI) according to the manufacturer's instructions. Cell extracts were normalized to each other based on total protein concentration and luciferase activity detected using a Luminometer from Turner Designs.

Western blotting. Immunoblottings were carried out as described (110). NF-κB2 levels were detected using an antibody from Upstate Biotechnology (#05-361; Charlottesville, VA). Actin levels were detected using the AC-15 antibody (Sigma; St. Louis, MO), p53 was detected using the p53 antibody PAb 1801, and Erk2 was detected using ERK2 (sc-154) antibody from Santa Cruz Biotechnology. Westerns blots were developed by the ECL method (GE Healthcare; Piscataway, NJ). SK-BR-3 blot was developed using Li-COR system as described by Sanka et al. 2011 (194).

Migration assays. Cell migration was carried out using wound-healing (scratch) assays, as previously described (195). Briefly, cells were trypsinized, plated in quadruplicate in 12-well

cell culture plates and incubated at 37°C until cells were completely confluent. At this time, a sterile pipette tip was used to scratch across the surface of the plate, removing the complete layer of cells within the scratch area. Following cell removal, each well was washed once with PBS and then replaced with growth medium. Immediately following, the width of the scratch was measured at six specific points under a 5x objective using a light microscope and AxioVision software (Carl Zeiss Microimaging, Thornwood, NY). Cells were incubated at 37°C from 20-60h depending on the cell line under study, at which time the scratch width was measured at the same position as at time 0.

Apoptosis-DNA Ladder Assay. H1048 cells stably expressing a shRNA against p53 or GFP were used for this assay along with a positive control (U937 cells treated with camptothecin, provided in the kit from Roche cat. # 11835246001). Two million cells were used for preparation of DNA for each sample. Cells were lysed with the binding/lysis buffer, and the lysate applied to filter tubes which contain glass fiber fleece to bind nucleic acids. Impurities are removed by washing and DNA is eluted off of the columns. 2µg of each DNA sample was run in a 1% agarose TBE gel and run at 70V until full separation of the positive control DNA ladder was visible.

Experimental Results.

Reducing p53 levels by RNAi in cancer cells with mutant p53 lowers gain of function activities. If mutant p53 expression in cancer cells induces GOF phenotypes such as increased tumorigenicity, growth rate, chemoresistance and motility, we hypothesized that by reducing the level of mutant p53 in those cells we should be able to see reduction of these properties. Therefore, we wanted to test whether that principle holds using cancer cell lines expressing

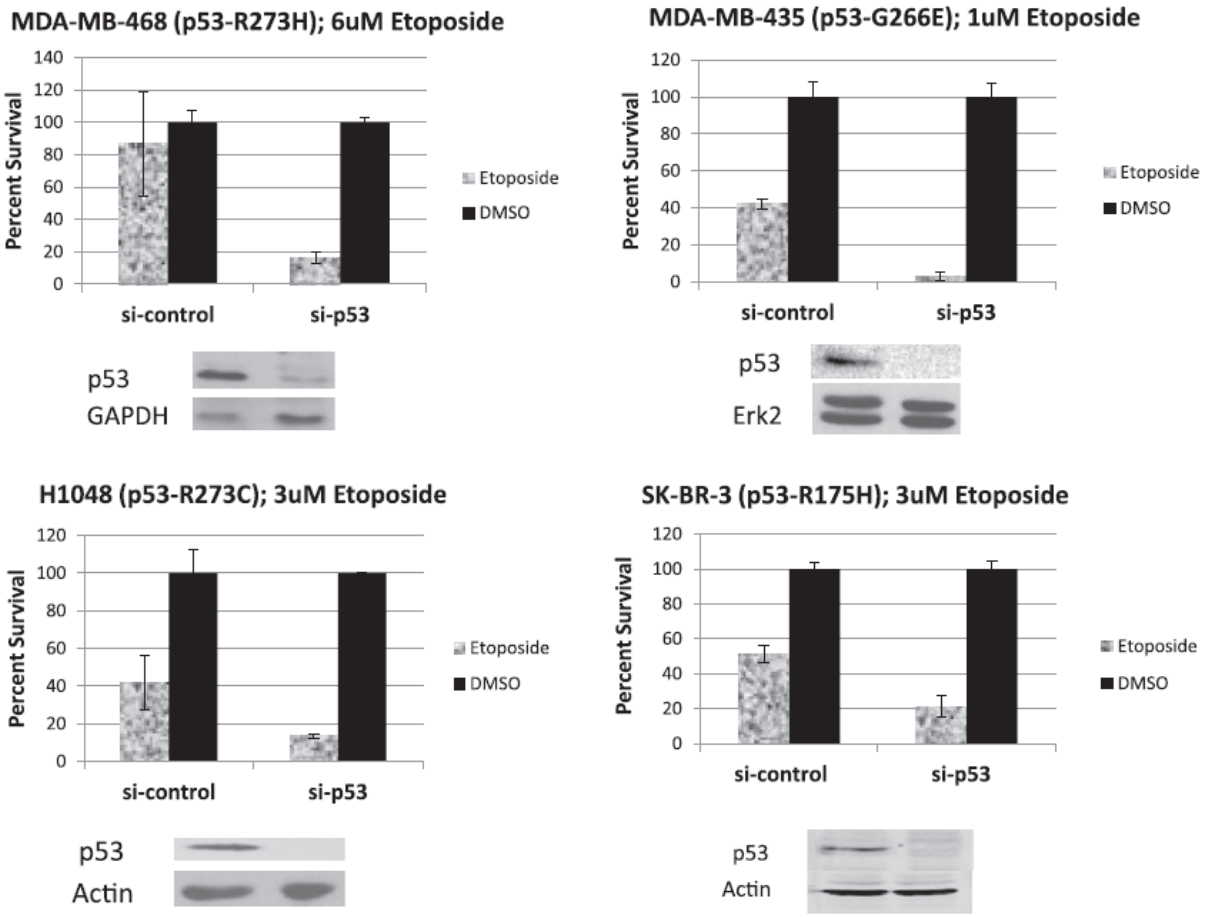

Figure 3. Reducing p53 levels in cancer cells with mutant p53 by RNAi lowers chemoresistance. The indicated cells were transfected with control (si-luc or si-scrambled) or p53-specific siRNA, treated with appropriate concentrations of etoposide for 48h depending on the sensitivity of the cell line to the drug, and colony formation determined (upper panels) as described in Materials and methods. Immunoblots show the efficacy of p53 siRNA treatment. Erk2, GAPDH, or actin was used as a loading control (lower panels). Percent survival is shown in the figures with error bars representing standard deviation from the average number of colonies. The percent survival was calculated by dividing the average number of drug-treated colonies by the average number of DMSO-treated colonies and multiplying by 100. Experiments were done in triplicate and data shown are representative of multiple independent repeats.

mutant p53. Figure 3 shows the results of colony formation assays performed with cell lines MDA-MB-468, MDA-MB-435, H1048 and SK-Br-3, after treatment with etoposide or DMSO. Cells were treated with the drug or DMSO for 48h after being nucleofected with siRNA specific for p53 or control (scrambled or luciferase) as described in Materials and methods. The data demonstrate that these cell lines become more chemosensitive when the mutant p53 level is reduced; thus the p53 mutants in these cells show GOF activity.

Figure 4 shows growth assay data of cancer lines H23, H1048 and H1437 when their p53 levels were reduced by lentivirus infection expressing shp53, compared to shGFP controls. The data indicate that reduction of mutant p53 level has a significant effect of reducing the growth rate of H23, H1048 and H1437 lung cancer cells. Thus mutant p53 controls the rate of growth of cancer cells expressing GOF mutant p53. We carried out a PARP cleavage assay (to detect apoptosis), which showed no significant differences between the shGFP and shp53 lentivirus infected H1048 cells (Figure 4D) suggesting slower proliferation instead of induction of apoptosis. We have also performed a DNA degradation assay using H1048 cells expressing shRNA for GFP or p53 to further detect apoptosis. The data shown in Figure 4E indicated that while in the positive control we could see DNA ladder formation, H1048 cells (expressing shRNA for GFP or p53) did not show any indication of ladder formation corroborating PARP cleavage data. This conclusively shows that mutant p53 reduction in H1048 cells does not cause apoptosis.

Another GOF phenotype of mutant p53 is an increase in cell motility. To determine the relationship between mutant p53 and motility, we performed migration assays of KNS-62 and H1437 lung cancer cells after their p53 levels were knocked down by lentivirus expressing p53 shRNA, compared to shGFP controls (Figure 5A). The data indicate that reduction of mutant

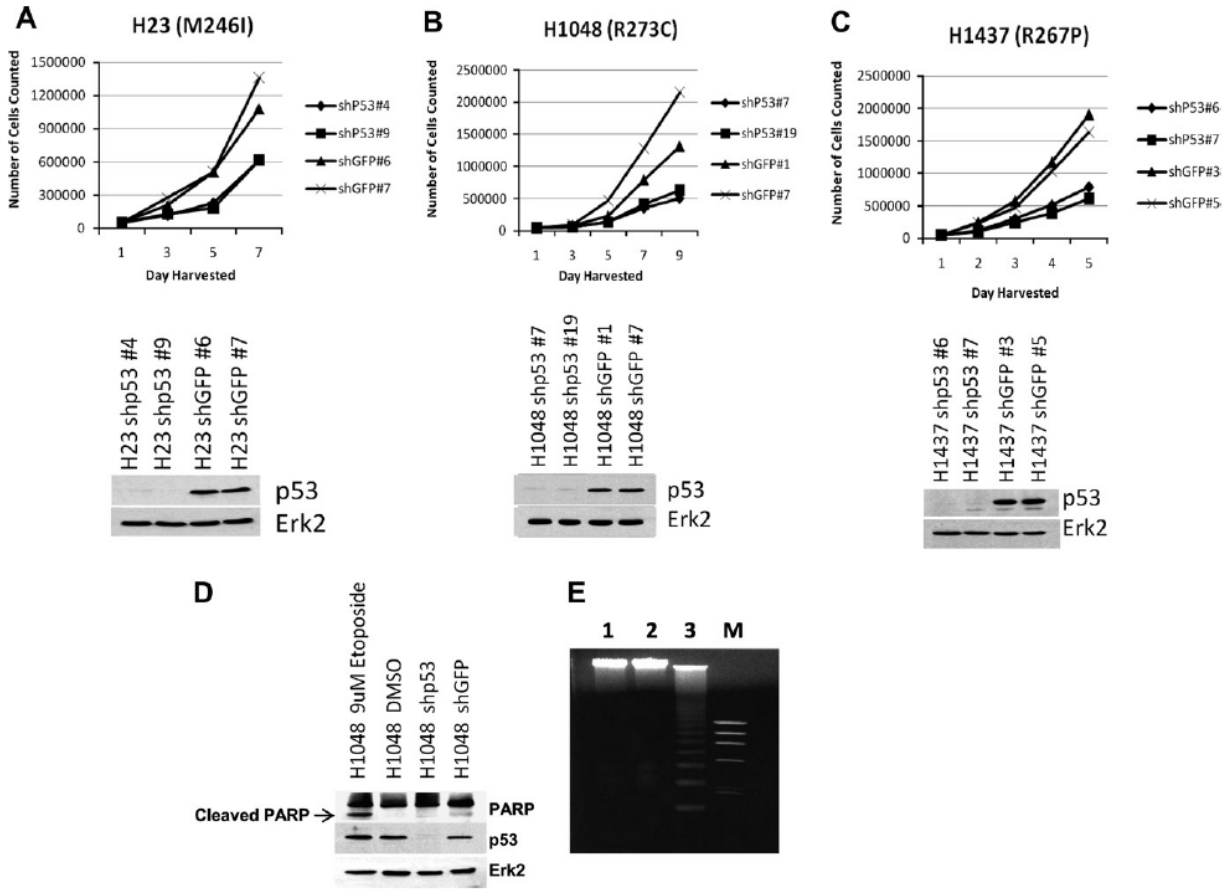

Figure 4. p53 knock down causes a decrease in growth rate in cancer cell lines. A-C. Mutant p53 levels were knocked down in three cancer cell lines by lentivirus expressing p53 shRNA as indicated and their growth rates measured in comparison to the corresponding cell lines generated after infecting with lentivirus expressing GFP shRNA as described in Materials and methods. Cells were harvested every other day and cell numbers were determined by Coulter Counter. Error bars represent standard deviation between the average cell counts. Experiments were done in triplicate, and data are representative of multiple independent repeats. Immunoblots show the efficacy of p53 knock down. ERK2 was used as a loading control. **D.** For the H1048 cells, we performed a PARP cleavage assay to check for caspase activation using PARP antibody from Cell Signaling. **E.** Apoptosis DNA-Ladder assay of H1048 cells stably transfected with shp53 (1) or shGFP (2). U937 cells after treatment with CAM (3) were used as a positive control for apoptosis.

p53 level decreases cell motility. Thus, mutant p53 contributes to the increased motility rate of cancer cells expressing GOF mutant p53.

Figure 5B shows a tumorigenicity assay of H1048 lung cancer cells when their p53 levels were knocked down by lentivirus expressing p53 shRNA, compared to shGFP controls. The data indicate that inhibiting mutant p53 expression leads to a reduction in tumor growth rate. Thus mutant p53 controls the rate of tumorigenicity of cancer cells expressing GOF mutant p53. We used three shp53 clones and two shGFP clones to rule out clonal variations, and got similar results. Taken together, these studies demonstrate that p53 mutants contribute to multiple GOF phenotypes in a wide range of cancer cell lines with endogenous p53 mutations.

Thus, different cell lines with different endogenous mutant p53 tested show similar loss of GOF activities in reducing the level of mutant p53 (Figures 3-5), suggesting the generality of the conclusion that mutant p53 levels determine GOF phenotypes.

Reducing NF-κB2 levels in cancer cells with mutant p53 by RNAi lowers GOF activity of mutant p53-expressing cells. We proposed earlier that mutant p53-induced GOF activity (such as chemoresistance) in cancer cells may be due to induction of NF-κB2 by mutant p53 (110). If that is the case then reducing the level of NF-κB2 in cancer cell lines with mutant p53 should reduce chemoresistance. Since reduction of mutant p53 levels reduces GOF activities in a number of cancer cell lines, we tested the idea whether a similar effect can be seen by reducing the level of NF-κB2. RNAi directed against NF-κB2 using MDA-MB-435 melanoma cells harboring a p53-G266E substitution (one of the cell lines tested in p53 knock down experiments above) was used for this. The data presented in Figure 6A and 6C show that RNAi directed against NF-κB2 reduces the level of expression of the corresponding gene, and reduces resistance to the chemotherapeutic drug etoposide substantially versus DMSO.

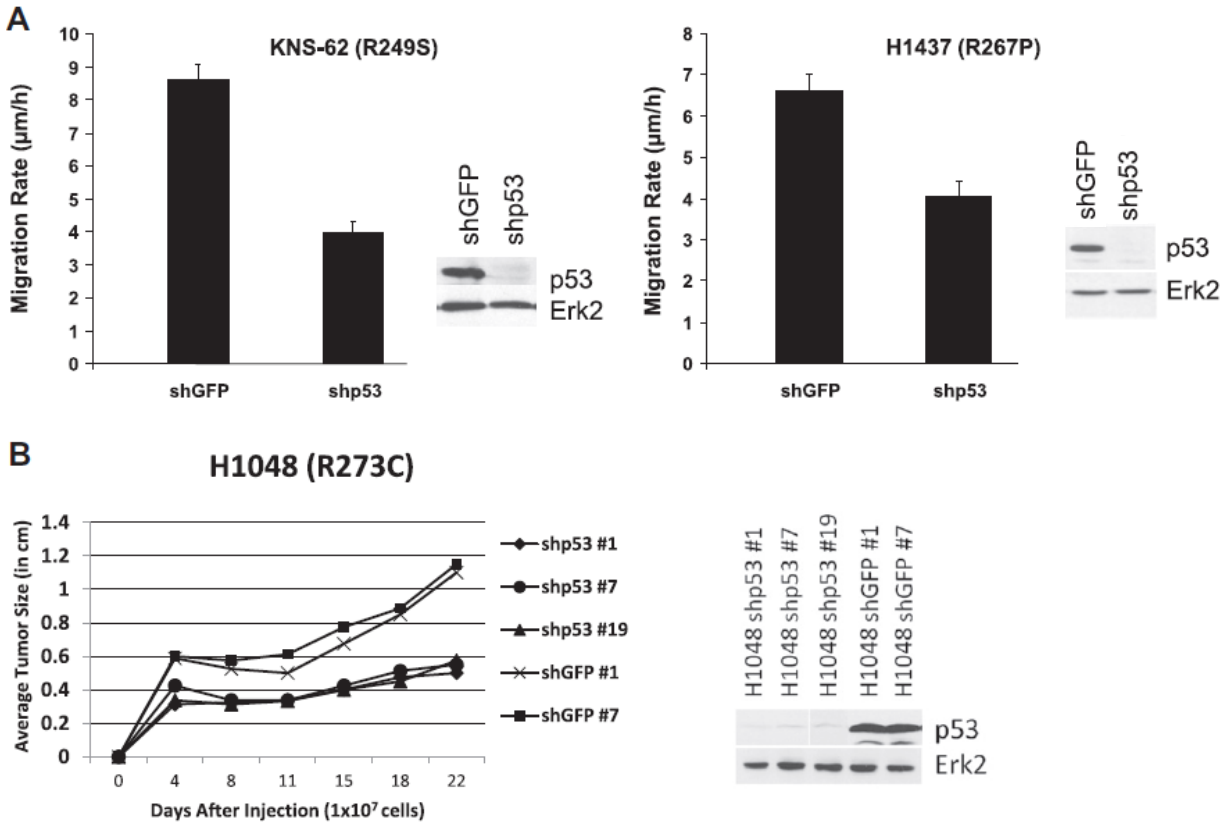

Figure 5. p53 knock down in cancer cell lines with naturally occurring p53 mutations causes a decrease in rate of motility and tumor growth. A. The indicated cell lines were cultured to confluence, a scratch made in the monolayer, and the distance measured as described in Methods. $p>0.001$ for both. Two mutant p53 knocked down clones (confirmed by Western blot, as indicated) were used for each cell line with the average migration between the clones shown. Error bars represent standard deviation from the mean migration rate. **B.** Mutant p53 levels were knocked down in the lung cancer cell line H1048 (p53-R273C) by lentivirus expressing p53 shRNA or GFP shRNA (used as our control cell line) as indicated and its tumorigenic ability measured after subcutaneous injection into nude mice. For all injections, $1x10^7$ cells/250µl media were used. Mice were injected subcutaneously and tumors allowed to grow to a maximum size of 1cm, measuring periodically as described before (17, 18). Three different clones of H1048 cells with mutant p53 levels knocked down by shRNA were used in comparison to two GFP shRNA control cell lines to eliminate clonal variations. Average tumor size was calculated by taking the average of the width and length of each tumor, then taking the average of all tumors from the particular cell line.

Furthermore, cell motility –another GOF p53 phenotype – is significantly inhibited by NF-κB2 RNAi (Figure 6B). p53 RNAi also inhibited cell migration (not shown), consistent with results with NF-κB2 RNAi. Thus, NF-κB2 at least partially regulates two mutant p53 GOF phenotypes in these cells, suggesting the possibility that mutant p53 may induce its GOF activity via NF-κB2 up-regulation, an observation we reached by expressing mutant p53 in H1299 lung carcinoma cell lines that are normally p53 null (196). Figures 6D and 6E show reduced resistance to the chemotherapeutic drug paclitaxel after treatment with RNAi against NF-κB2 in H1299 cells expressing either an empty vector (HC5) or the p53 mutant R273H, and Western analysis showing decreased NF-κB2 protein expression. Our data obtained by p53 knock down experiments, using cancer cell lines with naturally occurring mutant p53, and ectopic expression of mutant p53, in p53 null lung cancer cells, are similar, and interchangeable in this respect. Having determined that, we decided to determine the mechanism of up-regulation of NF-κB2 by mutant p53 using H1299 cell systems where mutant p53 was expressed.

Promoter deletion analysis by transient transcription assays does not indicate a mutant p53 response element. To test whether we could detect a mutant p53 response element on the NF-κB2 promoter, we generated a number of deletion mutants of the NF-κB2 promoter (110) by PCR and subcloned them into the pGL3-basic plasmid vector (Promega). We tested their promoter function by transient transfection in H1299 p53-null lung carcinoma cells as described (110). After harvesting the cells at 48 h post-transfection, we prepared extracts and determined the luciferase activity as described (110). Data presented in Figure 7 show that none of the deletion mutants eliminated the transactivation capacity of mutant p53–R273H. Some of the deletions resulted in higher transactivation by mutant p53 suggesting that some sequences of

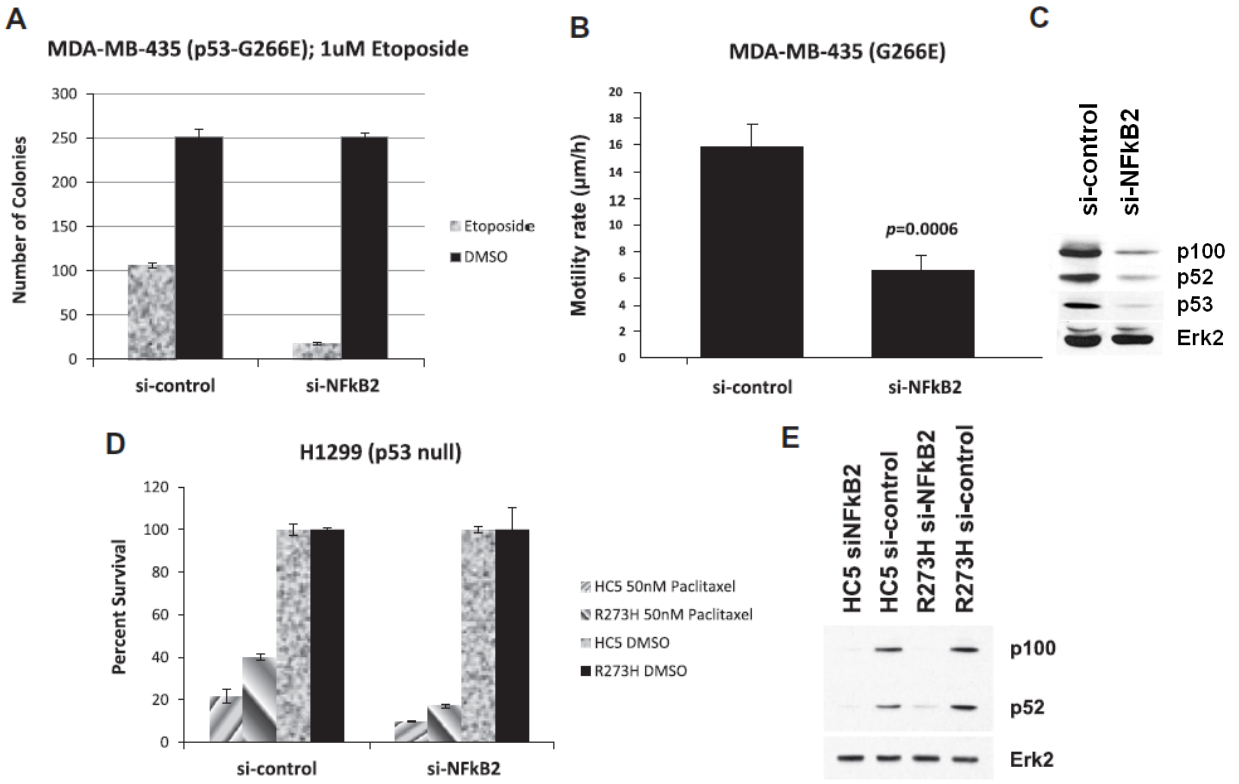

Figure 6. Reduction of NF-κB2 causes reduction of drug sensitivity, growth rate and rate of and motility. A. Chemoresistance of MDA-MB-435 cells depends on the NF-κB2 level. The indicated cells were transfected with control or NF-κB2-specific siRNA, treated with etoposide, and colony formation determined as described in Materials and methods (upper panels). Percent survival is shown in the figure with error bars representing standard deviation from the average number of colonies from triplicate samples. **B.** Motility of MDA-MB-435 cells depends on the NF-κB2 level. MDA-MB-435 cells were transfected as above. 48h later, standard Transwell migration assays were carried out, and migrating cells stained and counted as described (197). Bar = +/- 1 SD from the mean of 18 samples. **C.** NF-κB2 and Erk2 levels were determined by immunoblotting for MDA-MB-435 cells used in A and B. **D.** H1299 cells expressing an empty vector (HC5) or p53-R273H were treated with control (si-scrambled) or RNAi against NF-κB2, treated with paclitaxel, and colony formation determined as described in Materials and methods. **E.** Western analysis of NF-κB2 knock down by siRNA in H1299 cells expressing an empty vector or p53-R273H.

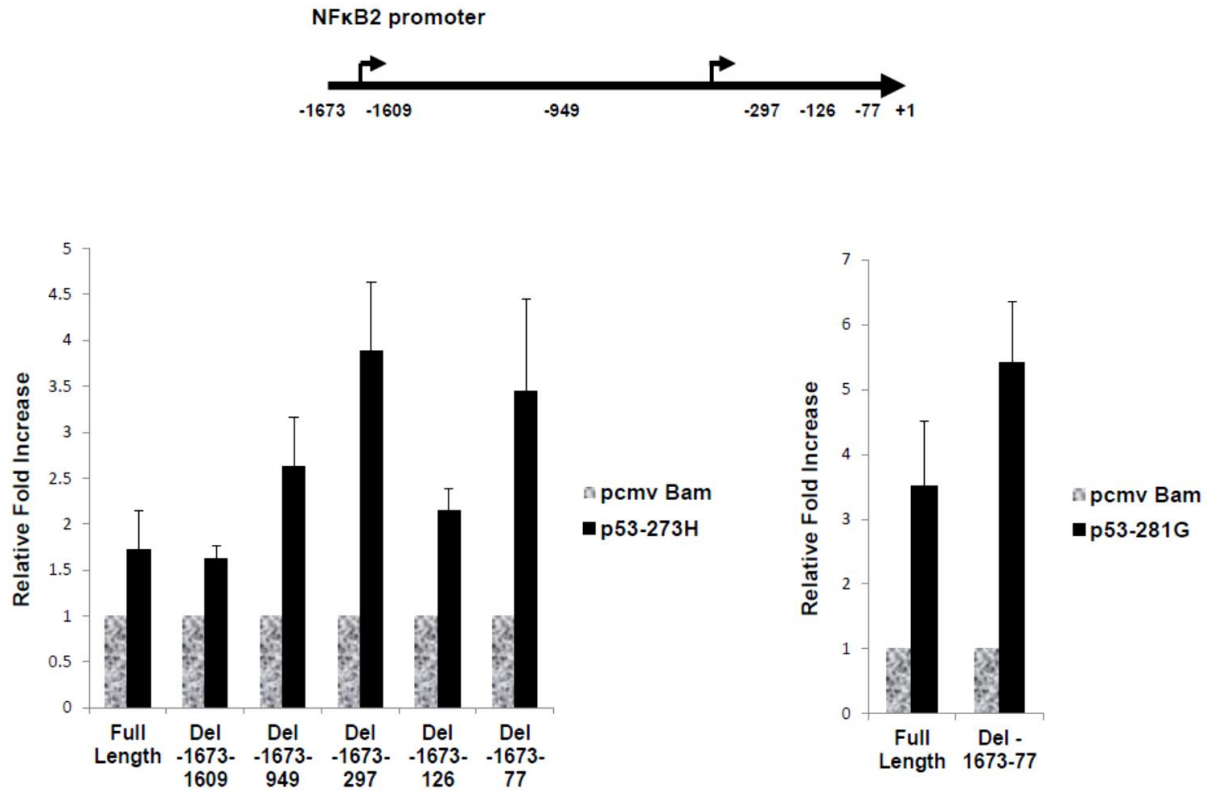

Figure 7. Promoter deletion analysis by transient transcription analysis does not indicate a mutant p53 response element. A number of NF-κB2 (110) promoter deletion mutants were generated by PCR, and their promoter activity was measured by transient transcriptional analysis using H1299 p53-null lung cancer cell lines transfected with empty vector as control, or p53-R273H (left panel) or p53-D281G (right panel). Forty-eight hours after transfection, cells were harvested and luciferase assays were performed using equal amounts of protein. The figure shows a representative example of multiple experiments. Relative fold of activation in luciferase activity has been plotted compared to that obtained by vector alone. Error bars indicate deviations in luciferase readings relative to vector transfected cells. All experiments were done in triplicate.

the promoter may act negatively on transactivation; the reason for that needs further investigation. We also tested another mutant, p53-D281G, with the full-length and the largest promoter deletion mutant. Both were transactivated, showing that this sequence independence has generality as far as mutant p53 is concerned. Thus, transient transcriptional analysis failed to provide evidence for a mutant p53 response element on the NF-κB2 promoter. These data are in agreement with those published by us earlier (198) where EGFR promoter deletion did not result in identification of a mutant p53 response element either. Therefore, it is possible that the gene specificity is attained at the chromatin level.

ChIP analysis indicates mutant p53 induced histone acetylation on the NF-κB2 promoter. Because mutant p53 can demonstrate GOF activity in different cells, and presumably one of its important targets is NF-κB2, we used H1299 cells in which mutant p53-R273H is expressed as a model to explore the mechanism of up-regulation. We wanted to test whether mutant p53 alters the chromatin structure on the endogenous NF-κB2 promoter in H1299 cells. Therefore, we performed ChIP assays using H1299 cells stably transfected with empty vector alone (HC5) or H1299 cells expressing mutant p53-R273H. The data shown in Figure 8A demonstrate that mutant p53 induces formation of acetylated histone H4 (but not acetylated histone H3) on the NF-κB2 promoter in H1299 cells expressing mutant p53–R273H, suggesting that the up-regulation of NF-κB2 expression by mutant p53 seen in these cells may occur via a transactivation role of mutant p53 executed at the NF-κB2 promoter through chromatin modification.

Mutant p53 induces binding of CBP and STAT2 on the NF-κB2 promoter. To extend our studies, we used ChIP analysis to determine whether there is preferential enhancement of interaction of one or more transcription factor(s) on the NF-κB2 promoter in

H1299 cells stably transfected with vector alone (HC5) or expressing mutant p53-R273H. The transcription factors CREB, NF-κB p65, STAT2, CBP, c-Rel, and p53 were chosen for ChIP analysis based on transcription factor binding sites located on the NF-κB2 promoter as well as known interactions. The data presented in Figure 8B show that STAT2 nucleation, as determined by ChIP assays, is significantly higher in mutant p53 expressing cells. Thus, our results demonstrate that mutant p53 induces STAT2 interaction on the NF-κB2 promoter, and suggests a STAT2-mutant p53 mechanism. Similarly, when tested by ChIP analysis, CBP, a histone acetyltransferase, is nucleated more on the NF-κB2 promoter in the presence of mutant p53 (Figure 8C). This suggests that CBP nucleation enhances histone acetylation on the promoter.

Figure 8 also shows no enhanced nucleation of mutant p53 on the NF-κB2 promoter as we did not observe any additional promoter fragments immunoprecipitated by p53 antibody DO1 or a p53 antibody recognizing the total protein (Figures 8C and 8D). This suggests that under the conditions of our assay, including cross-linking, we were unable to detect mutant p53 directly on the promoter. It is possible that by changing conditions we may be able to detect mutant p53 on the promoter if it is nucleated via protein-protein interactions.

Chapter Summary.

Since a previous report suggested part of mutant p53's chemoresistance was due in part to up-regulation of NF-κB2, this chapter investigated the role NF-κB2 plays in mutant p53 mediated gain of function. We report that p53-mediated NF-κB2 up-regulation significantly contributes to the aggressive oncogenic behavior of cancer cells. Lowering the level of mutant p53 in a number of cancer cell lines resulted in a loss of GOF phenotypes directly implicating

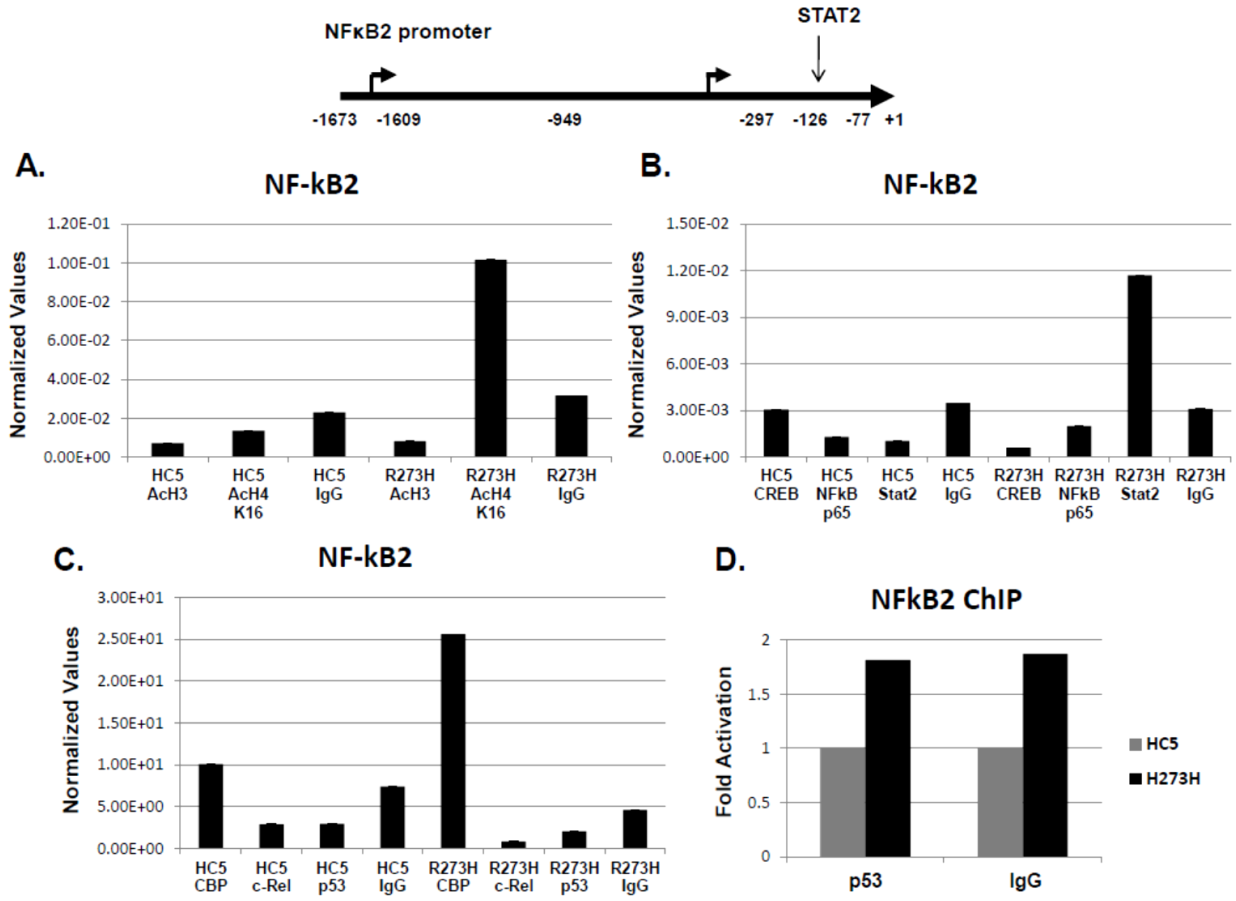

Figure 8. ChIP analysis indicates acetylation of histones on the NF-κB2 promoter. ChIP analysis was performed on H1299 cells stably transfected with vector (HC5) or mutant p53-R273H (R273H) to test whether histones are preferentially acetylated on the NF-κB2 promoter, and if different transcription factors and mutant p53 are nucleated on the promoter. **A.** ChIP performed with antibodies directed against acetylated histone H3 (acetylated at K9 and K14) and H4 (acetylated at K16). **B.** ChIP performed with antibodies directed against CREB, NF-κB p65, and STAT. **C.** ChIP performed with antibodies directed against CBP, cRel and p53 DO1. **D.** ChIP performed with an antibody against the total p53 protein

p53 mutants in the process. RNAi against NF-κB2 in naturally occurring cancer cell lines also lowered GOF activities. In H1299 cells expressing mutant p53, chromatin immunoprecipitation (ChIP) assays indicated that mutant p53 induces histone acetylation at specific sites on the regulatory regions of its target genes. ChIP assays using antibodies against transcription factors putatively capable of interacting with the NF-κB2 promoter show increased interaction of CBP and STAT2 in the presence of mutant p53. Thus, we propose that in H1299 cells, mutant p53 elevates expression of genes capable of enhancing cell proliferation, motility, and tumorigenicity by inducing acetylation of histones via recruitment of CBP and STAT2 on the promoters causing CBP-mediated histone acetylation.

Chapter 2

DETERMINE HOW MUTANT P53 UTILIZES RECEPTOR TYROSINE KINASE AXL TO ACCOMPLISH ITS GAIN-OF-FUNCTION ACTIVITY

The work presented in this chapter has been published in the Genes and Cancer research journal (Genes Cancer. 2012 Jul;3(7-8):491-502.). Microarray data was analyzed by Brad Windle, Western analysis of H1299 cells expressing different p53 mutants was performed by Shilpa Singh, the H1793 growth assay was performed by Becky Frum, and the motility assays were performed by Andrew Yeudall.

Introduction.

We and others have identified several genes important for their involvement in growth and oncogenesis that are regulated at the level of expression by GOF mutant p53 (110, 135, 178, 199-202). Receptor tyrosine kinases (RTKs) play an important role in growth and differentiation of normal cells, and represent a major class of protooncogenes, involved in the progression and metastasis of cancer (203). Axl/Mer/Sky represents a comparatively recent class of the RTK family that induces extracellular signals inside cells (204). Axl is a RTK with transforming activity (205, 206), and may be used as a target for therapy (207).

The antiapoptotic, cell adhesion, and chemotactic activities of Axl have been ascribed to an increased expression of Axl and its increased interaction with Gas6 (208). The invasiveness and metastasis in various cancer cell types is associated with an elevated level of Axl expression, which is also correlated with poor prognosis of patients with various cancers such as: myeloid leukemia (205, 209), metastatic lung cancer (210, 211), breast cancer (212), and gastric cancer (213). Use of RNAi techniques and dominant-negative receptor mutants of Axl have resulted in growth suppression of cancer cells in a xenograft model (214, 215).

Here, we show that the receptor protein tyrosine kinase Axl is up-regulated by p53 mutants in H1299 lung cancer cells expressing mutants p53-R175H, -R273H, and –D281G. Knock-down of Axl by Axl-specific RNAi caused reduction of GOF activities in lung cancer cells expressing endogenous mutant p53 suggesting that mutant p53 may induce Axl as one of the target genes to execute its GOF activities.

Materials and Methods.

Cell lines. Four human lung cancer cell lines: H1299 (p53-null, NSCLC), H1048 (p53-R273C, SCLC), H1437 (p53-R267P, NSCLC), ABC-1 (p53-P278S, Adenocarcinoma), and H1793 (p53-R273H, NSCLC) were used in these studies. H1299, H1048, and H1437 were grown in RPMI media supplemented with 10% fetal bovine serum while H1793 was grown in DMEM supplemented with 5% fetal bovine serum.

Generation of H1299 cells expressing GOF p53 mutants. To determine whether expression of mutant p53 leads to GOF phenotypes in human cells, we have used the H1299 (p53-null) lung cancer cell line and generated cells expressing p53 mutants -R175H, -R273H and -D281G (216). These GOF p53 mutants are commonly found in human cancer [http://www.iarc.fr/p53/Index.html].

Generation of mutant p53 knock down cell lines. The independent cell lines H1048, ABC-1, and H1437 were used to make stable p53 knock down cell lines. They were generated by using lentivirus expressing short hairpin RNA (shRNA) against p53 utilizing lentivirus systems (Open Biosystems) following the manufacturer's protocol. Clones were isolated using puromycin selection at 1 μg/ml.

Expression analysis. Microarray analysis was carried out as described previously (110, 134) after isolating RNA from H1299 cells expressing p53 mutants or stably transfected with vector alone. We analyzed expression of different genes using Affymetrix U95Av2 arrays as described earlier (110, 134). QPCR analysis of RNA levels were also done as described before (110, 134).

Tumor RNA analysis and p53 sequencing. Tumor RNAs were provided by the Tissue and Data Acquisition and Anaylsis Core repository under an Institutional Review Board approved protocol; cDNAs were prepared using the Superscript III cDNA synthesis kit (Invitrogen) and QPCR performed using primers specific for Axl (F: 5'-TGT TTG GTG TTT CTG GGA CA-3' and R: 5'-TCG CAG GAG AAA GAG GAT GT-3'). The degree of expression was quantitated using a relative standard curve and normalized to GAPDH (F: 5'-GTC AAC GGA TTT GGT CGT ATT-3' and R: 5'-GAT CTC GCT CCT GGA AGA TGG-3') corresponding to the cDNA batch. The p53 gene is sequenced following the method described by Sjogren *et al*. (217). Whenever a mutation is found, a new PCR reaction is performed and fragment re-sequenced to verify sequence information obtained previously. The AXL levels in the two populations of tumors, one with WT p53 and one with mutant p53, were compared and found to be different with a statistically significant difference based on the student t test.

siRNA transfection. Lung cancer cells were transfected with Lipofectamine 2000 (Invitrogen) two times (once every 24 hours) with RNAi directed against a specific or non-specific gene (luciferase) following the manufacture's protocol. Sequences used were: Control (C): 5'- CAU GUC AUG UGU CAC AUC ACT T -3' and 5'-GAG AUG UGA CAC AUG ACA UGT T -3' and Axl siRNA, was purchased from Santa Cruz Biotechnology. Forty eight hours

after transfection, cells were trypsinized, counted, plated and growth or migration assays were carried out as described below.

Growth assay. Growth assays were carried out as described by us earlier with slight modifications (110, 135). Cells were plated at 50,000 cells/6cm dish in triplicate for five time points and harvested after incubation with trypsin and counted using a Coulter Counter (Beckman). Each set of triplicate plates was counted three times, giving a total of nine counts per siRNA treatment per day. To determine the number of cell doublings, the ratio of Axl and control (Scram) siRNA treated cells was calculated to be the number of cells counted on day two divided by day one, and so on for the five time points. The ratios throughout the assay were averaged and plotted, and the standard deviation was calculated to be the difference in ratios from the triplicate counts per time point. All experiments were done in triplicate, and repeated multiple times. The cells lines were analyzed for growth rate using replicates based on exponential growth. The statistical difference between growth rates was determined by the student t test.

Axl promoter cloning and transient promoter assays. Axl promoter and its deletion mutants were cloned in pGL3-basic vector using sequence information available in NCBI. The primers used to generate the Axl promoter deletions were: 0-959: 5'-CCG GGG TAC CCG CAG GCA GCA GAT CTG CAA TAA C-3' and 0-200: 5'-CCG GTT ACC GGG AGT GAG GGA AGG AGG CAG GGG TGC TGA-3'. Transient transfection was performed with 200ng of promoter and 1ug of expression plasmid using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Luciferase analysis was carried out using the luciferase assay system (E1500) and instructions from Promega. Both transfection and luciferase assay were as described previously in triplicate (218).

Chromatin immunoprecipitation. Chromatin immunoprecipitations were performed as described (134). To crosslink protein and DNA, cell cultures were incubated in 2% formaldehyde for 10min at 37°C and then 200mM glycine was added for a further 10min. Cells were washed in cold phosphate buffered saline (PBS), scraped and centrifuged. Pellets were resuspended in lysis buffer containing 1% protease inhibitors and then sheared by six passages through a 27.5 gauge needle followed by 25min of sonication on ice such that the chromatin is fragmented to 500–2000bp in length. Following centrifugation, the protein content of the supernatants was determined and equal amounts used for immunoprecipitation overnight at 4ºC with gentle tilting with protein-specific antibodies or IgG as a control. Immune complexes were captured using Protein A-agarose, then washed sequentially in RIPA buffer (150mM NaCl, 50mM Tris pH8, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40), high salt buffer (500mM NaCl, 50mM Tris pH8, 0.1% SDS, 1% NP-40), twice in LiCl buffer (250mM LiCl, 50mM Tris pH8, 0.5% sodium deoxycholate, 1% NP-40) then twice in TE buffer. Protein was eluted from beads in fresh elution buffer (20% SDS, 10mM DTT, 100mM $NaHCO_3$), crosslinking reversed overnight at 65ºC in the presence of NaCl, and then samples were ethanol-precipitated. Following centrifugation, pellets were resuspended in TE buffer and incubated sequentially with 10mg/ml RNase A (30min) and 20mg/ml proteinase K (1h). Samples were phenol-extracted, ethanol-precipitated, and the pellets washed in 70% ethanol, dried and resuspended in sterile water. Acetyl histone H3 (17-615), acetyl histone H4 K8 (07-328), and normal rabbit IgG were from Millipore. p300 (sc-585), CREB (sc-186), E2F1 (sc-22820), p53 DO1 (sc-126), and p53 FL393 (sc-6243) were obtained from Santa Cruz. PCR primers used to analyze ChIP samples were: Axl (5kb) F: 5'-CCT TGA CTG AGG CTT TAC CA-3' and R: 5'-TTT TCA AAG TGC ACC GAC AT-3'; and Axl F: 5'-GAT GCA GCA GTT CCC AAA AT-3' and R: 5'-TAT CAT

CCC TTC TCC ATC GC-3'. The Axl promoter scanning primers are: Axl 2200-2000bp are the same primers indicated above, 1-400bp F: 5'-CCC CGT CTC TAC CAA AAA TA-3' and R: 5'-GGC CCT TCA CCG TTG T-3'; 401-800bp F: 5'-GAA GGG GCA GGT AGA AGA GA-3' and R: 5'-AGC CCT GAT CAT TCC ACT G-3'; 801-1200bp F: 5'-AGC GAT CCT CCC ACC TT-3' and R: 5'-ATC TTC AGA CAC GCC AAA AC-3'; 1201-1600bp F: 5'-TCT GCG TGT CTC TGC TTG TC-3' and R: 5'-TCT GGG CTC TGT GTC TGG TA-3'; and 1601-2000bp F: 5'-GGT CCC CTT CCC CCT CCT CA-3' and R: 5'-CCC AGC AGC CGC CTT CTC A-3'. Axl (5kb) corresponds to a sequence 5 kb upstream of the Axl gene without any regulatory sequences in it. This was used to normalize the QPCR values. The percent p53 binding was calculated by determining the fold activation of the Axl promoter by mutant p53 (versus control) for each region of the promoter. Five sets of ChIP experiments were averaged and the percent binding was calculated by setting the Axl ChIP primers (2200-2000bp) as equal to 100%. The standard deviation was calculated between the five sets of experiments and plotted.

Western blotting. Immunoblottings were carried out as described (110). Axl levels were detected using an antibody from Abnova (H00000558-M01). Erk2 (sc-154) and β-tubulin (sc-5274) levels were detected by antibodies from Santa Cruz. p53 was detected using the p53 antibody PAb 1801 (93). Westerns blots were developed by the ECL method (GE Healthcare; Piscataway, NJ).

Migration assays. Cell migration was carried out using wound-healing (scratch) assays, as previously described (219). Briefly, cells were trypsinized, plated in quadruplicate in 12-well cell culture plates and incubated at 37°C until cells were completely confluent. At this time, a sterile pipette tip was used to scratch across the surface of the plate, removing the complete layer of cells within the scratch area. Following cell removal, each well was washed once with PBS

and then replaced with growth medium. Immediately following, the width of the scratch was measured at six specific points under a 5x objective using a light microscope and AxioVision software (Carl Zeiss Microimaging, Thornwood, NY). Cells were incubated at 37°C from 20-60h depending on the cell line under study, at which time the scratch width was measured at the same position as at time 0.

Experimental Results.

The transactivation-deficient mutant p53-D281G (L22Q/W23S) is effective in up-regulating many mutant p53 target genes. We focused our attention on potential mechanisms of GOF mutant p53 by studying the role for p53's transactivation domain in gene regulation. We determined whether the triple amino acid substitution mutant p53-D281G (L22Q/W23S) has retained the ability to up-regulate expression of mutant p53 target genes identified by our expression analysis (110). We compared gene expression profiles of three H1299 cell lines stably transfected with vector alone (HC5), stably expressing p53 mutant – D281G, and the transactivation domain mutant -D281G (L22Q/W23S) using Affymetrix GeneChip arrays. The data shown in Table 1 shows a list of examples genes induced by p53 D281G and not affected by the 22Q, 23S mutations (p-value = $3x10^{-24}$). This indicates that a substantial part of the mutant p53 gene expression signature is independent of the transactivation domain at codons 22 and 23.

Interaction between mutant p53 and p63 has been demonstrated and shown to be independent of the transactivation domain mutants (220). The mutant p53 induced genes we identified that are not dependent on the transactivation domain might involve a mutant p53-p63 interaction. We analyzed mutant p53 induced genes for over-representation of genes that contain

Table 1. H1299 Cells Expressing Transactivation-Deficient Mutant p53-D281G (L22Q/W23S) Are Efficient in Up-Regulating Mutant p53 Targets

Symbol	Fold over the control	*P* value	Gene
ACTN4	5.47	0.001	Actinin, α 4
AXL	7.04	0.0007	AXL receptor tyrosine kinase
BTG1	0.49	0.0009	B-cell translocation gene 1, antiproliferative
CDC25B	0.51	0.001	Cell division cycle 25B
EEF1A2	0.28	0.0008	Eukaryotic translation elongation factor 1 α 2
ERCC1	5.23	1.04E-05	Excision repair cross-complementing rodent repair deficiency, complementation group 1
ERCC2	2.85	0.003	Excision repair cross-complementing rodent repair deficiency, complementation group 2
ERF	3.72	1.40E-05	Ets2 repressor factor
FN1	0.27	0.002	Fibronectin 1
GARS	5.26	0.002	Glycyl-tRNA synthetase
GDI2	0.29	0.001	GDP dissociation inhibitor 2
GPR126	0.43	0.0001	G protein–coupled receptor 126
IGFBP6	17.25	0.0004	Insulin-like growth factor binding protein 6
PRAME	0.28	0.0002	Preferentially expressed antigen in melanoma
PRKAR1A	0.19	8.78E-05	Protein kinase, cAMP-dependent, regulatory, type I, α (tissue-specific extinguisher 1)
PSMB6	0.31	0.0002	Proteasome (prosome, macropain) subunit, β type, 6
RAB31	0.22	0.001	RAB31, RAS oncogene family member
RELB	4.77	3.82E-05	v-Rel
SRPX	0.37	0.0009	Sushi repeat–containing protein, X-linked

putative p63 binding sites. The Axl gene was selected for further study based on the presence of a p63 binding site, it's known role in oncogenesis (205, 211, 214, 221, 222), and it's potential role in mutant p53 GOF activities. We first verified the microarray data by performing RT-QPCR quantitation of the Axl mRNA, confirming induction by mutant p53. Data presented in Figure 9A show presence of more Axl RNA in the H1299 cells expressing mutant p53 compared to vector transfected cells (compare lanes HC5 with R175H, R273H, D281G and D281G (L22Q/W23S)). We also tested the Axl expression at the protein level by performing immunoblot analysis as described in Materials and Methods. Protein expression analysis shown in Figure 9B demonstrates that cell lines expressing mutant p53 express higher levels of Axl protein compared to the vector transfected cells. This induction of Axl protein expression by mutant p53 remains when the transactivation domain mutations at codons 22 and 23 are present, confirming our RNA expression analysis. Similar data was also observed when we compared Axl levels in H1299 cells expressing p53-R273H and -R273H with additional mutations at codons 22 and 23 (Figures 9C and 9D).

We investigated if the converse was true, that reduced expression of endogenous mutant p53 would result in reduced expression of Axl. We studied the Axl RNA level in two lung cancer cell lines with endogenous mutant p53, H1437 (p53-R267P) and H1048 (p53-R273C), after knock-down of the mutant p53 levels in these lines by generating stable cell clones using lentivirus vectors expressing p53 shRNA with comparison to control cells expressing green fluorescence protein (GFP) shRNA. Data depicted in Figure 10A shows that reduction of mutant p53 levels is accompanied by a reduction of Axl levels suggesting, along with the mutant p53 induction studies, that mutant p53 regulates Axl expression.

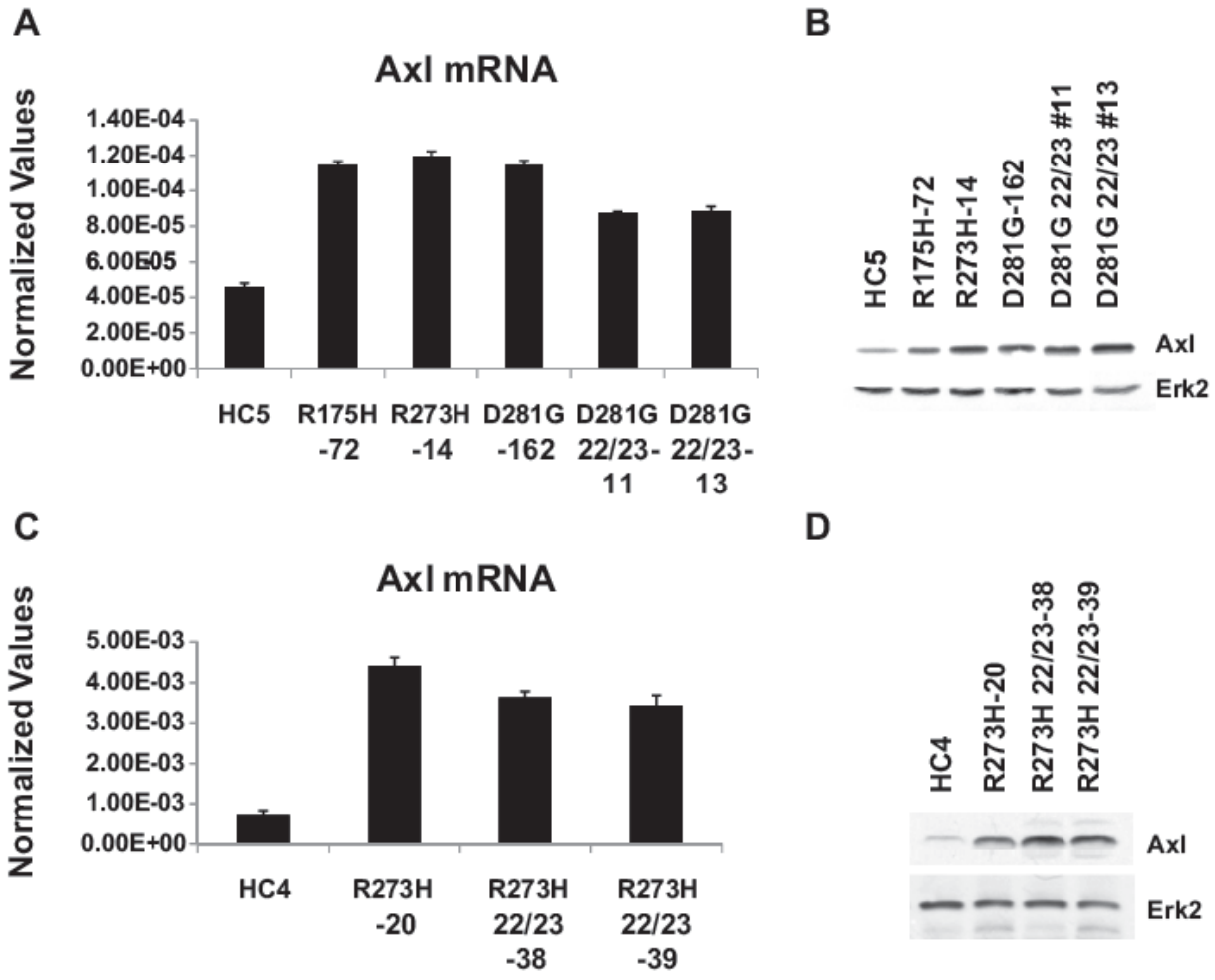

Figure 9. H1299 cells expressing gain of function mutant p53 up-regulate protein receptor tyrosine kinase Axl. **A and C.** Reverse transcriptase (RT) QPCR analysis of Axl levels in H1299 cells stably transfected with vector or mutant p53 expression plasmids. QPCR was performed for GAPDH and was used to normalize with Axl values. **B and D.** Western blot analysis of Axl levels in H1299 cells stably transfected with vector or mutant p53 expression plasmids. Data shows mutant p53 induced Axl expression both at RNA and protein levels in H1299 cells, and this induction is not disturbed by mutations at amino acids 22 and 23. Error bars showing standard deviations are indicated.

Lung tumors cells expressing mutant p53 show higher Axl levels. Since we observed that mutant p53 up-regulates Axl expression in lung cancer cell lines studied, we investigated whether this is also valid in human lung tumors expressing mutant p53. Figure 10B depicts the Axl level of different human lung tumors collected in Virginia Commonwealth University cancer tissue repository. On average, there was significantly more Axl expression in samples with mutant p53 versus samples with WT p53 (average 5.63-fold, p-value 0.013) corroborating our cell culture data that mutant p53 upregulates Axl expression.

Axl up-regulation mediates GOF activities of mutant p53. Axl is known to be involved in promoting growth and movement of cells (208, 210, 214, 215, 222-224). Therefore, we tested whether mutant p53-induced enhancement of growth and motility has any relationship with the fact that mutant p53 up-regulates expression of Axl. We used the lung cancer cell line H1048 expressing a mutant p53 (-R273C) for testing its growth rate and motility as described in Materials and Methods. To test whether the level of Axl affects the properties affected by GOF of mutant p53 expression, we transfected this cell line with siRNA against Axl (or control scrambled siRNA), and performed the growth and motility assays. Figure 11A shows a representative example of the growth effect showing a reduction of growth rate (shown as cell doubling/day) when the Axl level is reduced (p-value 0.0065). This suggests a correlation between mutant p53-mediated up-regulation of Axl level and the growth rate enhancement induced by mutant p53. We also tested the relationship between cell motility rate and Axl level in these cells. As shown in Figure 11B there is a significant reduction in cell mobility rate as

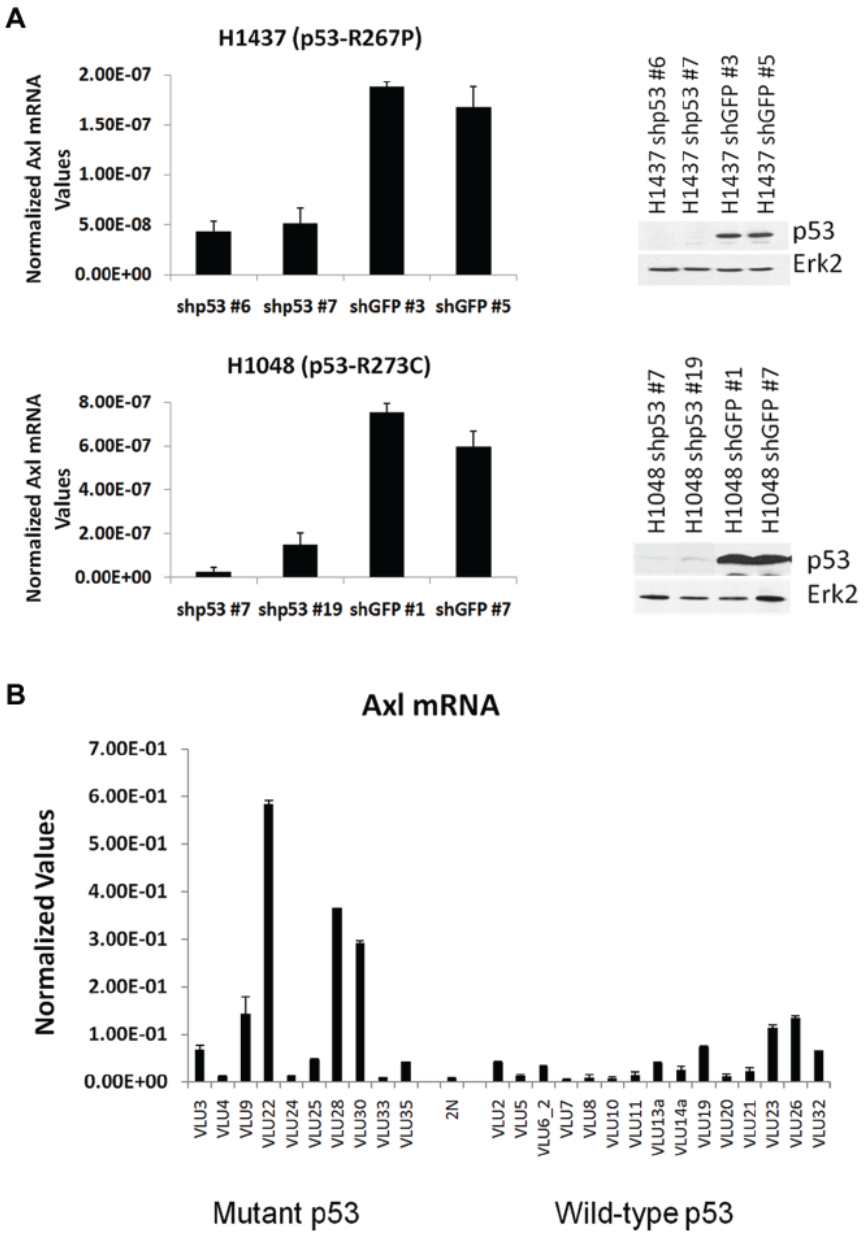

Figure 10. Lung tumor cells expressing p53 mutations show higher Axl levels. A. RT-QPCR of Axl mRNA levels in lung cancer cell lines H1437 and H1048. Lung cancer cell lines H1048 and H1437 were infected with control shRNA or p53 shRNA lentivirus to generate mutant p53 knock down cell lines. Western blot analysis was performed on isolated clones to identify p53 knock down clones. Erk2 was used as a loading control. These clones were further analyzed for Axl mRNA expression by QPCR using primers specific for the Axl gene and normalized by GAPDH. The data indicates lowering of mutant p53 levels lowers Axl levels. **B.** RT-QPCR of Axl levels in lung tumors. cDNA was prepared from human lung tumor RNAs (labeled VLU to protect patient identity) and a normal tissue sample (labeled 2N). The degree of expression was quantitated using a relative standard curve and normalized to GAPDH corresponding to the cDNA batch.

measured by scratch assays when the Axl level is reduced by the Axl siRNA even though the mutant p53 level remains unchanged. Similar data have been obtained from H1793 cell as well (Figures 11C, 11D). Thus, mutant p53 may induce some of its GOF activities via induction of Axl.

Mutant p53 up-regulates the Axl promoter. We have evidence that mutant p53 expressing cells have higher Axl levels both at RNA and protein levels (see above); therefore, we determined whether the upstream sequences of the human Axl gene can act as a faithful regulatory promoter in a transient promoter assay. We have cloned a 2000 bp long fragment encompassing the upstream sequences of the Axl into the pGL3 basic luciferase reporter vector (Promega), and tested its promoter activity by transient transfection analysis. We transfected p53-null human lung cancer H1299 cells with Axl-pGL3 basic in the presence and absence of different p53 expression plasmids and after 48 hours performed luciferase assays as described (110, 225). The data shown in Figure 4 indicate that mutant p53 up-regulates the Axl upstream sequences as expected. The Axl promoter sequence contains a putative p53/p63 binding site on the Axl upstream sequences raising the possibility of its involvement in mutant p53-mediated transactivation since some studies of GOF functions have implicated the p63-mutant p53 interactions (179, 180, 226, 227). Data shown in Figure 12 (and Figure 13, see below) also show that p63 (full length p63γ) and WT p53 could transactivate the Axl promoter depending on the presence of the p53/563 binding site.

Mutant p53 up-regulates the Axl promoter in H1299 cells independent of the p63 binding site. We generated two Axl promoter deletion mutants with and without the p63-binding site and tested whether transactivation by mutant p53 requires the p63-binding site. The data presented in Figure 13 shows that p63-mediated transactivation is lost by a deletion of the

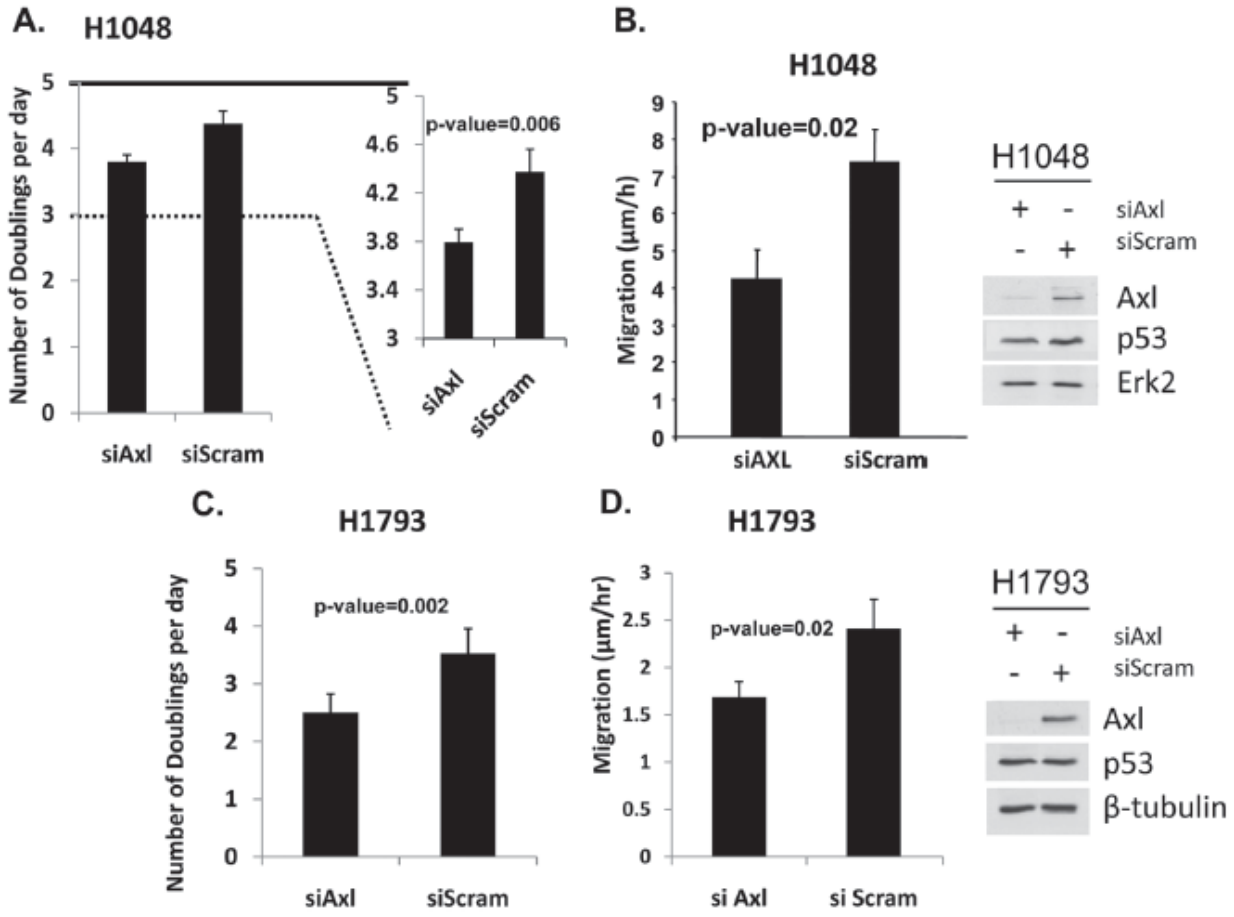

Figure 11. Axl up-regulation by mutant p53 has physiological significance. A and C. Growth rate of H1048 and H1793 lung cancer cells depends on the Axl level. H1048 and H1793 cells were transfected with control or Axl-specific siRNA, plated in equal numbers, and harvested each day for five days to determine the rate of doubling. In parallel, p53, Axl and Erk2 levels were determined by immunoblotting (right panel). **B and D.** Motility of H1048 and H1793 cells depends on the Axl level. H1048 and H1793 cells were transfected as above. 48h later, standard scratch assays were carried out as described in Materials and Methods, and migrating cells stained and counted. Bar = +/- 1 SD.

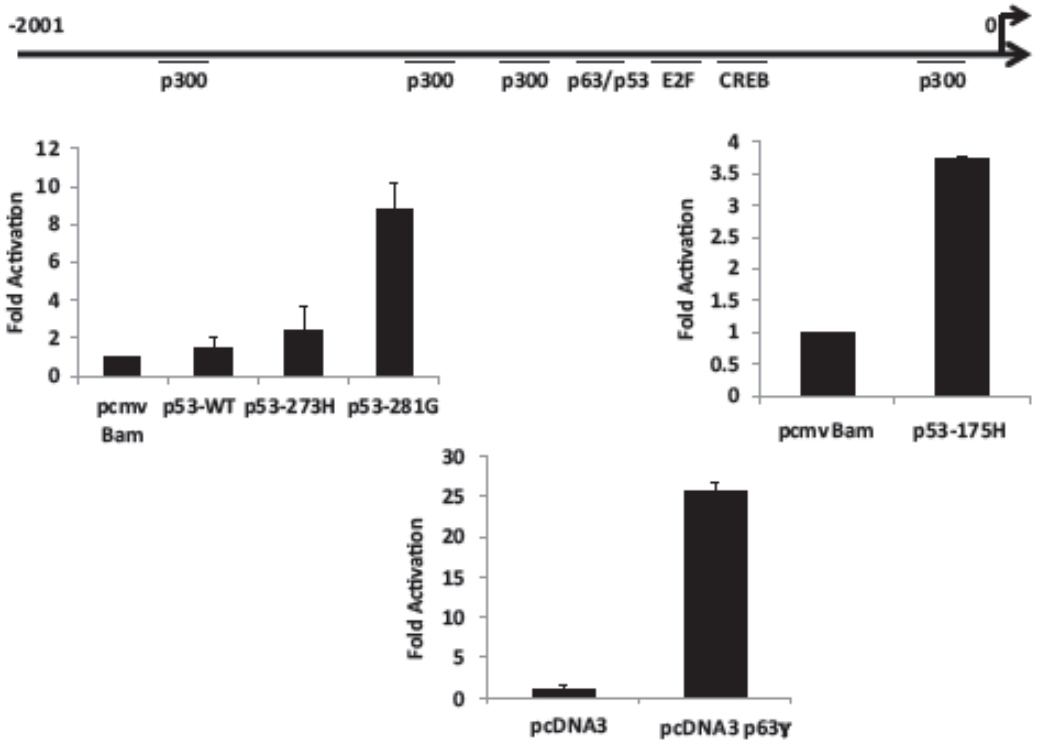

Figure 12. Mutant p53 up-regulates the Axl promoter in H1299 cells. H1299 cells were transfected with 200ng of pGL3-basic vector containing the Axl promoter upstream of the luciferase reporter gene, and 1ug of the indicated p53 expression plasmid. Cells lysates were prepared 48hr after transfection, and luciferase activity was determined. Data is shown as fold activation over control. Experiments were done in triplicate.

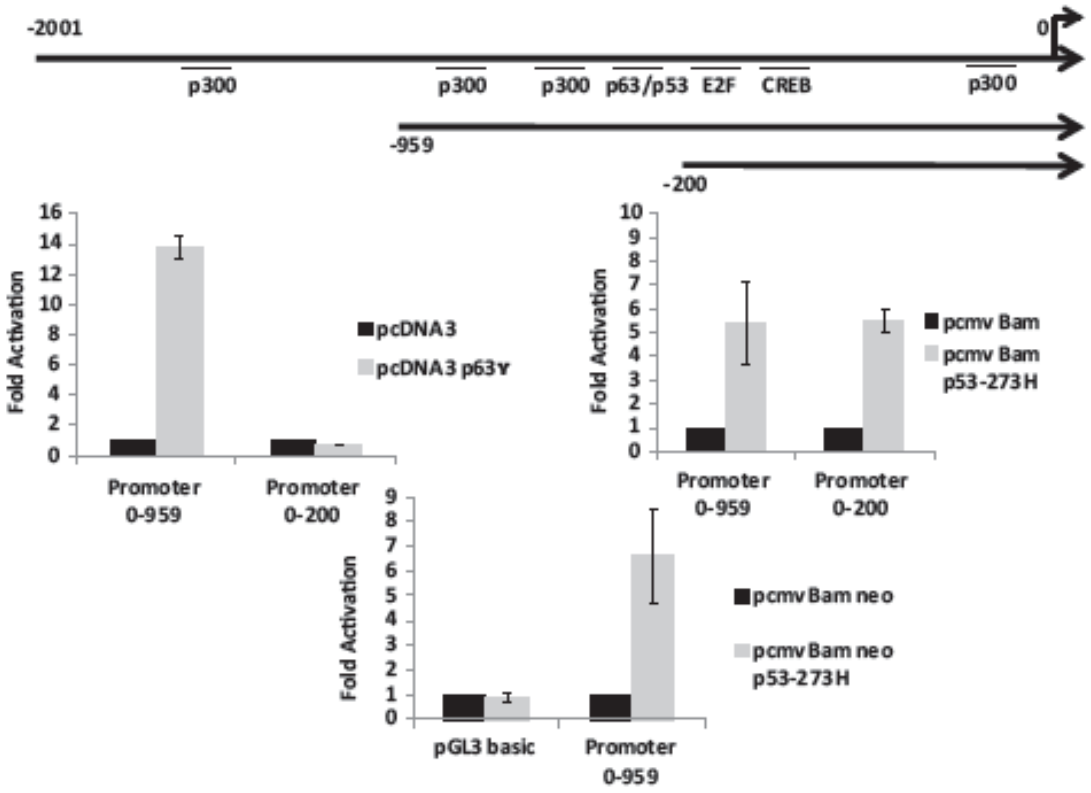

Figure 13. Mutant p53 up-regulates the Axl promoter in H1299 cells independent of the p63 site. Two deletion mutants of the Axl promoter were constructed and tested for promoter activity in H1299 cells in the presence and absence of mutant p53 and p63 to determine if the putative p63-binding site is required for mutant p53-mediated transactivation. The data indicates that mutant p53 does not require the p63 binding site to transactivate the Axl promoter. H1299 cells were also co-transfected with pGL3 basic or the larger Axl deletion mutant and empty vector or mutant p53 to ensure the transactivation capabilty is not lost. Data is shown as fold activation over control. Experiments were performed in triplicate.

p63-binding site; however, mutant p53-induced transactivation remained undisturbed even in the absence of the p63-binding site while the negative control (pGL3-basic alone) did not get activated at all. This suggests that GOF mutant p53 transactivates the Axl promoter independent of the p63 binding sites in transient transfection assays.

Mutant p53 induces acetylation of histones on the Axl promoter. We used H1299 cells expressing mutant p53-R273H as a model to study the mechanism of up-regulation of Axl expression by mutant p53. We performed ChIP assays using H1299 cells stably transfected with empty vector alone or H1299 cells expressing mutant p53-R273H using an antibody directed against acetylated histone to assay for the extent of histone modification as a measure of transcriptional activity at this gene. The data shown in Figure 14A and 14B demonstrate that mutant p53 induces enhanced formation of acetylated histone H3 and H4 on the Axl promoter in H1299 cells expressing mutant p53-R273H, suggesting that the up-regulation of Axl expression by mutant p53 seen in these cells may occur via a transactivation role of mutant p53 through chromatin modification at the Axl promoter location.

Knock-down of mutant p53 reduces histone acetylation on the Axl promoter. Since the overexpression of mutant p53 induces the acetylation of histones on the Axl promoter, we wanted to see if the reduction of mutant p53 would produce the opposite effect. We used two lung cancer cell lines, ABC-1 (p53-P278S) and H1048 (p53-R273C) after knock-down of their endogenous mutant p53 using a shRNA lentivirus directed against p53 or control GFP. The data shown in Figures 14C and 14D show a decrease of histone acetylation upon mutant p53 reduction compared to GFP control in both ABC-1 (Figure 14C) and H1048 (Figure 14D) cell lines. This further supports the idea that mutant p53 up-regulates Axl expression via chromatin modification.

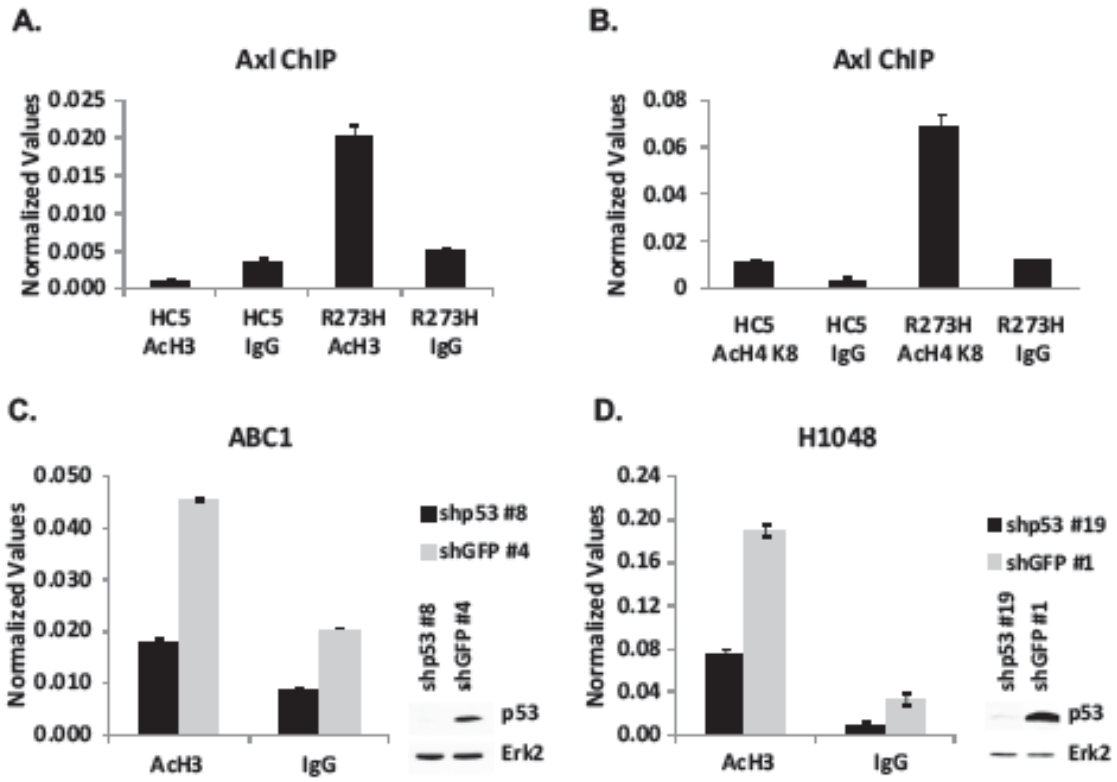

Figure 14. ChIP assay demonstrates increased acetylation of histones on the Axl promoter. ChIP analysis was performed on H1299 cells stably transfected with vector (HC5) or mutant p53-R273H (R273H) to test whether histones are preferentially acetylated on the Axl promoter. **A.** ChIP performed with an antibody directed against acetylated histone H3 (acetylated at K9 and K14). **B.** ChIP performed with an antibody directed against acetylated histone H4 (acetylated at K8). **C and D.** ChIP performed on ABC-1 and H1048 p53 knock-down cell lines using an antibody directed against acetylated histone H3 (acetylated at K9 and K14) shows a reduction in histone acetylation on the Axl promoter. Experiments were done in triplicate.

Nucleation of mutant p53 on the Axl promoter. Next we examined if we could locate mutant p53 on the Axl promoter sequences using the ChIP and H1299 cell systems described above (Figure 14). Here, chromatins were immunoprecipitated using p53 antibodies D01 and FL-393 (Santa Cruz), or corresponding antibody controls, as described in Materials and Methods. Immunoprecipitated DNA was quantified by QPCR, and data is shown in Figure 15A. It is clear that mutant p53 got localized on the sequences upstream to the Axl gene either directly or indirectly binding to DNA. To locate the site or sites of mutant p53 binding to the Axl promoter, the full length promoter was divided into five fragments and primers were designed. Fold activation by mutant p53 on the Axl promoter was determined and the percent of p53 binding was calculated (Figure 15B). The data shown indicate major mutant p53 interaction distal to ATG.

Transcription factor binding is enhanced by mutant p53 on the Axl promoter. Next, we wanted to check whether we could determine the transcription factor(s) that bind more efficiently on the Axl promoter in the presence of mutant p53 (Figure 16). For that purpose we performed ChIP assays using antibodies against transcription factors, p300, E2F1, and CREB, which putatively may interact with the Axl promoter. ChIP assay data carried out using vector transfected and mutant p53 (-R273H) expressing H1299 cells and corresponding antibodies against these transcription factors show that binding all transcription factors are increased: p300 2.5-fold, E2F-1 4.3-fold and CREB 8-fold. Increased binding of transcription factors is likely to contribute to the induced Axl expression mediated by mutant p53.

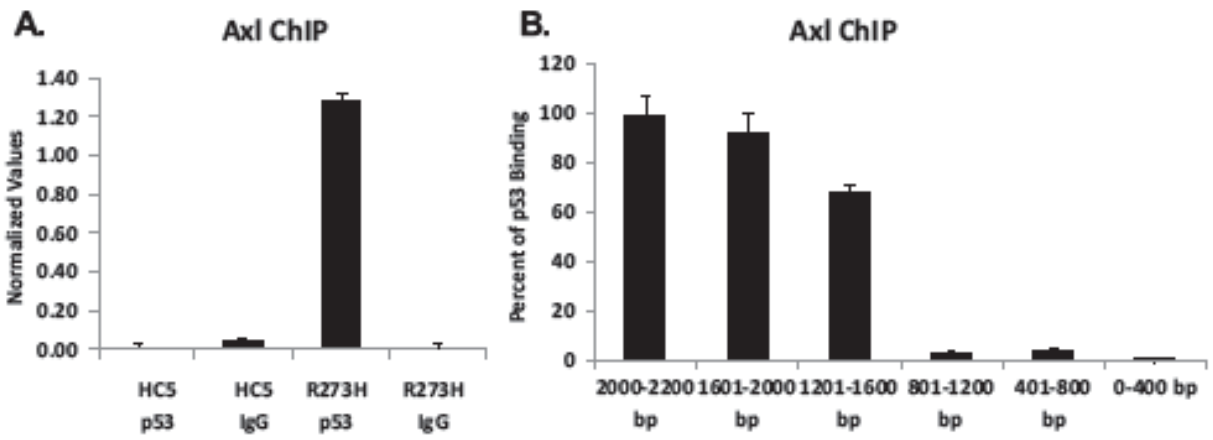

Figure 15. ChIP data showing p53-R273H binding in H1299 cells expressing this mutant to the Axl promoter region. **A.** ChIP was performed on H1299 cells stably transfected with vector (HC5) or mutant p53-R273H (R273H) to test whether mutant p53 is nucleated on the promoter. **B.** Primers were designed to amplify the full length Axl promoter in five regions: 2000-1601bp, 1201-1600bp, 801-1200bp, 401-800bp, and 1-400bp. QPCR was performed for each of these fragments on H1299 cells expressing either an empty vector (HC5) and p53-R273H that were used for ChIP with antibodies against p53 (D01 and FL393) or IgG as a control.

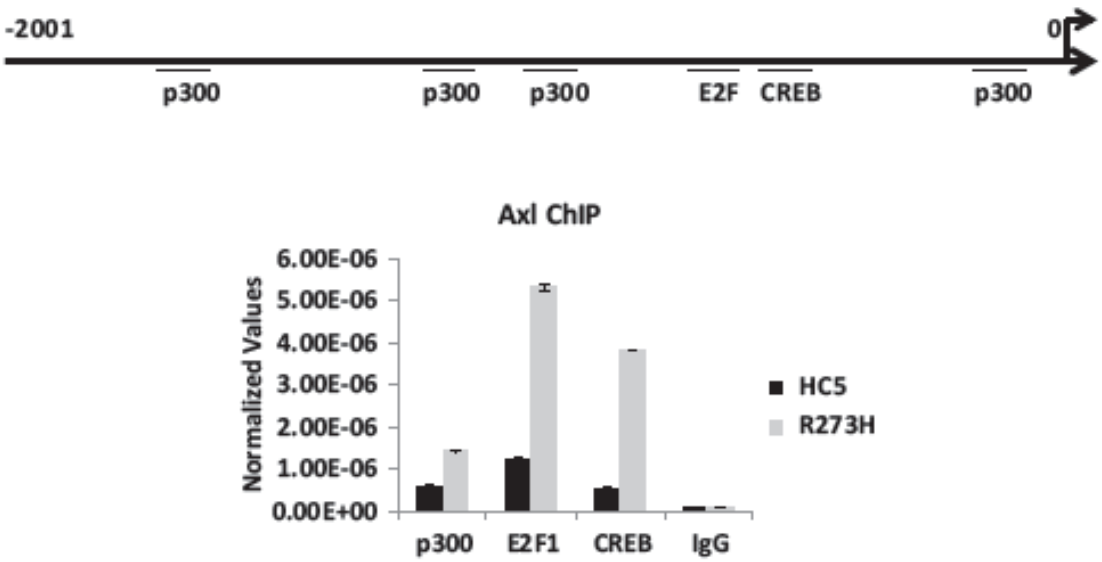

Figure 16. ChIP assay shows the mutant p53 mediated increase in transcription factor binding on the Axl promoter. ChIP analysis was performed on H1299 cells stably transfected with vector (HC5) or mutant p53-R273H (R273H) to test whether transcription factors are preferentially binding to the Axl promoter. ChIP was performed with antibodies directed against CREB, E2F1, and p300. Experiments were done in triplicate.

Chapter Summary.

Microarray data of H1299 lung adenocarcinoma cells expressing different p53 mutants showed up-regulation of genes that may affect growth and oncogenesis. We have investigated the receptor protein tyrosine kinase Axl, which is up-regulated by p53 mutants at both RNA and protein levels in H1299 lung cancer cells expressing mutants p53-R175H, -R273H, and –D281G. Knock-down of endogenous mutant p53 levels in human lung cancer cells H1048 (p53-R273C) and H1437 (p53-R267P) led to reduction of the level of Axl as well. This effect on Axl expression is refractory to the mutations at positions 22 and 23 of p53 suggesting that p53's transactivation domain may not play a critical role in the up-regulation of Axl gene expression. Chromatin immunoprecipitation (ChIP) assays carried out with acetylated histone antibodies demonstrated induced histone acetylation on the Axl promoter region by mutant p53. Direct mutant p53 nucleation on the Axl promoter was demonstrated by ChIP assays using antibodies against p53. The Axl promoter has a p53/p63 binding site, which however, is not required for mutant p53-mediated transactivation. Knock-down of Axl by Axl-specific RNAi caused reduction of gain of function (GOF) activities, reducing cell growth rate and motility rate in lung cancer cells expressing mutant p53. This indicates that for lung cancer cell lines with mutant p53, GOF activities are mediated in-part through Axl.

Chapter 3

DETERMINE HOW UP-REGULATION OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) AFFECTS ADDICTION OF LUNG CANCER CELLS TO GAIN-OF-FUNCTION MUTANT P53

The manuscript for the work presented in this chapter is currently under preparation. Growth assays and immunoprecipitations were performed by Isabella Pearsall, migration assays were performed by Andrew Yeudall, Western blot analysis on the different human lung cancer cell lines was performed by Shilpa Singh.

Introduction.

In earlier work, we have shown that GOF p53 transactivates the human EGFR promoter in transient transfection assays in the absence of specific DNA binding by p53 (198, 228). EGFR is involved in cell proliferation and motility (229) and its over-expression has been found to be implicated in various cancers including lung cancer (230). The mechanism through which GOF p53 up-regulates gene expression is not yet clear.

In this project, we show that lung cancer cells expressing GOF p53 are addicted to mutant p53; knock-down of p53 in lung cancer cells causes lowering of tumorigenicity and other GOF properties. We demonstrate that mutant p53 up-regulates epidermal growth factor receptor (EGFR) expression and activates the EGFR pathway. Knock-down of p53 lowers EGFR overexpression; however, the addiction to GOF p53 can be compensated by overexpressing EGFR suggesting that EGFR is in the GOF p53 pathway and plays a critical role in the addiction of lung cancer cells to GOF p53. Using chromatin immunoprecipitation (ChIP) assays we show that GOF p53 interacts with the EGFR promoter and increases H3 histone acetylation. ChIP and

ChIP-re-ChIP studies show docking of GOF p53 on Sp1 as well as increased binding of Sp1 and CBP on the EGFR promoter.

Materials and Methods.

Cells. H1299 (ATCC; Manassas, VA), KNS-62, VMRC (JCRB Cell Bank; Osaka, Japan), H1975, H23, H596, H1048, H1437, H1573, H1793, H2405, and ABC1 (ATCC) cell lines were all purchased from commercial sources and were maintained in media as suggested by the suppliers. Methods for lipofection, nucleofection, and generation of stable transfectants were as described (160, 231, 232). Clones were isolated using puromycin selection at 1μg/ml.

Generation of H1299 cells expressing GOF p53 mutants. To determine the influence of the transactivation domains on mutant p53-mediated transactivation, we have constructed 3 amino acid substitution mutants: p53-R273H (L22Q/W23S) using the Quikchange mutagenesis kit (Agilent; Santa Clara, CA), and expressed these p53 mutants in H1299 cells. Multiple clones were isolated with p53 expression equivalent to that of p53-R273H alone. We used these clones in comparison with vector transfected cell clones for our assays. We have also used H1299 cells expressing p53-R273H as described earlier (135).

EGFR promoter transient assays. The EGFR promoter-luciferase construct was obtained from Active Motif (Carlsbad, CA). The EGFR expression plasmid was created by cloning the EGFR cDNA sequence into the pWZL Hygro plasmid purchased from Addgene (Cambridge, MA). Transient transfection was performed with 500ng of promoter and 1ug of expression plasmid using Lipofectamine3000 (Invitrogen; Grand Island, NY) following the manufacturer's instructions. Luciferase analysis was carried out using the dual luciferase assay

system (E1500) according to the manufacturer's instructions (Promega; Madison, WI). Both transfection and luciferase assay were performed in triplicate as described previously (232).

siRNA Transfection. siRNAs were nucleofected into H1299 cells expressing p53-R273H or vector control following the manufacturer's instructions (Lonza; Walkersville, MD). Sequences used to target individual transcription factors were as follows: siCBP: 5'-UUGAGGAAUCAACAGCCGCtt-3' (233), siEGFR: 5'-GCAAAGUGUGUAACGGAAUAGGUAUtt-3 '(234), siEts1: 5'-ACUUGCUACCAUCCCGUACtt-3 '(235), sip63: 5'-AAAGCAGCAAGUUUCGGACAGtt-3 (236)', siSp1: 5'-GGUAGCUCUAAGUUUUGAUtt-3' (237), and siScrambled (control): 5'-CAUGUCAUGUGUCACAUUCtt-3 '(238).

Growth assays. Growth assays were carried out as described by us earlier with slight modifications (135). Cells were plated at a density of 50,000 cells/6cm dish in triplicate for five time points, harvested after incubation with trypsin and counted using a Coulter Counter (Beckman). For siRNA treatment of cells, siRNA transfection was carried out for two consecutive days before starting the growth assay. All experiments were done in triplicate.

Xenograft assay. Nu/J (Nude, Jackson Labs; Bar Harbor, ME) or NOD.CB17-*Prkdc*^{scid}/NcrCrl (Scid, Charles River Labs; Raleigh, NC) mice were used for the tumorigenicity studies. Mice were injected with $1x10^7$ cells subcutaneously on the flanks and tumors allowed to grow to a maximum size of 1cm, measuring periodically as described before (232). Two different clones of cells were used to rule out clonal variations. For the xenograft assays where transfections were done prior to injection, we counted the cells after transfection at the day of injection (48-72 h post transfection).

Western blotting. Immunoblotting was carried out as described earlier (135). Briefly, for a typical Western blot we have prepared extracts in Promega Lysis Buffer (Promega). For Western blots to detect phosphorylated proteins, we have prepared the extracts in RIPA buffer (see below) with the addition of phosphatase inhibitors. p53 was detected using the p53 antibody PAb 1801 (93), EGFR and Erk2 antibodies were from Santa Cruz Biotechnology (Dallas, TX) (sc-03 and sc-154 respectively), phospho-EGFR and phospho-Erk were from Cell Signaling (Danvers, MA) (2234 and 4370 respectively); transcription factors (TFs) were detected using respective antibodies from Santa Cruz Biotechnology: CBP (sc-369), Ets-1 (sc-350), p63 (sc-8431), and Sp1 (sc-59). Westerns blots were developed by the ECL method (GE Healthcare; Piscataway, NJ).

Tumor RNA analysis and p53 sequencing. Tumor RNAs were provided by the Tissue and Data Acquisition and Analysis Core repository under an Institutional Review Board approved protocol; cDNAs were prepared using the Superscript III cDNA synthesis kit (Invitrogen) and QPCR performed using primers specific for EGFR (F: 5'-AAGTGTAAGAAGTGCGAAGG-3' and R: 5'-GGAGGAGTATGTGTGAAGGA-3'). The degree of expression was quantified using a relative standard curve and normalized to GAPDH (F: 5'-GTCAACGGATTTGGTCGTATT-3' and R: 5'-GATCTCGCTCCTGGAAGATGG-3') corresponding to the cDNA batch. The p53 gene is sequenced following the method described previously (160). Whenever a mutation was found, a new PCR reaction was performed and fragment re-sequenced to verify sequence information obtained previously. The EGFR levels in the two populations of tumors, one with WT p53 and one with mutant p53, were compared and found to be different with a statistically significant difference based on the student t-test.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed as described earlier (135). Antibodies used for ChIP were: p53 (DO1: sc-126 and FL-393: sc-6243, Santa Cruz), acetylated histone H3 that recognizes acetylated lysine at positions 9 and 14 (17-615, Millipore; Billerica, MA), total histone H3 (06-755, Millipore), TFs (CBP (sc-369), Ets-1 (sc-350), p63 (sc-8431), Sp1 (sc-59), and USF1 (sc-229), Santa Cruz Biotechnology) and IgG (normal mouse: sc-2025 and normal rabbit: sc-2027, Santa Cruz). Quantitative PCR (QPCR) was used to quantify precipitated DNA using promoter specific primers. The following primers were used: GAPDH ChIP (F: 5'-GTATTCCCCCAGGTTTACAT-3' and R: 5'-TTCTGTCTTCCACTCACTCCT-3'), EGFR ChIP (F: 5'-CCCGCGCGAGCTAGACGTCC-3' and R: 5'-GCTCGCTCCGGCTCTCCC-3'), EGFR ChIP set 2 (F: 5'-ACTATGAAGGCTGTTGTCTC-3' and R: 5'-ACAACAGTGGAACATAAAAT-3'), EGFR ChIP set 3 (F: 5'-TCTGTGTTTCTACGGACTGC-3' and R: 5'-ATGTTTGTGCCTGGGTCT-3'), and EGFR ChIP set 4 (F: 5'-AAAGATGTAAGGTTGCTCCC-3' and R: 5'-TTGGCCAAAAGAAACTGAG-3'). ChIP-re-ChIP was performed following the method described (239) by incubating equal amounts of extracts with p53 and IgG antibodies overnight and then incubating with BSA and sonicated salmon sperm saturated protein A agarose beads for one hour at 4°C. The DNA-protein-antibody complexes were then washed once with RIPA (150mM NaCl, 50mM Tris pH8, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40), once with High Salt Buffer (500mM NaCl, 50mM Tris pH 8, 0.1% SDS, 1% NP-40), once with LiCl Buffer (250mM LiCl, 50mM Tris pH 8, 0.5% sodium deoxycholate, 1% NP-40), and once with 1X TE. DNA-protein complexes were eluted from the protein A agarose beads by incubation at 37°C for 30min in 10mM DTT in 1X TE. Eluants were then incubated with the indicated second antibody overnight, and BSA and sonicated salmon sperm saturated protein A agarose beads

were added for one hour at 4°C the following day. The DNA-protein-antibody complexes were then washed once with RIPA, once with High Salt Buffer, once with LiCl Buffer, and once with 1X TE. DNA-protein complexes were eluted at 65°C overnight in fresh elution buffer (20% SDS, 10mM DTT, 100mM $NaHCO_3$), RNase and proteinase K digested, phenol/chloroform extracted, and QPCR was performed with specific primers.

Immunoprecipitation assays. Co-immunoprecipitation (IP) of proteins as an indication of protein-protein interactions were carried out as described earlier (240, 241). Briefly, immunoprecipitations were carried out as follows: cells were washed with 1X PBS and harvested in NP-40 Buffer (50mM Tris pH 7.5, 150mM NaCl, 2mM EDTA, 0.5% NP-40 supplemented with PMSF and protease inhibitors). Cells were lysed for 30min on ice and passaged through a 27 G needle three times. Lysates were centrifuged and protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific; Waltham, MA). Equal protein amounts were used for IP. Protein extracts were precleared with protein A agarose rocking at 4°C for one hour. The extract/bead mix was centrifuged and the supernatant was transferred to new tubes. Extracts were then incubated with an antibody against p53 (PAb 421) or CBP (sc-369, Santa Cruz) and protein A agarose beads while rocking at 4°C overnight. The following morning the extract/bead/antibody mix was centrifuged and the beads were washed three times with NP-40 Buffer. The buffer was removed and equal volume 2XLLB was added and boiled for ten minutes. Extracts were then run in a PAGE gel. Additionally, a small aliquot of the IP supernatant was set aside and run in a gel to show the loading control.

Cell migration assays. Cell migration was determined by wound closure assays described previously (125). Briefly, cells were trypsinized, counted, plated in both chambers of tissue culture inserts (Ibidi GmbH, Martinsried, Germany), and then grown to confluence. The

insert was removed, and the distance across the cell-free zone measured (Axiovision software; Carl Zeiss Microimaging, Thornwood, NY). Cultures were returned to the incubator, allowed to migrate for 8h, and the width of the cell-free zone re-measured. Migration rate was determined by subtraction of the final measurement of distance from the initial measurement, divided by time.

Statistical Analysis. All statistical analyses were calculated using the student's t-test. Data was considered significant if the p-value was below 0.05.

Experimental Results.

Tumor-derived gain-of function mutant p53 binds on the upstream region of the EGFR gene and induces histone acetylation. In order to decipher the mechanism of activation of gene expression by GOF p53 we first identified promoter sequences bound by GOF p53-R273H in H1299 cells expressing p53-R273H by performing ChIP-Seq [to be communicated separately, (242)]. In this analysis, we identified EGFR as a candidate gene whose promoter is bound by GOF p53. Figure 17A shows mutant p53 (R273H) ChIP-Seq driven peak analysis of mutant binding on the EGFR promoter with Figure 17B giving the sequence where maximal mutant p53 binding occurs (indicated by brackets surrounding the peaks). Some of the known TF binding sites are identified in the sequence. We verified mutant p53 binding on the promoter region of the EGFR gene by ChIP assays followed by QPCR (Figure 17C). We have also carried out ChIP assays with an antibody against acetylated histone H3 (AcH3) (Figure 17D). Figure 17D shows increased binding of K9/K14 acetylated histone H3 to the EGFR promoter upon expression of mutant p53. It is possible that p53-R273H binds to one or more of the TFs indicated (Figure 17B) and gets nucleated on the promoter, thereby leading to increased

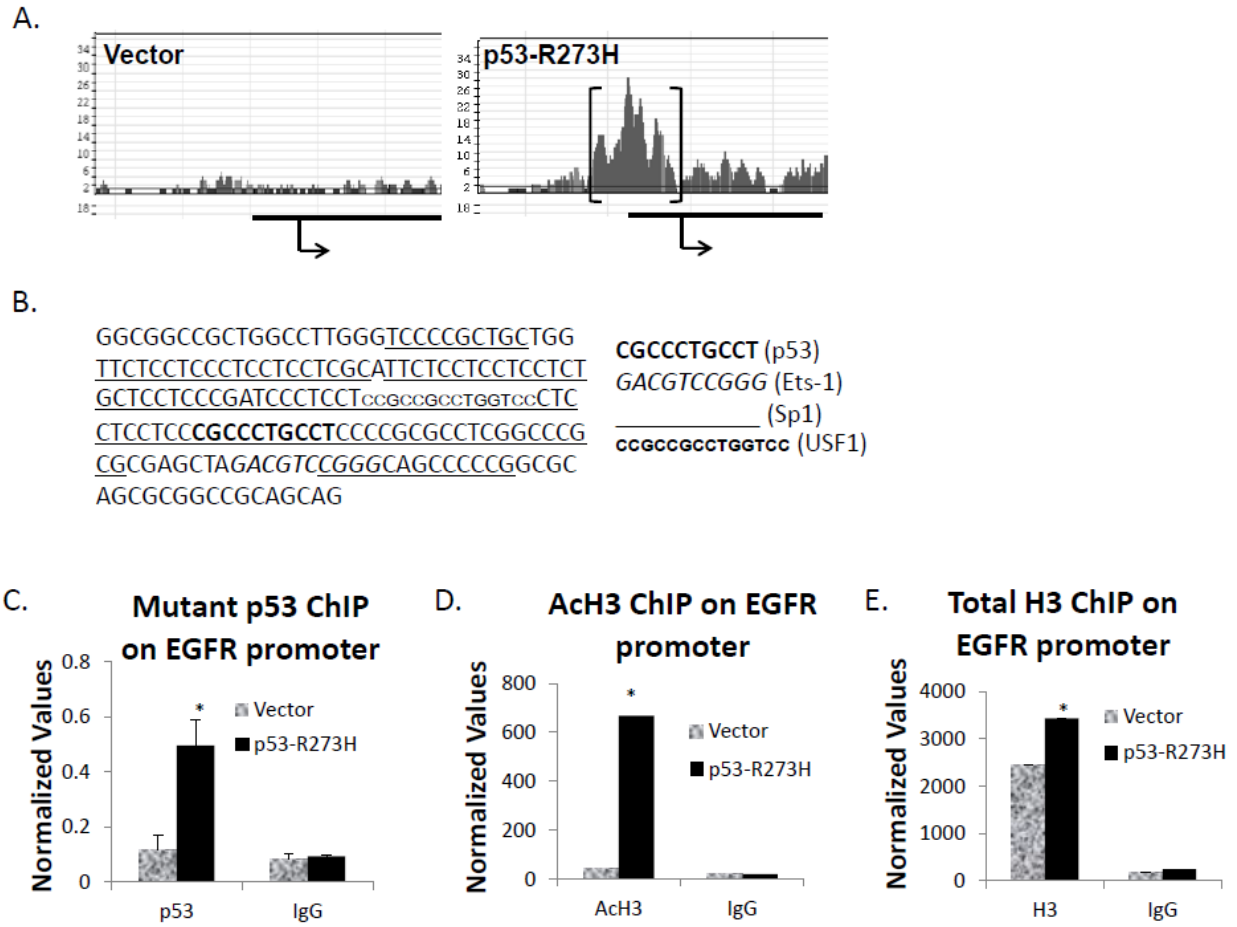

Figure 17. p53 and AcH3 ChIP sequence peaks on EGFR gene upstream sequences and QPCR verification of ChIP on the EGFR promoter. Details of ChIP seq analysis and their verifications have been previously (150). **A.** The peaks representing areas under which maximal p53-R273H binding occurs as apparent by next generation sequence analysis (242). **B.** Sequence of the major peak (shown above red brackets in A) where mutant p53 binding occurs along with some of the prominent TF binding sites. **C** and **D.** ChIP assay results showing p53-R273H and AcH3 binding to the EGFR promoter. **E.** ChIP assay result showing total histone H3 binding to the EGFR promoter. Asterisks indicate a p-value of less than 0.05.

AcH3 binding on the EGFR promoter. Figure 17E shows a marginal increase of total histone H3 binding to the promoter, strengthening the case for mutant p53 causing an enhanced amount of acetylated histone H3 binding to the EGFR promoter.

Tumor-derived gain-of function mutant p53 induces expression of the EGFR gene. Once we found that mutant p53 binds to the EGFR promoter region, coupled with the knowledge that mutant p53 also transactivates the EGFR promoter (198, 228), we tested whether H1299 cells expressing p53-R175H and -R273H show higher levels of EGFR mRNA compared to vector transfected cells. We prepared RNA from these cells and determined EGFR mRNA levels in samples prepared from two individual clones per transfection. RT-QPCR data shown in Figure 18 demonstrates that EGFR expression is up-regulated by the p53 mutants in each case in multiple stable clones. Figure 18B shows an example of a Western blot with higher level of EGFR in H1299 cells expressing p53 mutants.

EGFR is a target of GOF p53. Next, we wanted to determine if EGFR behaves as a mutant p53 inducible gene in lung cancer cells expressing endogenous GOF p53. Thus, we generated p53 knocked-down derivatives from lung cancer cells H1975 (p53-R273H) and KNS-62 (p53-R249S) using lentiviral vectors carrying p53 shRNA. Figure 19 indicates knock-down of the endogenous p53 in stable clones of H1975 and KNS-62 cell lines and shows that the EGFR level is reduced upon mutant p53 knockdown consistent with EGFR being a mutant p53 target gene. Figure 19B shows the results of RT-QPCR experiments to assay for EGFR levels in the cell clones generated (as shown in Figure 19A).

Since GOF p53 transactivates the EGFR promoter and induces EGFR expression, we tested whether it results in enhanced phosphorylation of EGFR, which is indicative of the activation of EGFR pathway (229). We tested the level of these proteins in H1299 cells

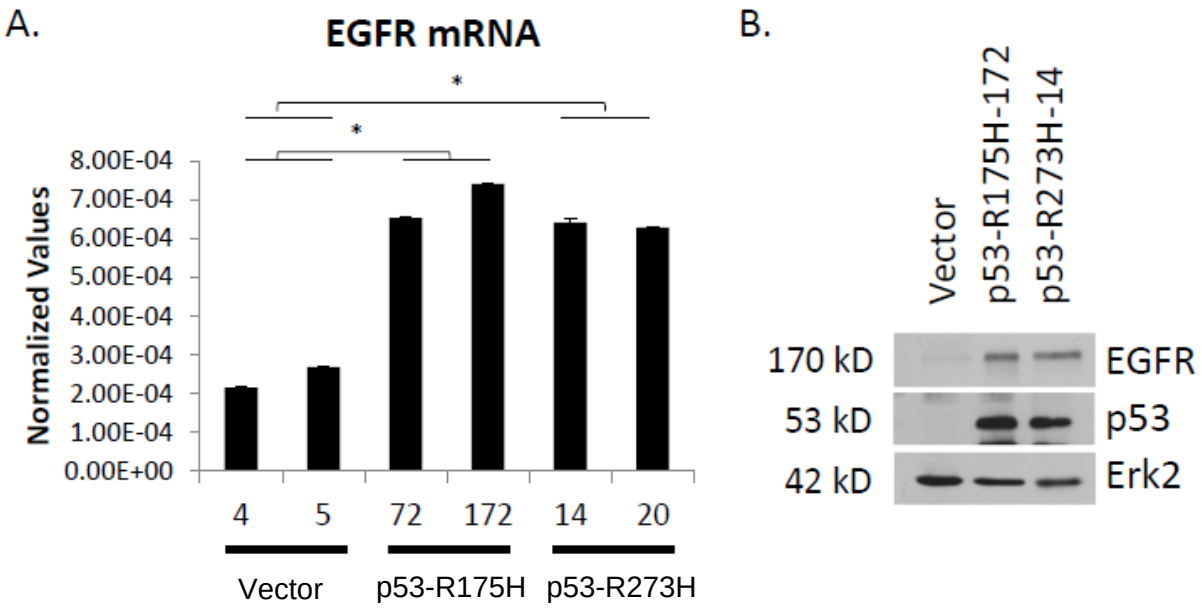

Figure 18. Gain-of-function mutant p53 up-regulates expression of EGFR in H1299 lung cancer cells. H1299 cells have been stably transfected to express p53 mutants -R175H and -R273H (or vector alone). **A.** RT-QPCR was used to assay for EGFR levels in different cell clones. The data presented show that GOF p53 up-regulates EGFR mRNA expression. Different cell clones are indicated by clone numbers. Experiments were done in technical triplicates. Asterisks indicate a p-value of less than 0.05. **B.** Example of Western analysis showing EGFR and mutant p53 levels in different cell clones.

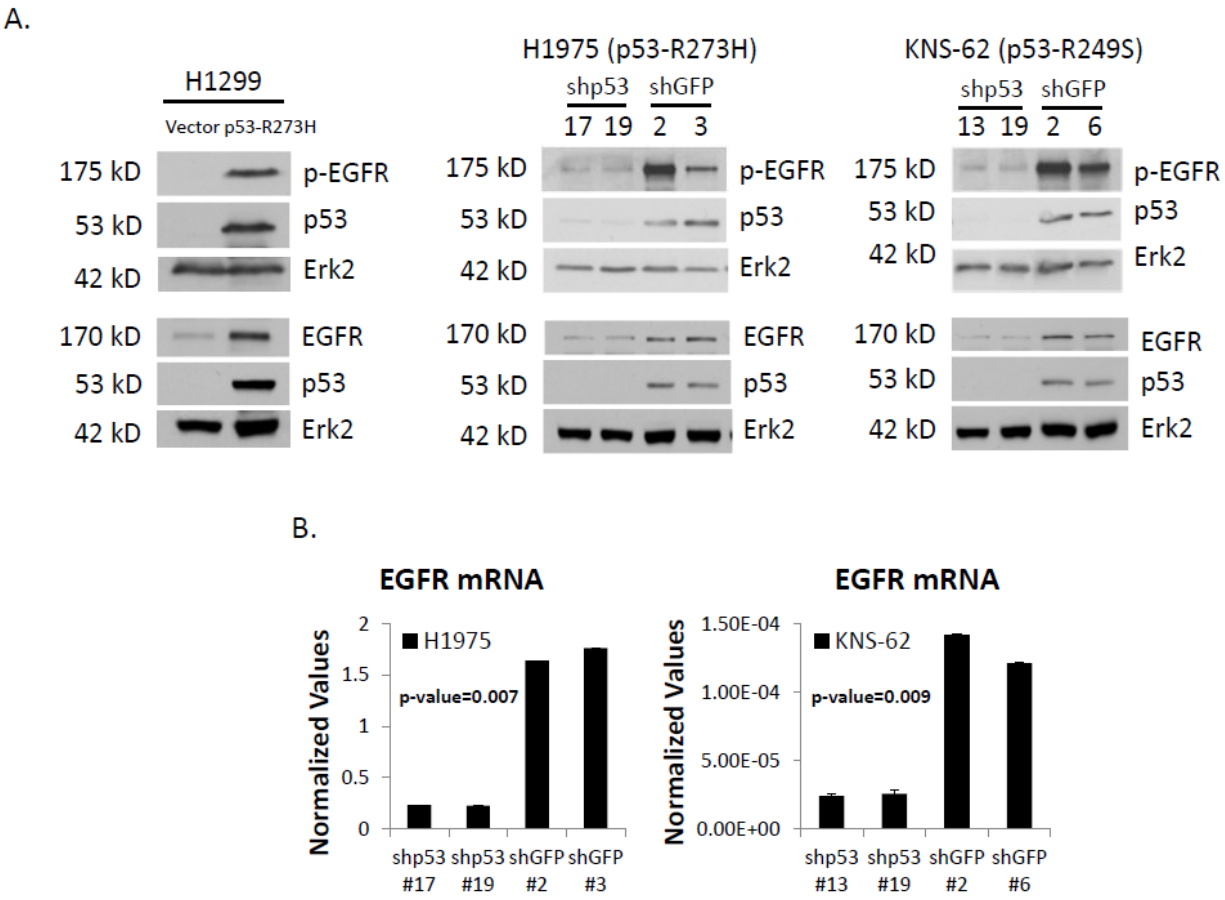

Figure 19. p53 knock-down in H1975 and KNS-62 cells reduces EGFR levels. **A.** Western blot showing phospho-EGFR, EGFR, p53, and Erk2 levels in H1299 cells expressing either an empty vector or the p53 mutant R273H as well as different cell clones used generated by recombinant lentiviruses expressing p53 shRNA or control GFP shRNA in H1975 and KNS-62 cells. **B.** RT-QPCR data for EGFR in different cell lines under study. Different cell clones are indicated by clone numbers. Experiments were done in technical triplicates.

expressing p53-R273H (or vector control) as well as H1975 and KNS-62 cells p53 knock-down (and GFP knock-down control). Data shown in Figure 19A shows that p53-R273H led to an increase of phospho-EGFR. These data are corroborated by our observations in p53 knock-down cells.

Lung tumor cells expressing mutant p53 show higher EGFR levels. Since we observed that mutant p53 up-regulates EGFR expression (see above), we tested if this is also true for other lung cancer cell lines and human lung tumors expressing mutant p53. Figure 20A shows p53 and EGFR protein levels and Figure 20B shows EGFR mRNA levels in different lung cancer cell lines. Figure 20C depicts EGFR mRNA levels of different human lung tumors collected in Virginia Commonwealth University's cancer tissue repository. On average, there was more EGFR expression in samples with mutant p53 versus samples with WT p53 (average 2.1-fold, p-value 0.03), corroborating our cell culture data that mutant p53 up-regulates EGFR expression. Thus, overall there is an increased expression of EGFR in human lung tumors with mutant p53.

Lung cancer cells with endogenous GOF p53 are addicted to mutant p53. We tested whether reduction of p53 would cause significant reduction in oncogenic functions of lung cancer cells as measured by tumorigenicity in immunodeficient mice. Thus, we performed tumorigenicity assays in nude or SCID mice as described in Materials and Methods. Figure 21A shows that p53 knock-down results in remarkable reduction of tumorigenicity, demonstrating that these lung cancer cells are addicted to mutant p53 for effective tumor formation.

Reduction of mutant p53 and EGFR in lung cancer cells retards tumorigenicity growth rate and cell motility. To determine the effect of reducing expression of the endogenous mutant p53 in the H1975 and KNS-62 cell lines, we performed tumorigenicity

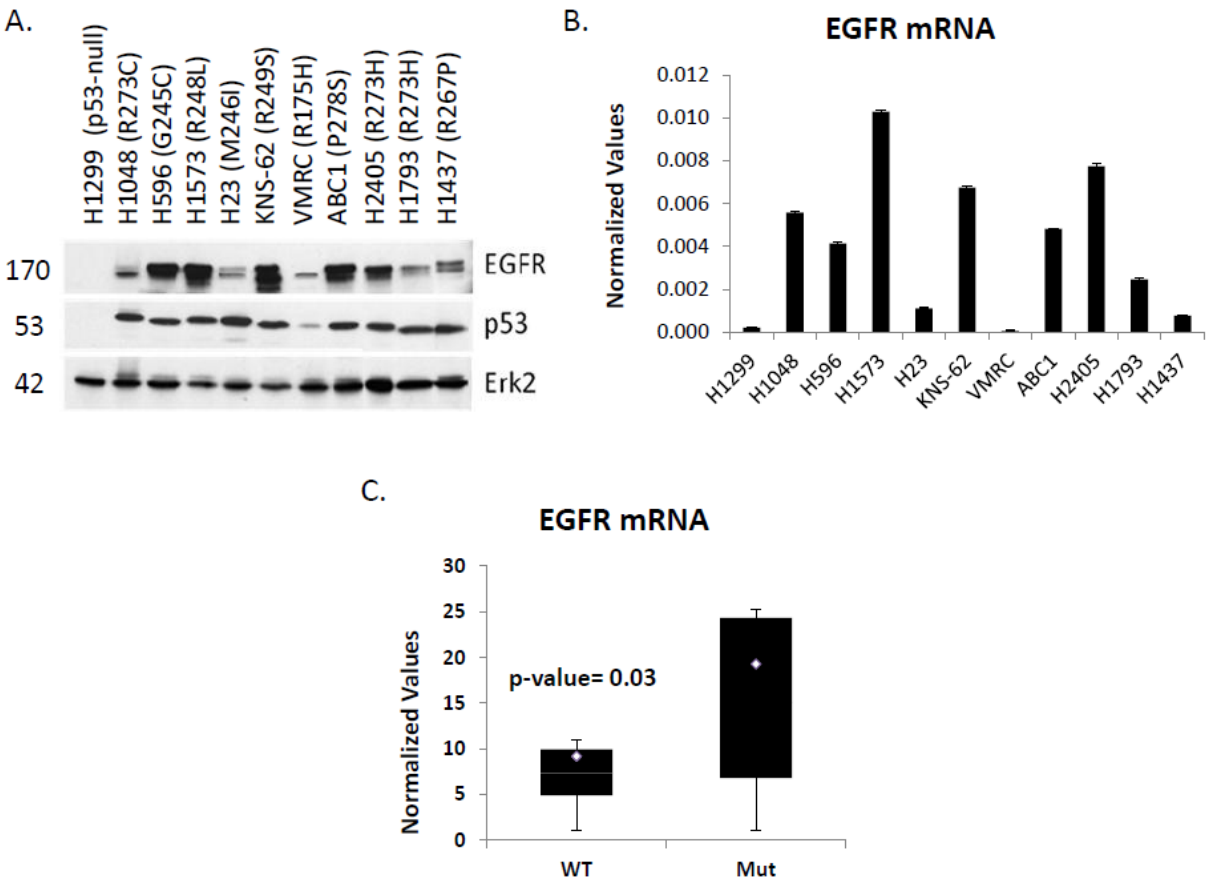

Figure 20. Lung tumor cells expressing mutant p53 show higher EGFR levels. **A.** EGFR levels in various lung cancer cell lines. Western blot analysis of protein extracts of different lung cancer cell lines were assayed by immunoblot analysis. Many of these lines expressing mutant p53 also express higher levels of EGFR. **B.** EGFR mRNA levels in the lung cancer cell lines presented in A. **C.** RT-QPCR of EGFR levels in lung tumors. Experiments were done in technical triplicates as described in the text.

assays in nude mice where the stable knockdown cell clones were subcutaneously injected. We then wanted to test whether reduction of p53 can be mimicked by EGFR knock-down in terms of reduction of oncogenicity as measured by tumorigenicity as well as proliferation and motility rate of lung cancer cells. Thus, we performed growth assays as described in Materials and Methods. Similarly, we transiently transfected H1975 cells with EGFR siRNA (or scrambled siRNA) and performed nude mice tumorigenicity and cell growth assays. Tumorigenicity data shown in Figure 21B indicate a drastic effect on the tumor growth of H1975 cells suggesting a strong dependence of the growth of the tumor cells on EGFR even when GOF p53 is present. Data shown in Figures 21C show that knock-down of either p53 or EGFR reduced the growth rate significantly. This result suggests the possibility that GOF p53 regulates cell growth, at least in part, through EGFR expression. In parallel, we performed wound closure assays to determine the impact of reducing GOF p53 and EGFR on cell motility. As shown in Figures 21E and 21F respectively, EGFR and GOF p53 knockdown resulted in a decrease in cell motility.

We hypothesize that GOF p53 may execute (some of) its oncogenic function via the EGFR pathway; if that is true, then the defects encountered by knock-down of GOF p53 should be compensated by EGFR overexpression in those cells. Therefore, we tested whether EGFR overexpression can restore the growth and tumorigenicity defect encountered by knock-down of GOF p53. Figures 21D and 21G show that expression of EGFR by transfection of H1975 (p53-R273H) p53 knock-down cells with an EGFR expression plasmid compensates for the reduced growth rate and, more importantly, tumorigenicity, respectively, in nude mice. This suggests that EGFR plays a crucial role in mediating the effects of the mutant p53 GOF pathway.

TFs are involved in inducing binding of acetylated histone H3 on the EGFR promoter. In order to decipher the mechanism used by GOF p53 in inducing upregulation of

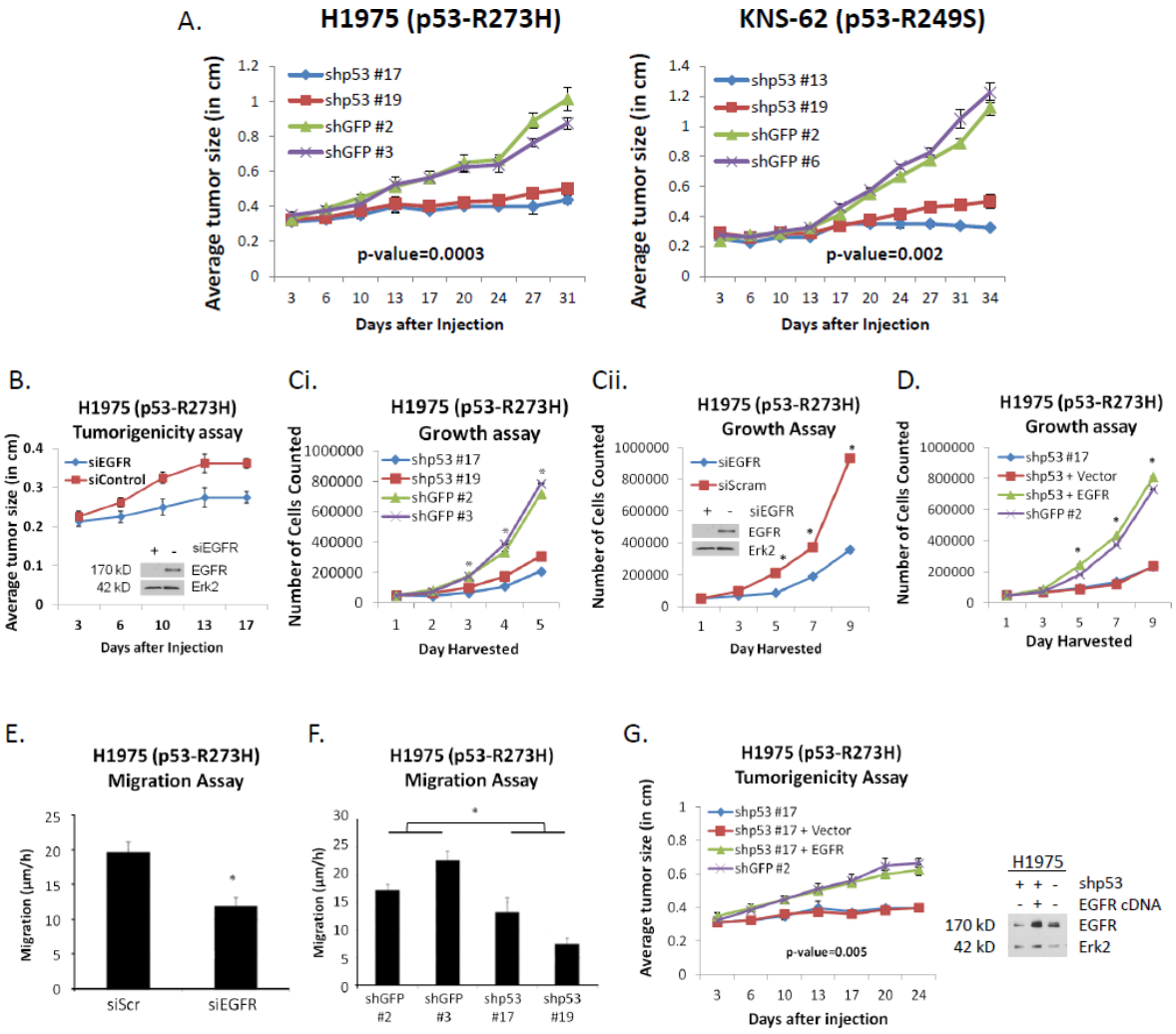

Figure 21. Reduction of mutant p53 and EGFR in lung cancer cells retards tumorigenicity, growth rate and cell motility. A. H1975 and KNS-62 cell clones stably expressing shRNA against p53 were injected into nude (H1975) or SCID (KNS-62) mice. **B.** H1975 lung cancer cells were transfected with siRNA targeting EGFR and subsequently injected into nude mice. **C.** (i) Growth assay of H1975 cells knocked-down for p53 (and control) generated by recombinant lentivirus expressing p53 shRNA. (ii) Growth rate of H1975 cells depends on the EGFR level. H1975 cells were transfected with control or EGFR-specific siRNA. Asterisks indicate a p-value of less than 0.05. **D.** H1975 p53 knock-down cells were transfected with an EGFR expression plasmid to compensate for the EGFR expression loss, and growth assays were performed. Asterisks indicate a p-value of less than 0.05. **E.** Migration of H1975 after transient transfection of RNAi against EGFR shows a reduced migration rate. Asterisk indicates a p-value of less than 0.05. **F.** H1975 cells show a reduction in migration when the endogenous mutant p53 is stably knocked-down. Asterisk indicates a p-value of less than 0.05. **G.** H1975 p53 knock-down cells were transfected with an EGFR expression plasmid to compensate for the EGFR expression loss, and tumorigenicity (in nude mice) assays were performed. EGFR expression recovers GOF activity loss observed on knock-down of mutant p53 of H1975 cells. Western blot showing EGFR levels of a representative experiment is shown. Experiments were done in triplicate.

EGFR expression, we investigated the transcriptional machinery at the EGFR promoter which might be positively influenced by GOF p53. One such mechanism could be to promote chromatin opening through histone acetylation. To determine which TFs may be involved in influencing binding of AcH3 on the EGFR promoter, we transfected mutant p53-R273H expressing cells (and control) with individual siRNAs targeting different TFs (as well as a nonspecific control), and performed AcH3 ChIP to test if AcH3 binding on the EGFR promoter has been changed along with decrease in the TF levels. If a particular TF satisfies this criterion, this would indicate the involvement of that particular TF in the induction of acetylated H3 histone binding to the EGFR promoter. Figure 22A depicts RNAi experimental data showing that siRNAs against CBP, Ets-1 and Sp1 had significant effects on binding of AcH3 on the EGFR promoter whereas RNAi against p63 had limited effects. Figure 22B shows immunoblots depicting reduction of TF levels after treatment with the respective siRNA. Thus, our results shown in Figure 22 suggest an involvement of Ets1, Sp1 and CBP in the activation of the EGFR promoter by GOF p53.

Sp1, CBP and Ets1 affect mutant p53 binding on the EGFR promoter. Since the data presented in Figure 22A indicated the involvement of TFs Ets1, Sp1 and the histone acetyl transferase (HAT) CBP, we wanted to test whether these factors are also required for interaction of mutant p53 on the promoter. To test this, once again we performed TF-directed RNAi experiments and carried out ChIP for mutant p53 to determine if lowering the levels of any of these TFs impacts mutant p53 binding. Figure 22C shows that although nonspecific siRNA did not affect the level of TFs or the binding of mutant p53 on the EGFR promoter, siRNA directed against Sp1, Ets1 and CBP indeed drastically inhibited the interaction of p53-R273H with the EGFR promoter. Figure 22D shows that the individual transcription factor expression levels

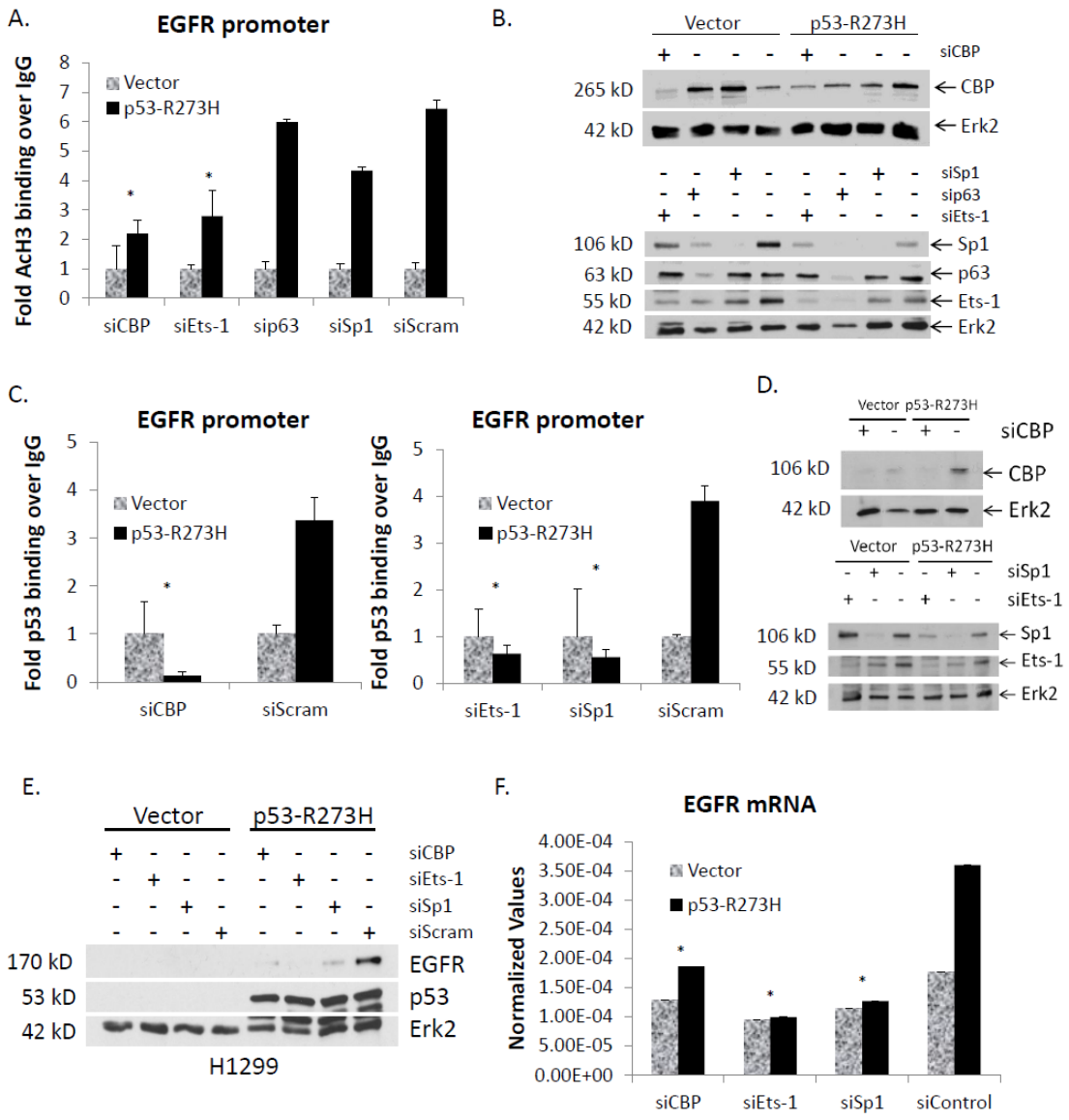

Figure 22. TFs are involved in inducing binding of acetylated histone H3 and p53 on the EGFR promoter. A. AcH3 ChIP analysis was performed on H1299 cells expressing p53-R273H (or vector alone) transfected with RNAi against TFs suspected of binding to the EGFR promoter (or scrambled siRNA). Normalized values for each siRNA treatment were divided by the normalized IgG value to calculate fold binding over IgG. The vector was then set to 1 in each set to be able to compare AcH3 binding between the different transcription factor knockdowns. Asterisks indicate a p-value of less than 0.05. **B.** Western blot shows extent of knock-down of different TFs levels. **C.** ChIP assays to determine the extent of TF-mediated p53 binding on the EGFR promoter. H1299 cells expressing p53-R273H (or vector alone) were transfected with RNAi against Sp1, CBP and Ets1 (or scrambled siRNA). Normalized values for each siRNA treatment were divided by the normalized IgG value to calculate fold binding over IgG. The vector was then set to 1 in each set to be able to compare p53 binding between the different transcription factor knockdowns. Asterisks indicate a p-value of less than 0.05. **D.**

Western blot analysis of cells used in C showing the effect of TF siRNA on their expression levels. **E.** Western analysis of p53 and EGFR expression in siRNA treated cells used for ChIP in A and C. **F.** EGFR mRNA expression in siRNA treated cells used for ChIP in A and C. Asterisks indicate a p-value of less than 0.005.

were indeed reduced without changing the level of mutant p53 (Figure 22E). This suggests that these TFs are involved in nucleating mutant p53 on the promoter. Transcription factor silencing also affects mutant p53-mediated EGFR transactivation. Through expression analysis after transfection of different siRNAs we show a reduction of EGFR expression (Figure 22F).

Facilitation of TF interactions on the EGFR promoter. We wanted to determine if GOF p53 facilitates interaction of one or more TFs on the EGFR promoter. Therefore, we carried out ChIP assays as described (160) using antibodies against TFs with H1299 cell lines expressing p53-R273H and vector control. We wanted to determine any difference in interaction of TFs with the EGFR promoter in the presence of mutant p53. Data in Figure 23A show that mutant p53 induces an increased interaction of a number of TFs on the EGFR promoter, suggesting cooperative interactions between these TFs and GOF p53. *In vivo* GOF p53-TF interactions were studied by immunoprecipitation analysis using procedures described previously (240, 243).

Data shown in Figure 23B support GOF p53 interactions with Sp1, Ets1, and CBP. Mutant p53-transcription factor cooperation is particularly high in cases of Ets1, Sp1 and CBP suggesting that GOF p53 induces nucleation of CBP on the EGFR promoter thorough Sp1 and Ets1. It is also possible that mutant p53 may stabilize or activate certain TFs and as a result up-regulate EGFR gene expression (Figure 23B).

We next used ChIP-re-ChIP experiments to determine if any of these mutant p53-TF interactions are occurring on the chromatin itself. Figure 23C shows ChIP-re-ChIP data investigating the interaction of CBP and Sp1 and mutant p53 on the promoter, and demonstrates Sp1 as a strong candidate in multiple assays, while CBP also showed significant interactions on

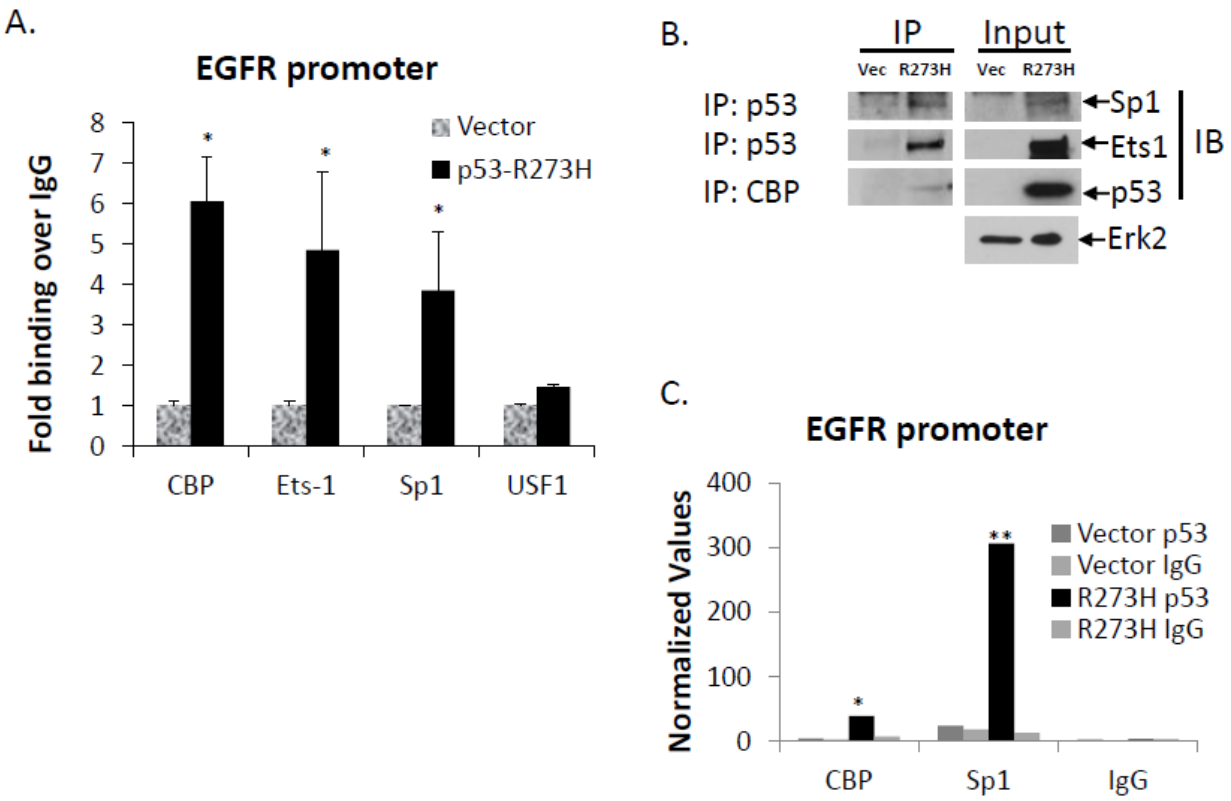

Figure 23. GOF p53 facilitates interaction of TFs on the EGFR promoter. A. ChIP of individual TFs on the EGFR promoter in the presence and absence of GOF p53 shows enhanced interaction of different TFs on the promoter. Normalized values for each transcription factor were divided by the normalized IgG value to calculate fold binding over IgG. The vector was then set to 1 in each set to be able to compare binding between the different transcription factors. Asterisks indicate a p-value of less than 0.05. **B.** *In vivo* interactions between different TFs were carried out in H1299 cells expressing p53-R273H (or vector alone) without transfection of TFs by immunoprecipitation analysis. Immunoprecipitation of p53-R273H from mutant p53 expressing cells shows binding of mutant p53 with CBP, Ets1, and Sp1. **C.** ChIP-re-ChIP assay showing an increased interaction between mutant p53 and CBP as well as mutant p53 and Sp1. Antibodies used for the first immunoprecipitation are indicated in the body of the figure, and antibodies used for the second immunoprecipitation are shown on the X-axis. Asterisks indicate a p-value of less than 0.05 for CBP and less than 0.005 for Sp1.

the promoter under the conditions of the assay. These data demonstrate that mutant p53 may nucleate on the EGFR promoter through Sp1 and, to some extent, CBP.

EGFR transactivation by GOF p53 can withstand mutations in the p53 transactivation domain. To determine if the transactivation domain (TAD) of GOF p53 is needed for up-regulation of EGFR expression, we stably transfected H1299 cells with p53-R273H or p53-R273H (22Q/23S) (or vector alone) and isolated independent cell clones. Figure 24A shows Western blot analysis of EGFR and p53 levels of the clones being used in our assays. Next, we isolated RNA from each cell clone, and performed quantitative RT-QPCR to determine the level of EGFR mRNA in the different cell lines. The data presented in Figure 24B show that the particular TAD mutations did not affect EGFR induction by mutant p53-R273H. To ensure that our TAD mutants do in fact transactivate the EGFR promoter we performed a transient transactivation assay and found (Figure 24C) that the presence of TAD mutations do not inhibit EGFR promoter activation.

TAD mutations differentially affect GOF p53 interactions and binding of acetylated histones on the EGFR promoter. Since TAD is an important component of the transactivation machinery and is where TFs have a tendency to contact p53 (244), we wanted to test if TAD mutations affect transactivation by GOF p53 via effects on mutant p53 binding on the EGFR promoter and/or effects on histone acetylation. Therefore, we used QPCR to quantitatively determine the effects of TAD mutations on mutant p53-mediated activation of EGFR transcription (assayed by RT-QPCR) as well as nucleation of p53-R273H and AcH3 on its promoters (assayed by ChIP) (Figure 25A). TAD mutations did indeed show a significant reduction of mutant p53 and TF interactions on the region examined (shown in Figure 17B).

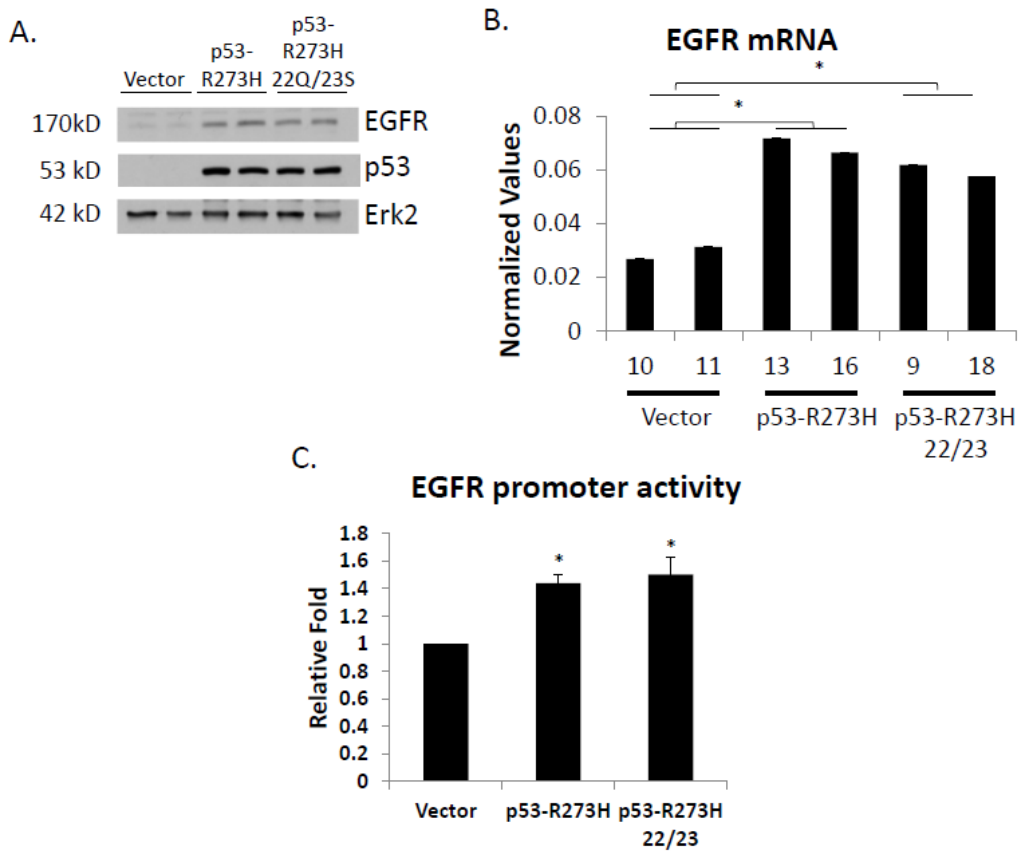

Figure 24. EGFR transactivation by GOF p53 can withstand mutations in the transactivation domain of GOF p53. H1299 cells were stably transfected with expression plasmids containing either the p53 mutant R273H, the p53 TAD mutant R273H (L22Q/W23S), or vector alone and cell clones were isolated. **A.** Western blots show expression levels of mutant EGFR and p53. **B.** RT-QPCR analysis showing levels of EGFR in different cell lines. Asterisks indicate a p-value of less than 0.005. **C.** Luciferase assay showing transactivation of the EGFR promoter by both p53-R273H as well as p53-R273H 22/23. Asterisks indicate a p-value of less than 0.05.

This is accompanied by a reduction of histone H3 acetylation and the data are shown in Figure 25A. Next we looked at the ability of our TAD mutant cell line to recruit transcription factors to the EGFR promoter. Figure 25B shows a reduction of transcription factor binding that was similar to the reduction of mutant p53 and histone H3 acetylation binding in Figure 25A at the region shown in Figure 17B. This suggested to us that mutant p53 with TAD mutations might be efficiently interacting at one or more different sites other than that shown in Figure 17B. We have performed ChIP using PCR primers corresponding to sequences spanning different regions on the EGFR promoter as shown on the schematic. Figure 25D shows data supporting our hypothesis that mutant p53-R273H and its TAD mutant both interact significantly with multiple locations on the EGFR promoter (identified by ChIP Seq), although the TAD mutant failed to interact in the primary site identified by ChIP Seq; these interactions perhaps result in activation of histone H3 acetylation where TFs are also successfully recruited (Figure 25C). It is possible that TAD interacts with different sequences of the EGFR promoter region using motifs defined by amino acids other than those mutated in the present construct (amino acids 22 and 23).

Chapter Summary.

Earlier work has shown that GOF p53 transactivates the EGFR promoter using transient transfection assays. Microarray and RNA sequencing have also shown up-regulation of EGFR by mutant p53. Here, we show that lung cancer cells expressing GOF p53 are addicted to mutant p53; knock-down of p53 in H1975 (p53-R273H) and KNS-62 (p53-R249S) lung cancer cells causes reduction of tumorigenicity and other GOF properties. Using H1299 cells expressing p53-R273H we demonstrate that mutant p53 up-regulates epidermal growth factor receptor (EGFR) expression and activity. Human lung tumors expressing mutant p53 also show higher

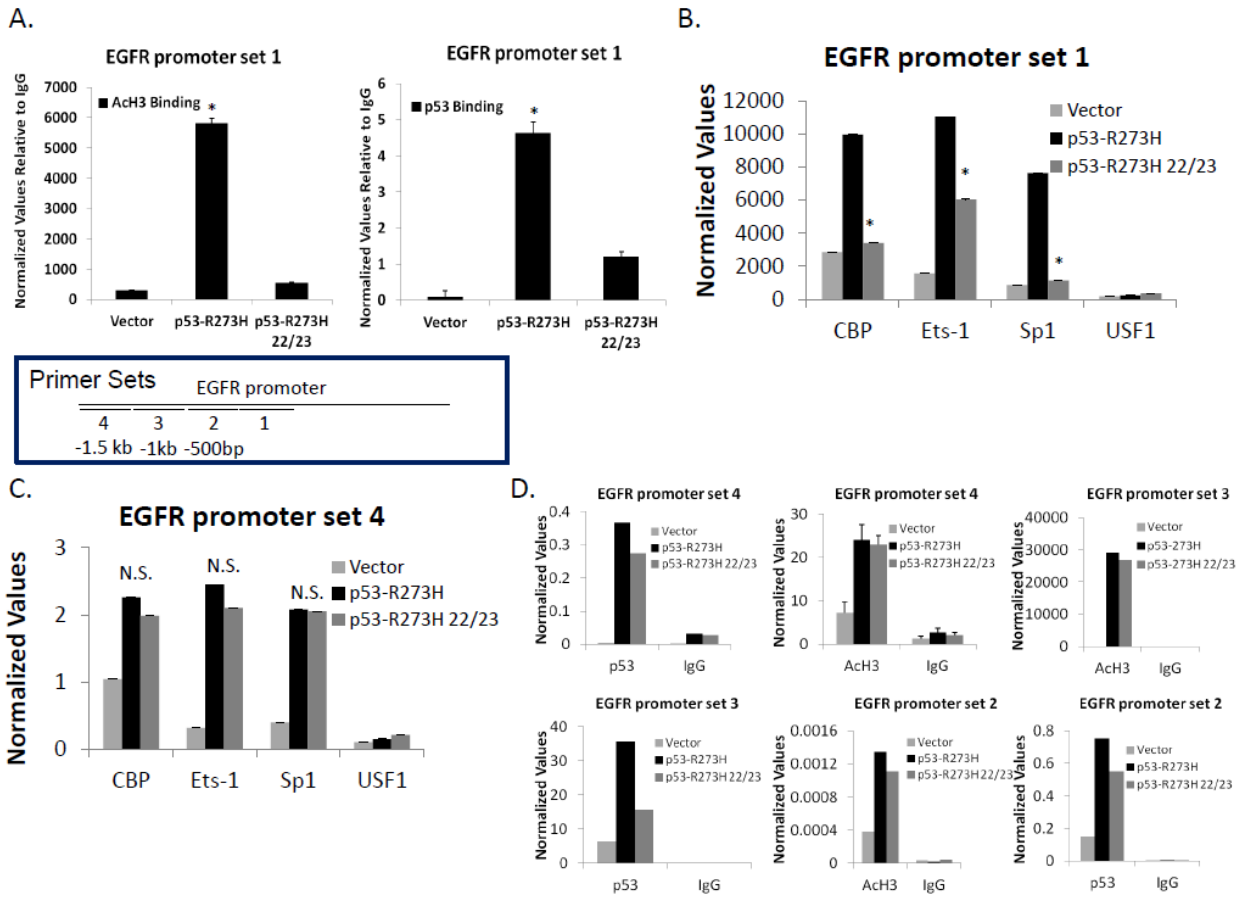

Figure 25. TAD mutations differentially affect GOF p53 interactions and binding of acetylated histones with the EGFR promoter. A. ChIP assays showing mutations in TAD alter a majority of interactions of mutant p53 and enhanced binding of acetylated histone H3 to the EGFR promoter. Normalized values were divided by the normalized IgG. Asterisks indicate a p-value of less than 0.05. **B.** ChIP assays showing mutations in TAD alter binding of different transcription factors to the EGFR promoter. Asterisks indicate a p-value of less than 0.05. **C.** ChIP samples in Figure 6B were assayed using a different set of primers on the EGFR promoter about 1.5kb upstream of set 1 to show mutant p53 and its TAD mutant have a similar binding pattern at a distant location (as shown in the diagram). N.S. indicates no significant difference. **D.** ChIP samples in Figure 6A were assayed using three different sets of primers on the EGFR promoter about 500bp-1.5kb upstream of set 1 to show mutant p53 and its TAD mutant have a similar binding pattern at a distant locations. Positions of the primer sets are shown in the figure. N.S. indicates no significant difference.

EGFR levels. Knock-down of p53 in H1975 and KNS-62 cells lowers EGFR overexpression; however, the addiction to GOF p53 can be compensated in H1975 cells by overexpressing EGFR, suggesting that EGFR is in the GOF p53 pathway and plays a critical role in addiction of lung cancer cells to GOF p53. Chromatin immunoprecipitation (ChIP) assays show that p53-R273H interacts with the EGFR promoter and increases histone H3 acetylation, indicating a mechanism whereby mutant p53 enhances chromatin opening for improved access to transcription factors. ChIP and ChIP-re-ChIP studies show docking of GOF p53 on Sp1, leading to increased binding of Sp1 and CBP on the EGFR promoter. Using a transactivation domain (TAD) mutant of GOF p53, we show that TAD-mutated GOF p53 is efficient in up-regulating EGFR. Although TAD mutations cause disruption of mutant p53 interactions at a major binding site, mutant p53-promoter interactions at multiple sites appear to be responsible for significant mutant p53-mediated EGFR transactivation capability which suggests multiple contacts of TAD with transcription factors.

Discussion

p53 plays a significant role in oncogenesis, whether it is present in the cell as the wild-type or mutant form of the protein. WT p53 has long been shown to be key to maintaining the integrity of the cell and therefore preventing oncogenesis; meanwhile mutant p53 causes the acquisition of new oncogenic functions. Both WT and mutant p53 have biological activities that are dependent on its transcriptional ability, and both proteins have been demonstrated to repress and activate expression of a number of genes. We and others have shown that expression of tumor-derived mutant p53 in cells leads to upregulated expression of a set of genes, some of which are involved in increased cell growth rate, cell motility, tumorigenicity, and loss of sensitivity to chemotherapeutic drugs (107, 110, 245) as an indicator of GOF activity.

We have suggested earlier that, in the case of H1299 lung carcinoma cells, the chemoresistance observed by the expression of mutant p53 can be attributed at least in part through mutant p53-mediated transactivation of the NF-κB2 gene (110). Here, using cancer cell lines containing endogenous GOF p53, we have demonstrated that the presence of p53 mutants leads to GOF phenotypes as shown by an increase in cell growth rate, cell motility, tumorigenicity, and loss of sensitivity to chemotherapeutic drugs (Figures 3-5). We have further shown that the GOF activities are dependent on the level of NF-κB2 (Figure 6), verifying that mutant p53-induced GOF functions utilize NF-κB2 to accomplish these phenotypes, at least in part.

We next examined the mechanism of up-regulation of NF-κB2 by mutant p53 since transient transcriptional analysis could not detect any mutant p53 response element (Figure 7).

This was not unexpected as previous studies also failed to detect any (163, 198). Furthermore, ChIP experiments showed that mutant p53 induces chromatin changes via histone acetylation that may cause activation of the NF-κB2 promoter (Figure 8). This histone acetylation perhaps leads to increased interaction of the transcription factor STAT2 to a STAT binding site on the NF-κB2 promoter which may be the reason for increased NF-κB2 promoter activity in the presence of mutant p53 (Figure 8). Data also show an interaction of CBP with the NF-κB2 promoter (Figure 8). Since CBP/p300 has histone acetylase activity associated with it, these data indicate that mutant p53 may be enhancing histone acetylation through the use of CBP/p300. Interestingly, interactions of CBP/p300 and STAT, p53 and STAT as well as p53 and CBP/p300 have been reported (246-248). Since the data was generated, several reports have been published illustrating STAT regulation via acetylation (249). In fact, STAT2 was even shown to recruit HATs and cause transient acetylation of histones even though p300/CBP is not required for STAT function (248).

With that information in hand, we speculate that the STAT1/STAT2 complex on the NF-κB2 promoter interacts with CBP/p300 to open up chromatin and pull down mutant p53. We further speculate that mutant p53 then stimulates further opening up of chromatin via its interaction with CBP/p300 with a positive feed-back loop.

Earlier, we had suggested that the GOF activities observed in H1299 cells after expression of mutant p53 can be explained in part by mutant p53's ability to enhance expression of NF-κB2 (110). Axl has been identified as another player via microarray analysis of H1299 cells stably expressing different p53 mutants. Axl has previously been implicated in oncogenesis (206) and is significantly up-regulated by tumor derived p53 mutants in lung cancer cell lines and human lung tumors (Figures 9 and 10). One interesting and important aspect of Axl being

up-regulated by p53 mutants is the fact that p53 mutants do not require an intact transactivation domain for this up-regulation (Figure 9). Thus, this mutant p53 target seems to be a good candidate gene that is up-regulated by mutant p53 perhaps through interaction with p63/p73 (155). We note that there is a second transactivation subdomain that may contribute to transactivation, and further studies would need to be performed to investigate the role the second transactivation domain plays in Axl upregulation by mutant p53.

Using human lung cancer cell lines and lung tumor samples expressing mutant p53 we demonstrate that expression of GOF mutant p53 induces Axl expression (Figure 10). We found that knocking down Axl levels by RNAi in H1048 cells led to a decrease in cell motility and growth rate (Figure 11) while mutant p53 level remained constant. This suggests that GOF mutant p53 may induce part of its GOF activity via induction of Axl.

We have examined the promoter sequences of Axl and identified several transcription factor binding sites (Figure 12 top), and have performed transient transcriptional analysis to determine the mechanism of activation of the promoter by GOF mutant p53. Transient transfections showed that mutant p53 indeed transactivates the Axl promoter in H1299 cells (Figure 12), however, our promoter deletion analysis indicated that the p53/p63-binding site present is not needed for transactivation by mutant p53 in H1299 cells (Figure 13). This shows that GOF p53 mutants transactivate the Axl promoter without the necessity of p63 binding to the promoter.

ChIP assay analysis demonstrated that GOF mutant p53 induces histone acetylation at the Axl promoter (Figure 14A-D) suggesting that mutant p53 causes chromatin modifications on the promoter indicative of increased transcriptional activity. Transcription factor ChIP analyses indicated that mutant p53 induced interaction between p300, CREB, and E2F1 on the Axl

promoter (Figure 16), which suggests their involvement in general in transactivation of Axl by mutant p53. The observed increased histone acetylation may lead to increased interaction of the transcription factors p300, CREB, and E2F1 with their binding sites on the Axl promoter, which then leads to an increase in Axl promoter activity. Since CBP/p300 has histone acetylase activity associated with it, these data suggest that mutant p53 may be enhancing histone acetylation of the Axl promoter through the use of CBP/p300. Interestingly, interactions of CBP/p300 and E2F1 and CREB (250) may further nucleate p300 on the Axl promoter and lead to further acetylation of histones increasing its promoter activity.

We have demonstrated that there is more mutant p53-mediated activation of the Axl promoter further away from the transcription start site indicating mutant p53 may bind to an enhancer-like region to regulate transcription (Figure 15). Further in vivo and in vitro work is needed to clarify the mechanism of transactivation by mutant p53.

We next examined the mechanism of up-regulation of EGFR expression by GOF p53 and demonstrated that EGFR is elevated at the mRNA level by GOF p53 in H1299, KNS-62 and H1975 cells. Through RNAi experiments in H1975 cells we show that reduction of mutant p53 or EGFR levels lowers the proliferation rate of these cells, indicating that both genes are in a pathway that controls cell proliferation. Since mutant p53 up-regulates EGFR, this also suggests that this particular GOF activity of mutant p53 is through EGFR up-regulation, at least in part. This concept has been strengthened further by restoration of GOF activity lost by reduction of p53 levels on overexpression of EGFR (Figure 21). However, multiple mutant p53 targets have been identified that may be responsible for induction of proliferation (84, 160, 251). Thus, a thorough functional-genomic approach is needed to fully understand which genes are required for different GOF activities.

We also show that mutant p53 expression leads to enhanced binding of GOF p53 on the EGFR promoter, and importantly it induces enhanced interaction of TFs on the EGFR promoter including the HAT, CBP and Sp1 (Figure 23). ChIP for AcH3 indicates enhanced acetylation of histone H3 in the presence of mutant p53, indicative of induced opening of the chromatin near the mutant p53 binding site. Thus, the mechanism by which mutant p53 activates EGFR transcription may depend upon nucleation of mutant p53 that then induces acetylation of histone H3, opening chromatin and activating transcription.

We examined the contribution of the transactivation domain in transactivation of EGFR and nucleation of mutant p53 and AcH3 (as assayed by ChIP). Interestingly, mutations in TAD affected nucleation of mutant p53 and AcH3 at the major binding site (Figure 25) but not transactivation of EGFR (Figure 24). Since our data show no absolute requirements of integrity of amino acids at positions 22 and 23, it perhaps points to the possible interactions of TFs included in extended regions of TAD. However, we showed that transactivation by GOF p53 requires the presence of TAD (198) suggesting an important contribution of TAD in transactivation. The scanning ChIP QPCR data shown in Figure 25 suggest that the promoter sequences defined by primer set 1 requires integrity of amino acids 22 and 23 of TAD to be precise, perhaps through a direct interaction of TFs whereas TAD can contact other regions via other TAD sequences. It is possible in that case sequences in the second transactivation domain may prove to be important (244).

It is important to note that we find that mutant p53 induces enhanced interaction of TFs on the EGFR promoter. It is possible that this results in enhanced binding of CBP/p300 to the EGFR promoter and consequently higher levels of acetylation of histone H3. This is expected to impact the chromatin structure in a positive manner, paving the way for a higher rate of

transcription. In the future, it needs to be ascertained if the information exchange between the proteins happens while they remain on the promoter or when they are unbound from the DNA. Involvement of the Sp1 and Ets group of transcription factors and mutant p53 in mutant p53-mediated transactivation as a component of its GOF activity has been suggested by us and others in the past (166, 242, 252).

Since NF-κB2, Axl, and EGFR all show enhanced histone acetylation and transcription factor binding to their promoters in the presence of GOF p53, it is possible that mutant p53 utilizes a similar mechanism to activate transcription and thus upregulate expression of these three genes. We have shown that mutant p53 enhances Sp1 and CBP binding for both the Axl and EGFR promoters, however, the NF-κB2 promoter also has Sp1 binding sites which have not been explored yet. It would be interesting to know whether we can also see enhanced Sp1 binding in the presence of mutant p53 on the NF-κB2 promoter as well. In addition, Sp1 and Ets1 are known to interact, and in the case of the EGFR promoter ChIP-reChIP, Sp1 was seen to interact with mutant p53 at the promoter whereas Ets1 was not (data not shown). In this case, it is possible that the enhanced Ets1 binding to that particular promoter may be due at least in part to interaction with Sp1 since there are many more binding sites for Sp1 than Ets1.

The work presented here shows a possible mechanism of up-regulation of mutant p53 target gene expression with the involvement of chromatin modifications. In the case of NF-κB2, this chromatin modification is brought into action most likely by CBP which is found to be nucleated on the promoter. As a result of the histone acetylation, we can observe increased STAT interaction on the promoter that might be the reason for enhanced transcription of the mutant p53 target, NF-κB2.

We suggest a model (Figure 26) in which mutant p53 interacts on the target promoters via interaction with multiple TFs: Ets1, Sp1 and perhaps others (Figure 25). Possibly, GOF p53 docks with Sp1 and CBP. p300/CBP may become involved in the process either because of direct interaction of p53 and CBP/p300 or through Ets1-CBP/p300 and/or Sp1-CBP/p300 interactions (253-256). Sp1 and Ets1 interactions with CBP/p300 have been suggested to facilitate acetylation of histones (257, 258). This increased histone acetylation then translates into chromatin opening and increased transcription.

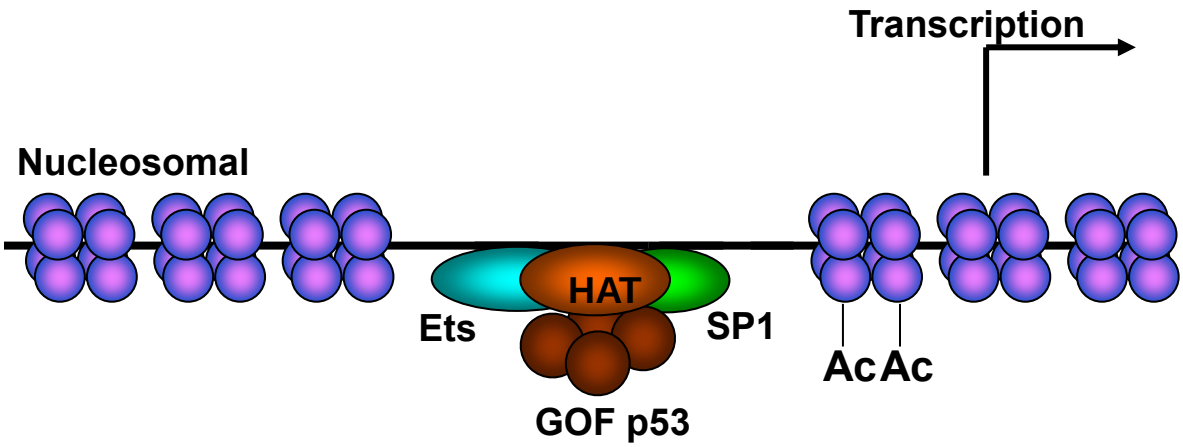

Figure 26. Proposed model for GOF p53 nucleating on a target promoter.

Literature Cited

1. Chang C, Simmons DT, Martin MA, Mora PT. Identification and partial characterization of new antigens from simian virus 40-transformed mouse cells. J Virol. 1979;31(2):463-71. Epub 1979/08/01. PubMed PMID: 225565; PMCID: 353469.
2. DeLeo AB, Jay G, Appella E, Dubois GC, Law LW, Old LJ. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proceedings of the National Academy of Sciences of the United States of America. 1979;76(5):2420-4. Epub 1979/05/01. PubMed PMID: 221923; PMCID: 383613.
3. Kress M, May E, Cassingena R, May P. Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. J Virol. 1979;31(2):472-83. Epub 1979/08/01. PubMed PMID: 225566; PMCID: 353470.
4. Lane DP, Crawford LV. T antigen is bound to a host protein in SV40-transformed cells. Nature. 1979;278(5701):261-3. Epub 1979/03/15. PubMed PMID: 218111.
5. Linzer DI, Levine AJ. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell. 1979;17(1):43-52. Epub 1979/05/01. PubMed PMID: 222475.
6. Melero JA, Stitt DT, Mangel WF, Carroll RB. Identification of new polypeptide species (48-55K) immunoprecipitable by antiserum to purified large T antigen and present in SV40-infected and -transformed cells. Virology. 1979;93(2):466-80. Epub 1979/03/01. PubMed PMID: 222051.
7. Matlashewski G, Banks L, Pim D, Crawford L. Analysis of human p53 proteins and mRNA levels in normal and transformed cells. European journal of biochemistry / FEBS. 1986;154(3):665-72. PubMed PMID: 2419131.
8. Prokocimer M, Shaklai M, Bassat HB, Wolf D, Goldfinger N, Rotter V. Expression of p53 in human leukemia and lymphoma. Blood. 1986;68(1):113-8. PubMed PMID: 3521760.
9. Jenkins JR, Rudge K, Currie GA. Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. Nature. 1984;312(5995):651-4. Epub 1984/12/13. PubMed PMID: 6095117.
10. Eliyahu D, Raz A, Gruss P, Givol D, Oren M. Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature. 1984;312(5995):646-9. Epub 1984/12/13. PubMed PMID: 6095116.
11. Parada LF, Land H, Weinberg RA, Wolf D, Rotter V. Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. Nature. 1984;312(5995):649-51. Epub 1984/12/13. PubMed PMID: 6390217.
12. Hinds P, Finlay C, Levine AJ. Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J Virol. 1989;63(2):739-46. Epub 1989/02/01. PubMed PMID: 2642977; PMCID: 247745.
13. Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi-Kimhi O, Oren M. Wild-type p53 can inhibit oncogene-mediated focus formation. Proceedings of the National Academy of Sciences of

the United States of America. 1989;86(22):8763-7. Epub 1989/11/01. PubMed PMID: 2530586; PMCID: 298370.
14. Finlay CA, Hinds PW, Levine AJ. The p53 proto-oncogene can act as a suppressor of transformation. Cell. 1989;57(7):1083-93. PubMed PMID: 2525423.
15. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 1992;356(6366):215-21. PubMed PMID: 1552940.
16. Matlashewski G, Lamb P, Pim D, Peacock J, Crawford L, Benchimol S. Isolation and characterization of a human p53 cDNA clone: expression of the human p53 gene. EMBO J. 1984;3(13):3257-62. PubMed PMID: 6396087; PMCID: 557846.
17. McBride OW, Merry D, Givol D. The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). Proceedings of the National Academy of Sciences of the United States of America. 1986;83(1):130-4. PubMed PMID: 3001719; PMCID: 322805.
18. Kern SE, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C, Vogelstein B. Identification of p53 as a sequence-specific DNA-binding protein. Science (New York, NY. 1991;252(5013):1708-11. PubMed PMID: 2047879.
19. Kraiss S, Quaiser A, Oren M, Montenarh M. Oligomerization of oncoprotein p53. J Virol. 1988;62(12):4737-44. PubMed PMID: 3054153; PMCID: 253589.
20. Stenger JE, Mayr GA, Mann K, Tegtmeyer P. Formation of stable p53 homotetramers and multiples of tetramers. Mol Carcinog. 1992;5(2):102-6. PubMed PMID: 1554407.
21. Friedman PN, Chen X, Bargonetti J, Prives C. The p53 protein is an unusually shaped tetramer that binds directly to DNA. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(8):3319-23. PubMed PMID: 8475074; PMCID: 46291.
22. Donehower LA, Bradley A. The tumor suppressor p53. Biochimica et biophysica acta. 1993;1155(2):181-205. PubMed PMID: 8357826.
23. Lane DP. p53 and human cancers. Br Med Bull. 1994;50(3):582-99. PubMed PMID: 7987642.
24. Levine AJ, Chang A, Dittmer D, Notterman DA, Silver A, Thorn K, Welsh D, Wu M. The p53 tumor suppressor gene. The Journal of laboratory and clinical medicine. 1994;123(6):817-23. PubMed PMID: 8201258.
25. Prives C, Bargonetti J, Farmer G, Ferrari E, Friedlander P, Wang Y, Jayaraman L, Pavletich N, Hubscher U. DNA-binding properties of the p53 tumor suppressor protein. Cold Spring Harb Symp Quant Biol. 1994;59:207-13. PubMed PMID: 7587071.
26. Farmer G, Bargonetti J, Zhu H, Friedman P, Prywes R, Prives C. Wild-type p53 activates transcription in vitro. Nature. 1992;358(6381):83-6. doi: 10.1038/358083a0. PubMed PMID: 1614538.
27. Subler MA, Martin DW, Deb S. Inhibition of viral and cellular promoters by human wild-type p53. J Virol. 1992;66(8):4757-62. PubMed PMID: 1352831.
28. el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. Nature genetics. 1992;1(1):45-9. PubMed PMID: 1301998.
29. Bienz-Tadmor B, Zakut-Houri R, Libresco S, Givol D, Oren M. The 5' region of the p53 gene: evolutionary conservation and evidence for a negative regulatory element. EMBO J. 1985;4(12):3209-13. PubMed PMID: 3004941; PMCID: 554644.

30. Oren M. The p53 cellular tumor antigen: gene structure, expression and protein properties. Biochimica et biophysica acta. 1985;823(1):67-78. PubMed PMID: 3902087.
31. O'Rourke RW, Miller CW, Kato GJ, Simon KJ, Chen DL, Dang CV, Koeffler HP. A potential transcriptional activation element in the p53 protein. Oncogene. 1990;5(12):1829-32. Epub 1990/12/01. PubMed PMID: 2284102.
32. Raycroft L, Wu HY, Lozano G. Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science (New York, NY. 1990;249(4972):1049-51. Epub 1990/08/31. PubMed PMID: 2144364; PMCID: 2935288.
33. Sturzbecher HW, Brain R, Addison C, Rudge K, Remm M, Grimaldi M, Keenan E, Jenkins JR. A C-terminal alpha-helix plus basic region motif is the major structural determinant of p53 tetramerization. Oncogene. 1992;7(8):1513-23. PubMed PMID: 1321401.
34. Fields S, Jang SK. Presence of a potent transcription activating sequence in the p53 protein. Science (New York, NY. 1990;249(4972):1046-9. PubMed PMID: 2144363.
35. Bargonetti J, Manfredi JJ, Chen X, Marshak DR, Prives C. A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. Genes & development. 1993;7(12B):2565-74. PubMed PMID: 8276239.
36. Halazonetis TD, Kandil AN. Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. EMBO J. 1993;12(13):5057-64. PubMed PMID: 8262048; PMCID: 413766.
37. Wang Y, Reed M, Wang P, Stenger JE, Mayr G, Anderson ME, Schwedes JF, Tegtmeyer P. p53 domains: identification and characterization of two autonomous DNA-binding regions. Genes & development. 1993;7(12B):2575-86. PubMed PMID: 8276240.
38. Subler MA, Martin DW, Deb S. Overlapping domains on the p53 protein regulate its transcriptional activation and repression functions. Oncogene. 1994;9(5):1351-9. PubMed PMID: 8152795.
39. Clore GM, Omichinski JG, Sakaguchi K, Zambrano N, Sakamoto H, Appella E, Gronenborn AM. High-resolution structure of the oligomerization domain of p53 by multidimensional NMR. Science (New York, NY. 1994;265(5170):386-91. PubMed PMID: 8023159.
40. Soussi T, Caron de Fromentel C, May P. Structural aspects of the p53 protein in relation to gene evolution. Oncogene. 1990;5(7):945-52. PubMed PMID: 2142762.
41. Pavletich NP, Chambers KA, Pabo CO. The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. Genes & development. 1993;7(12B):2556-64. PubMed PMID: 8276238.
42. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell. 1993;75(4):817-25. PubMed PMID: 8242752.
43. Lowe SW, Bodis S, Bardeesy N, McClatchey A, Remington L, Ruley HE, Fisher DE, Jacks T, Pelletier J, Housman DE. Apoptosis and the prognostic significance of p53 mutation. Cold Spring Harb Symp Quant Biol. 1994;59:419-26. PubMed PMID: 7587096.
44. Del Sal G, Murphy M, Ruaro E, Lazarevic D, Levine AJ, Schneider C. Cyclin D1 and p21/waf1 are both involved in p53 growth suppression. Oncogene. 1996;12(1):177-85. PubMed PMID: 8552389.
45. el-Deiry WS. Regulation of p53 downstream genes. Seminars in cancer biology. 1998;8(5):345-57. PubMed PMID: 10101800.

46. Prives C, Hall PA. The p53 pathway. The Journal of pathology. 1999;187(1):112-26. PubMed PMID: 10341712.
47. Kannan K, Kaminski N, Rechavi G, Jakob-Hirsch J, Amariglio N, Givol D. DNA microarray analysis of genes involved in p53 mediated apoptosis: activation of Apaf-1. Oncogene. 2001;20(26):3449-55. PubMed PMID: 11423996.
48. Funk WD, Pak DT, Karas RH, Wright WE, Shay JW. A transcriptionally active DNA-binding site for human p53 protein complexes. Mol Cell Biol. 1992;12(6):2866-71. PubMed PMID: 1588974.
49. Zauberman A, Barak Y, Ragimov N, Levy N, Oren M. Sequence-specific DNA binding by p53: identification of target sites and lack of binding to p53 - MDM2 complexes. The EMBO journal. 1993;12(7):2799-808. Epub 1993/07/01. PubMed PMID: 8334996; PMCID: 413530.
50. Pietenpol JA, Tokino T, Thiagalingam S, el-Deiry WS, Kinzler KW, Vogelstein B. Sequence-specific transcriptional activation is essential for growth suppression by p53. Proceedings of the National Academy of Sciences of the United States of America. 1994;91(6):1998-2002. Epub 1994/03/15. PubMed PMID: 8134338; PMCID: 43296.
51. Sahin E, DePinho RA. Axis of ageing: telomeres, p53 and mitochondria. Nature reviews. 2012;13(6):397-404. Epub 2012/05/17. doi: 10.1038/nrm3352. PubMed PMID: 22588366.
52. Reinhardt HC, Schumacher B. The p53 network: cellular and systemic DNA damage responses in aging and cancer. Trends Genet. 2012;28(3):128-36. Epub 2012/01/24. doi: 10.1016/j.tig.2011.12.002. PubMed PMID: 22265392.
53. Ryan KM. p53 and autophagy in cancer: guardian of the genome meets guardian of the proteome. Eur J Cancer. 2011;47(1):44-50. Epub 2010/11/30. doi: 10.1016/j.ejca.2010.10.020. PubMed PMID: 21112207.
54. Aylon Y, Oren M. p53: guardian of ploidy. Molecular oncology. 2011;5(4):315-23. Epub 2011/08/20. doi: 10.1016/j.molonc.2011.07.007. PubMed PMID: 21852209; PMCID: 3164924.
55. Maddocks OD, Vousden KH. Metabolic regulation by p53. J Mol Med (Berl). 2011;89(3):237-45. Epub 2011/02/23. doi: 10.1007/s00109-011-0735-5. PubMed PMID: 21340684; PMCID: 3043245.
56. Hermeking H. MicroRNAs in the p53 network: micromanagement of tumour suppression. Nat Rev Cancer. 2012;12(9):613-26. Epub 2012/08/18. doi: 10.1038/nrc3318. PubMed PMID: 22898542.
57. Vilborg A, Bersani C, Wilhelm MT, Wiman KG. The p53 target Wig-1: a regulator of mRNA stability and stem cell fate? Cell Death Differ. 2011;18(9):1434-40. Epub 2011/03/12. doi: 10.1038/cdd.2011.20. PubMed PMID: 21394102; PMCID: 3178426.
58. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature. 1997;387(6630):296-9. PubMed PMID: 9153395.
59. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature. 1997;387(6630):299-303. PubMed PMID: 9153396.
60. Freedman DA, Levine AJ. Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. Mol Cell Biol. 1998;18(12):7288-93. PubMed PMID: 9819415.
61. Roth J, Dobbelstein M, Freedman DA, Shenk T, Levine AJ. Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the

human immunodeficiency virus rev protein. EMBO J. 1998;17(2):554-64. doi: 10.1093/emboj/17.2.554. PubMed PMID: 9430646; PMCID: 1170405.
62. Lain S, Xirodimas D, Lane DP. Accumulating active p53 in the nucleus by inhibition of nuclear export: a novel strategy to promote the p53 tumor suppressor function. Experimental cell research. 1999;253(2):315-24. doi: 10.1006/excr.1999.4672. PubMed PMID: 10585254.
63. Tao W, Levine AJ. Nucleocytoplasmic shuttling of oncoprotein Hdm2 is required for Hdm2-mediated degradation of p53. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(6):3077-80. PubMed PMID: 10077639; PMCID: 15897.
64. Chen J, Marechal V, Levine AJ. Mapping of the p53 and mdm-2 interaction domains. Mol Cell Biol. 1993;13(7):4107-14. PubMed PMID: 7686617.
65. Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature. 1993;362(6423):857-60. PubMed PMID: 8479525.
66. Maltzman W, Czyzyk L. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol Cell Biol. 1984;4(9):1689-94. PubMed PMID: 6092932; PMCID: 368974.
67. Fu L, Minden MD, Benchimol S. Translational regulation of human p53 gene expression. EMBO J. 1996;15(16):4392-401. PubMed PMID: 8861966; PMCID: 452163.
68. Mayo LD, Donner DB. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(20):11598-603. PubMed PMID: 11504915.
69. Abraham AG, O'Neill E. PI3K/Akt-mediated regulation of p53 in cancer. Biochem Soc Trans. 2014;42(4):798-803. doi: 10.1042/BST20140070. PubMed PMID: 25109960.
70. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nature cell biology. 2001;3(11):973-82. PubMed PMID: 11715018.
71. Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, Masuyama N, Gotoh Y. Akt enhances Mdm2-mediated ubiquitination and degradation of p53. The Journal of biological chemistry. 2002;277(24):21843-50. PubMed PMID: 11923280.
72. Ashcroft M, Ludwig RL, Woods DB, Copeland TD, Weber HO, MacRae EJ, Vousden KH. Phosphorylation of HDM2 by Akt. Oncogene. 2002;21(13):1955-62. doi: 10.1038/sj.onc.1205276. PubMed PMID: 11960368.
73. Gottlieb TM, Leal JF, Seger R, Taya Y, Oren M. Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis. Oncogene. 2002;21(8):1299-303. doi: 10.1038/sj.onc.1205181. PubMed PMID: 11850850.
74. Lill NL, Grossman SR, Ginsberg D, DeCaprio J, Livingston DM. Binding and modulation of p53 by p300/CBP coactivators. Nature. 1997;387(6635):823-7. Epub 1997/06/19. doi: 10.1038/42981. PubMed PMID: 9194565.
75. Grossman SR. p300/CBP/p53 interaction and regulation of the p53 response. European journal of biochemistry / FEBS. 2001;268(10):2773-8. Epub 2001/05/19. PubMed PMID: 11358491.
76. Brooks CL, Gu W. The impact of acetylation and deacetylation on the p53 pathway. Protein & cell. 2011;2(6):456-62. doi: 10.1007/s13238-011-1063-9. PubMed PMID: 21748595; PMCID: 3690542.

77. Scolnick DM, Chehab NH, Stavridi ES, Lien MC, Caruso L, Moran E, Berger SL, Halazonetis TD. CREB-binding protein and p300/CBP-associated factor are transcriptional coactivators of the p53 tumor suppressor protein. Cancer Res. 1997;57(17):3693-6. PubMed PMID: 9288775.
78. Luo J, Su F, Chen D, Shiloh A, Gu W. Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature. 2000;408(6810):377-81. doi: 10.1038/35042612. PubMed PMID: 11099047.
79. Beckerman R, Prives C. Transcriptional regulation by p53. Cold Spring Harbor perspectives in biology. 2010;2(8):a000935. Epub 2010/08/04. doi: 10.1101/cshperspect.a000935. PubMed PMID: 20679336.
80. Seto E, Usheva A, Zambetti GP, Momand J, Horikoshi N, Weinmann R, Levine AJ, Shenk T. Wild-type p53 binds to the TATA-binding protein and represses transcription. Proceedings of the National Academy of Sciences of the United States of America. 1992;89(24):12028-32. PubMed PMID: 1465435.
81. Ginsberg D, Mechta F, Yaniv M, Oren M. Wild-type p53 can down-modulate the activity of various promoters. Proceedings of the National Academy of Sciences of the United States of America. 1991;88(22):9979-83. Epub 1991/11/15. PubMed PMID: 1946467; PMCID: 52850.
82. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. The Journal of biological chemistry. 2002;277(5):3247-57. Epub 2001/11/21. doi: 10.1074/jbc.M106643200. PubMed PMID: 11714700.
83. Wilkinson DS, Tsai WW, Schumacher MA, Barton MC. Chromatin-bound p53 anchors activated Smads and the mSin3A corepressor to confer transforming-growth-factor-beta-mediated transcription repression. Mol Cell Biol. 2008;28(6):1988-98. Epub 2008/01/24. doi: 10.1128/MCB.01442-07. PubMed PMID: 18212064; PMCID: 2268392.
84. Oren M, Rotter V. Mutant p53 gain-of-function in cancer. Cold Spring Harbor perspectives in biology. 2010;2(2):a001107. Epub 2010/02/26. doi: 10.1101/cshperspect.a001107. PubMed PMID: 20182618; PMCID: 2828285.
85. Ozbun MA, Butel JS. Tumor suppressor p53 mutations and breast cancer: a critical analysis. Adv Cancer Res. 1995;66:71-141. PubMed PMID: 7793321.
86. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science (New York, NY. 1991;253(5015):49-53. PubMed PMID: 1905840.
87. Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith-Sorensen B, Montesano R, Harris CC. Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic acids research. 1994;22(17):3551-5. PubMed PMID: 7937055.
88. Soussi T, Kato S, Levy PP, Ishioka C. Reassessment of the TP53 mutation database in human disease by data mining with a library of TP53 missense mutations. Hum Mutat. 2005;25(1):6-17. doi: 10.1002/humu.20114. PubMed PMID: 15580553.
89. Petitjean A, Achatz MI, Borresen-Dale AL, Hainaut P, Olivier M. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene. 2007;26(15):2157-65. PubMed PMID: 17401424.
90. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res. 1994;54(18):4855-78. PubMed PMID: 8069852.

91. Hollstein M, Shomer B, Greenblatt M, Soussi T, Hovig E, Montesano R, Harris CC. Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. Nucleic acids research. 1996;24(1):141-6. PubMed PMID: 8594564.
92. Morris SM. A role for p53 in the frequency and mechanism of mutation. Mutat Res. 2002;511(1):45-62. Epub 2002/03/22. PubMed PMID: 11906841.
93. Rivlin N, Brosh R, Oren M, Rotter V. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. Genes & cancer. 2011;2(4):466-74. doi: 10.1177/1947601911408889. PubMed PMID: 21779514; PMCID: 3135636.
94. Hamada Y, Mizoguchi M, Suzuki SO, Iwaki T. Accumulation of class I mutant p53 and apoptosis induced by carboplatin in a human glioma cell line. Brain tumor pathology. 1998;15(2):77-82. PubMed PMID: 10328543.
95. Ryan KM, Vousden KH. Characterization of structural p53 mutants which show selective defects in apoptosis but not cell cycle arrest. Mol Cell Biol. 1998;18(7):3692-8. PubMed PMID: 9632751; PMCID: 108951.
96. Ory K, Legros Y, Auguin C, Soussi T. Analysis of the most representative tumour-derived p53 mutants reveals that changes in protein conformation are not correlated with loss of transactivation or inhibition of cell proliferation. EMBO J. 1994;13(15):3496-504. PubMed PMID: 8062826; PMCID: 395253.
97. Kikuchi-Yanoshita R, Tanaka K, Muraoka M, Konishi M, Kawashima I, Takamoto S, Hirai H, Miyaki M. Malignant transformation of rat embryo fibroblasts by cotransfection with eleven human mutant p53 cDNAs and activated H-ras gene. Oncogene. 1995;11(7):1339-45. Epub 1995/10/05. PubMed PMID: 7478555.
98. Zerdoumi Y, Aury-Landas J, Bonaiti-Pellie C, Derambure C, Sesboue R, Renaux-Petel M, Frebourg T, Bougeard G, Flaman JM. Drastic effect of germline TP53 missense mutations in Li-Fraumeni patients. Human mutation. 2013;34(3):453-61. Epub 2012/11/23. doi: 10.1002/humu.22254. PubMed PMID: 23172776.
99. Allred DC, Clark GM, Elledge R, Fuqua SA, Brown RW, Chamness GC, Osborne CK, McGuire WL. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. J Natl Cancer Inst. 1993;85(3):200-6. Epub 1993/02/03. PubMed PMID: 8423624.
100. Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C, Levine AJ. Gain of function mutations in p53. Nature genetics. 1993;4(1):42-6. PubMed PMID: 8099841.
101. Pohl J, Goldfinger N, Radler-Pohl A, Rotter V, Schirrmacher V. p53 increases experimental metastatic capacity of murine carcinoma cells. Mol Cell Biol. 1988;8(5):2078-81. PubMed PMID: 3290647.
102. Hsiao M, Low J, Dorn E, Ku D, Pattengale P, Yeargin J, Haas M. Gain-of-function mutations of the p53 gene induce lymphohematopoietic metastatic potential and tissue invasiveness. Am J Pathol. 1994;145(3):702-14. PubMed PMID: 8080050.
103. Lassus P, Bertrand C, Zugasti O, Chambon JP, Soussi T, Mathieu-Mahul D, Hibner U. Anti-apoptotic activity of p53 maps to the COOH-terminal domain and is retained in a highly oncogenic natural mutant. Oncogene. 1999;18(33):4699-709. doi: 10.1038/sj.onc.1202841. PubMed PMID: 10467417.
104. Lanyi A, Deb D, Seymour RC, Ludes-Meyers JH, Subler MA, Deb S. 'Gain of function' phenotype of tumor-derived mutant p53 requires the oligomerization/nonsequence-specific nucleic acid-binding domain. Oncogene. 1998;16(24):3169-76. PubMed PMID: 9671396.

105. Lin J, Teresky AK, Levine AJ. Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants. Oncogene. 1995;10(12):2387-90. PubMed PMID: 7784087.
106. Shi XB, Nesslinger NJ, Deitch AD, Gumerlock PH, deVere White RW. Complex functions of mutant p53 alleles from human prostate cancer. Prostate. 2002;51(1):59-72. PubMed PMID: 11920959.
107. Blandino G, Levine AJ, Oren M. Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. Oncogene. 1999;18(2):477-85. PubMed PMID: 9927204.
108. Bristow RG, Peacock J, Jang A, Kim J, Hill RP, Benchimol S. Resistance to DNA-damaging agents is discordant from experimental metastatic capacity in MEF ras-transformants-expressing gain of function MTp53. Oncogene. 2003;22(19):2960-6. PubMed PMID: 12771947.
109. Lai SL, Perng RP, Hwang J. p53 gene status modulates the chemosensitivity of non-small cell lung cancer cells. J Biomed Sci. 2000;7(1):64-70. PubMed PMID: 10644891.
110. Scian MJ, Stagliano KE, Anderson MA, Hassan S, Bowman M, Miles MF, Deb SP, Deb S. Tumor-derived p53 mutants induce NF-kappaB2 gene expression. Mol Cell Biol. 2005;25(22):10097-110. PubMed PMID: 16260623.
111. Bossi G, Lapi E, Strano S, Rinaldo C, Blandino G, Sacchi A. Mutant p53 gain of function: reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression. Oncogene. 2006;25(2):304-9. Epub 2005/09/20. doi: 10.1038/sj.onc.1209026. PubMed PMID: 16170357.
112. Deb D, Scian M, Roth KE, Li W, Keiger J, Chakraborti AS, Deb SP, Deb S. Hetero-oligomerization does not compromise 'gain of function' of tumor-derived p53 mutants. Oncogene. 2002;21(2):176-89. PubMed PMID: 11803461.
113. Di Agostino S, Strano S, Emiliozzi V, Zerbini V, Mottolese M, Sacchi A, Blandino G, Piaggio G. Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer cell. 2006;10(3):191-202. PubMed PMID: 16959611.
114. Duan W, Gao L, Jin D, Otterson GA, Villalona-Calero MA. Lung specific expression of a human mutant p53 affects cell proliferation in transgenic mice. Transgenic research. 2008;17(3):355-66. Epub 2007/10/31. doi: 10.1007/s11248-007-9154-3. PubMed PMID: 17968669.
115. Haupt S, di Agostino S, Mizrahi I, Alsheich-Bartok O, Voorhoeve M, Damalas A, Blandino G, Haupt Y. Promyelocytic leukemia protein is required for gain of function by mutant p53. Cancer Res. 2009;69(11):4818-26. Epub 2009/06/03. doi: 10.1158/0008-5472.CAN-08-4010. PubMed PMID: 19487292.
116. Murphy KL, Dennis AP, Rosen JM. A gain of function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model. Faseb J. 2000;14(14):2291-302. PubMed PMID: 11053251.
117. Murphy KL, Rosen JM. Mutant p53 and genomic instability in a transgenic mouse model of breast cancer. Oncogene. 2000;19(8):1045-51. PubMed PMID: 10713688.
118. Iwamoto KS, Mizuno T, Ito T, Tsuyama N, Kyoizumi S, Seyama T. Gain-of-function p53 mutations enhance alteration of the T-cell receptor following X-irradiation, independently of the cell cycle and cell survival. Cancer Res. 1996;56(17):3862-5. Epub 1996/09/01. PubMed PMID: 8752146.

119. El-Hizawi S, Lagowski JP, Kulesz-Martin M, Albor A. Induction of gene amplification as a gain-of-function phenotype of mutant p53 proteins. Cancer Res. 2002;62(11):3264-70. PubMed PMID: 12036943.
120. Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B, Solari A, Bobisse S, Rondina MB, Guzzardo V, Parenti AR, Rosato A, Bicciato S, Balmain A, Piccolo S. A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell. 2009;137(1):87-98. PubMed PMID: 19345189.
121. Muller PA, Caswell PT, Doyle B, Iwanicki MP, Tan EH, Karim S, Lukashchuk N, Gillespie DA, Ludwig RL, Gosselin P, Cromer A, Brugge JS, Sansom OJ, Norman JC, Vousden KH. Mutant p53 drives invasion by promoting integrin recycling. Cell. 2009;139(7):1327-41. Epub 2010/01/13. doi: 10.1016/j.cell.2009.11.026. PubMed PMID: 20064378.
122. Solomon H, Madar S, Rotter V. Mutant p53 gain of function is interwoven into the hallmarks of cancer. The Journal of pathology. 2011;225(4):475-8. Epub 2011/10/26. doi: 10.1002/path.2988. PubMed PMID: 22025211.
123. Taylor WR, Egan SE, Mowat M, Greenberg AH, Wright JA. Evidence for synergistic interactions between ras, myc and a mutant form of p53 in cellular transformation and tumor dissemination. Oncogene. 1992;7(7):1383-90. PubMed PMID: 1620551.
124. Wang SP, Wang WL, Chang YL, Wu CT, Chao YC, Kao SH, Yuan A, Lin CW, Yang SC, Chan WK, Li KC, Hong TM, Yang PC. p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. Nature cell biology. 2009;11(6):694-704. Epub 2009/05/19. doi: 10.1038/ncb1875. PubMed PMID: 19448627.
125. Yeudall WA, Vaughan CA, Miyazaki H, Ramamoorthy M, Choi MY, Chapman CG, Wang H, Black E, Bulysheva AA, Deb SP, Windle B, Deb S. Gain-of-function mutant p53 upregulates CXC chemokines and enhances cell migration. Carcinogenesis. 2012;33(2):442-51. Epub 2011/11/25. doi: 10.1093/carcin/bgr270. PubMed PMID: 22114072.
126. Fontemaggi G, Dell'Orso S, Trisciuoglio D, Shay T, Melucci E, Fazi F, Terrenato I, Mottolese M, Muti P, Domany E, Del Bufalo D, Strano S, Blandino G. The execution of the transcriptional axis mutant p53, E2F1 and ID4 promotes tumor neo-angiogenesis. Nature structural & molecular biology. 2009;16(10):1086-93. Epub 2009/09/29. doi: 10.1038/nsmb.1669. PubMed PMID: 19783986.
127. Cooks T, Pateras IS, Tarcic O, Solomon H, Schetter AJ, Wilder S, Lozano G, Pikarsky E, Forshew T, Rozenfeld N, Harpaz N, Itzkowitz S, Harris CC, Rotter V, Gorgoulis VG, Oren M. Mutant p53 prolongs NF-kappaB activation and promotes chronic inflammation and inflammation-associated colorectal cancer. Cancer cell. 2013;23(5):634-46. Epub 2013/05/18. doi: 10.1016/j.ccr.2013.03.022. PubMed PMID: 23680148; PMCID: 3657134.
128. Liu G, Parant JM, Lang G, Chau P, Chavez-Reyes A, El-Naggar AK, Multani A, Chang S, Lozano G. Chromosome stability, in the absence of apoptosis, is critical for suppression of tumorigenesis in Trp53 mutant mice. Nature genetics. 2004;36(1):63-8. PubMed PMID: 14702042.
129. Wang XJ, Greenhalgh DA, Jiang A, He D, Zhong L, Brinkley BR, Roop DR. Analysis of centrosome abnormalities and angiogenesis in epidermal-targeted p53172H mutant and p53-knockout mice after chemical carcinogenesis: evidence for a gain of function. Mol Carcinog. 1998;23(3):185-92. PubMed PMID: 9833779.
130. Terzian T, Suh YA, Iwakuma T, Post SM, Neumann M, Lang GA, Van Pelt CS, Lozano G. The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. Genes & development. 2008;22(10):1337-44. PubMed PMID: 18483220.

131. Heinlein C, Krepulat F, Lohler J, Speidel D, Deppert W, Tolstonog GV. Mutant p53(R270H) gain of function phenotype in a mouse model for oncogene-induced mammary carcinogenesis. Int J Cancer. 2008;122(8):1701-9. Epub 2007/12/20. doi: 10.1002/ijc.23317. PubMed PMID: 18092324.
132. Tan EH, Morton JP, Timpson P, Tucci P, Melino G, Flores ER, Sansom OJ, Vousden KH, Muller PA. Functions of TAp63 and p53 in restraining the development of metastatic cancer. Oncogene. 2013. Epub 2013/07/23. doi: 10.1038/onc.2013.287. PubMed PMID: 23873029.
133. Hanel W, Marchenko N, Xu S, Xiaofeng Yu S, Weng W, Moll U. Two hot spot mutant p53 mouse models display differential gain of function in tumorigenesis. Cell Death Differ. 2013. Epub 2013/03/30. doi: 10.1038/cdd.2013.17. PubMed PMID: 23538418.
134. Scian MJ, Stagliano KE, Deb D, Ellis MA, Carchman EH, Das A, Valerie K, Deb SP, Deb S. Tumor-derived p53 mutants induce oncogenesis by transactivating growth-promoting genes. Oncogene. 2004;23(25):4430-43. PubMed PMID: 15077194.
135. Scian MJ, Stagliano KE, Ellis MA, Hassan S, Bowman M, Miles MF, Deb SP, Deb S. Modulation of gene expression by tumor-derived p53 mutants. Cancer Res. 2004;64(20):7447-54. PubMed PMID: 15492269.
136. Yan W, Liu G, Scoumanne A, Chen X. Suppression of inhibitor of differentiation 2, a target of mutant p53, is required for gain-of-function mutations. Cancer Res. 2008;68(16):6789-96. PubMed PMID: 18701504.
137. Vikhanskaya F, Lee MK, Mazzoletti M, Broggini M, Sabapathy K. Cancer-derived p53 mutants suppress p53-target gene expression--potential mechanism for gain of function of mutant p53. Nucleic acids research. 2007;35(6):2093-104. Epub 2007/03/09. doi: 10.1093/nar/gkm099. PubMed PMID: 17344317; PMCID: 1874625.
138. Zalcenstein A, Stambolsky P, Weisz L, Muller M, Wallach D, Goncharov TM, Krammer PH, Rotter V, Oren M. Mutant p53 gain of function: repression of CD95(Fas/APO-1) gene expression by tumor-associated p53 mutants. Oncogene. 2003;22(36):5667-76. Epub 2003/08/29. doi: 10.1038/sj.onc.1206724. PubMed PMID: 12944915.
139. Gurova KV, Rokhlin OW, Budanov AV, Burdelya LG, Chumakov PM, Cohen MB, Gudkov AV. Cooperation of two mutant p53 alleles contributes to Fas resistance of prostate carcinoma cells. Cancer Res. 2003;63(11):2905-12. Epub 2003/06/05. PubMed PMID: 12782597.
140. Wong RP, Tsang WP, Chau PY, Co NN, Tsang TY, Kwok TT. p53-R273H gains new function in induction of drug resistance through down-regulation of procaspase-3. Molecular cancer therapeutics. 2007;6(3):1054-61. PubMed PMID: 17363498.
141. Zalcenstein A, Weisz L, Stambolsky P, Bar J, Rotter V, Oren M. Repression of the MSP/MST-1 gene contributes to the antiapoptotic gain of function of mutant p53. Oncogene. 2006;25(3):359-69. PubMed PMID: 16170349.
142. Schilling T, Kairat A, Melino G, Krammer PH, Stremmel W, Oren M, Muller M. Interference with the p53 family network contributes to the gain of oncogenic function of mutant p53 in hepatocellular carcinoma. Biochemical and biophysical research communications. 2010;394(3):817-23. Epub 2010/03/18. doi: 10.1016/j.bbrc.2010.03.082. PubMed PMID: 20233581.
143. Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P, Moll UM. p53 has a direct apoptogenic role at the mitochondria. Molecular cell. 2003;11(3):577-90. PubMed PMID: 12667443.

144. Moll UM, Zaika A. Nuclear and mitochondrial apoptotic pathways of p53. FEBS Lett. 2001;493(2-3):65-9. PubMed PMID: 11286997.
145. Boehden GS, Akyuz N, Roemer K, Wiesmuller L. p53 mutated in the transactivation domain retains regulatory functions in homology-directed double-strand break repair. Oncogene. 2003;22(26):4111-7. PubMed PMID: 12821945.
146. Boehden GS, Restle A, Marschalek R, Stocking C, Wiesmuller L. Recombination at chromosomal sequences involved in leukaemogenic rearrangements is differentially regulated by p53. Carcinogenesis. 2004;25(8):1305-13. Epub 2004/01/27. doi: 10.1093/carcin/bgh092. PubMed PMID: 14742315.
147. Strano S, Fontemaggi G, Costanzo A, Rizzo MG, Monti O, Baccarini A, Del Sal G, Levrero M, Sacchi A, Oren M, Blandino G. Physical interaction with human tumor-derived p53 mutants inhibits p63 activities. The Journal of biological chemistry. 2002;277(21):18817-26. Epub 2002/03/15. doi: 10.1074/jbc.M201405200. PubMed PMID: 11893750.
148. Weisz L, Oren M, Rotter V. Transcription regulation by mutant p53. Oncogene. 2007;26(15):2202-11. PubMed PMID: 17401429.
149. Quante T, Otto B, Brazdova M, Kejnovska I, Deppert W, Tolstonog GV. Mutant p53 is a transcriptional co-factor that binds to G-rich regulatory regions of active genes and generates transcriptional plasticity. Cell cycle (Georgetown, Tex. 2012;11(17):3290-303. Epub 2012/08/17. doi: 10.4161/cc.21646. PubMed PMID: 22894900; PMCID: 3466528.
150. Vaughan CA, Deb SP, Deb S, Windle B. Preferred binding of gain-of-function mutant p53 to bidirectional promoters with coordinated binding of ETS1 and GABPA to multiple binding sites. Oncotarget. 2014;5(2):417-27. Epub 2014/02/01. PubMed PMID: 24481480; PMCID: 3964217.
151. Botcheva K, McCorkle SR. Cell context dependent p53 genome-wide binding patterns and enrichment at repeats. PLoS ONE. 2014;9(11):e113492. doi: 10.1371/journal.pone.0113492. PubMed PMID: 25415302; PMCID: 4240582.
152. Botcheva K, McCorkle SR, McCombie WR, Dunn JJ, Anderson CW. Distinct p53 genomic binding patterns in normal and cancer-derived human cells. Cell cycle (Georgetown, Tex. 2011;10(24):4237-49. doi: 10.4161/cc.10.24.18383. PubMed PMID: 22127205; PMCID: 3272258.
153. Tebaldi T, Zaccara S, Alessandrini F, Bisio A, Ciribilli Y, Inga A. Whole-genome cartography of p53 response elements ranked on transactivation potential. BMC genomics. 2015;16:464. doi: 10.1186/s12864-015-1643-9. PubMed PMID: 26081755; PMCID: 4470028.
154. Cordani N, Pozzi S, Martynova E, Fanoni D, Borrelli S, Alotto D, Castagnoli C, Berti E, Vigano MA, Mantovani R. Mutant p53 subverts p63 control over KLF4 expression in keratinocytes. Oncogene. 2011;30(8):922-32. Epub 2010/10/26. doi: 10.1038/onc.2010.474. PubMed PMID: 20972454.
155. Gaiddon C, Lokshin M, Ahn J, Zhang T, Prives C. A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol Cell Biol. 2001;21(5):1874-87. Epub 2001/03/10. doi: 10.1128/MCB.21.5.1874-1887.2001. PubMed PMID: 11238924; PMCID: 86759.
156. Li Y, Prives C. Are interactions with p63 and p73 involved in mutant p53 gain of oncogenic function? Oncogene. 2007;26(15):2220-5. PubMed PMID: 17401431.

157. Liu K, Ling S, Lin WC. TopBP1 mediates mutant p53 gain of function through NF-Y and p63/p73. Mol Cell Biol. 2011;31(22):4464-81. Epub 2011/09/21. doi: 10.1128/MCB.05574-11. PubMed PMID: 21930790; PMCID: 3209258.
158. Muller PA, Vousden KH. p53 mutations in cancer. Nature cell biology. 2013;15(1):2-8. Epub 2012/12/25. doi: 10.1038/ncb2641. PubMed PMID: 23263379.
159. Osada M, Park HL, Nagakawa Y, Yamashita K, Fomenkov A, Kim MS, Wu G, Nomoto S, Trink B, Sidransky D. Differential recognition of response elements determines target gene specificity for p53 and p63. Mol Cell Biol. 2005;25(14):6077-89. Epub 2005/07/01. doi: 10.1128/MCB.25.14.6077-6089.2005. PubMed PMID: 15988020; PMCID: 1168821.
160. Vaughan CA, Singh S, Windle B, Yeudall WA, Frum R, Grossman SR, Deb SP, Deb S. Gain-of-Function Activity of Mutant p53 in Lung Cancer through Up-Regulation of Receptor Protein Tyrosine Kinase Axl. Genes & cancer. 2012;3(7-8):491-502. Epub 2012/12/25. doi: 10.1177/1947601912462719. PubMed PMID: 23264849; PMCID: 3527987.
161. Neilsen PM, Noll JE, Suetani RJ, Schulz RB, Al-Ejeh F, Evdokiou A, Lane DP, Callen DF. Mutant p53 uses p63 as a molecular chaperone to alter gene expression and induce a pro-invasive secretome. Oncotarget. 2011;2(12):1203-17. Epub 2011/12/29. PubMed PMID: 22203497; PMCID: 3282078.
162. Subler MA, Martin DW, Deb S. Activation of the human immunodeficiency virus type 1 long terminal repeat by transforming mutants of human p53. J Virol. 1994;68(1):103-10. PubMed PMID: 8254719.
163. Deb S, Jackson CT, Subler MA, Martin DW. Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. J Virol. 1992;66(10):6164-70. PubMed PMID: 1356162.
164. Borellini F, Glazer RI. Induction of Sp1-p53 DNA-binding heterocomplexes during granulocyte/macrophage colony-stimulating factor-dependent proliferation in human erythroleukemia cell line TF-1. The Journal of biological chemistry. 1993;268(11):7923-8. PubMed PMID: 8463313.
165. Fogal V, Hsieh JK, Royer C, Zhong S, Lu X. Cell cycle-dependent nuclear retention of p53 by E2F1 requires phosphorylation of p53 at Ser315. Embo J. 2005;24(15):2768-82. Epub 2005/07/23. doi: 10.1038/sj.emboj.7600735. PubMed PMID: 16037820; PMCID: 1182237.
166. Sampath J, Sun D, Kidd VJ, Grenet J, Gandhi A, Shapiro LH, Wang Q, Zambetti GP, Schuetz JD. Mutant p53 cooperates with ETS and selectively up-regulates human MDR1 not MRP1. The Journal of biological chemistry. 2001;276(42):39359-67. PubMed PMID: 11483599.
167. Giebler HA, Lemasson I, Nyborg JK. p53 recruitment of CREB binding protein mediated through phosphorylated CREB: a novel pathway of tumor suppressor regulation. Mol Cell Biol. 2000;20(13):4849-58. PubMed PMID: 10848610.
168. Lee CW, Ferreon JC, Ferreon AC, Arai M, Wright PE. Graded enhancement of p53 binding to CREB-binding protein (CBP) by multisite phosphorylation. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(45):19290-5. Epub 2010/10/22. doi: 10.1073/pnas.1013078107. PubMed PMID: 20962272; PMCID: 2984141.
169. Ito M, Yuan CX, Malik S, Gu W, Fondell JD, Yamamura S, Fu ZY, Zhang X, Qin J, Roeder RG. Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. Molecular cell. 1999;3(3):361-70. Epub 1999/04/13. PubMed PMID: 10198638.

170. Meyer KD, Lin SC, Bernecky C, Gao Y, Taatjes DJ. p53 activates transcription by directing structural shifts in Mediator. Nature structural & molecular biology. 2010;17(6):753-60. Epub 2010/05/11. doi: 10.1038/nsmb.1816. PubMed PMID: 20453859; PMCID: 2932482.
171. Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell. 1996;87(5):953-9. Epub 1996/11/29. PubMed PMID: 8945521.
172. Bannister AJ, Kouzarides T. The CBP co-activator is a histone acetyltransferase. Nature. 1996;384(6610):641-3. Epub 1996/12/19. doi: 10.1038/384641a0. PubMed PMID: 8967953.
173. Weinstein IB. Cancer. Addiction to oncogenes--the Achilles heal of cancer. Science (New York, NY. 2002;297(5578):63-4. PubMed PMID: 12098689.
174. Vaughan CA, Frum R, Pearsall I, Singh S, Windle B, Yeudall A, Deb SP, Deb S. Allele specific gain-of-function activity of p53 mutants in lung cancer cells. Biochemical and biophysical research communications. 2012;428(1):6-10. Epub 2012/09/20. doi: 10.1016/j.bbrc.2012.09.029. PubMed PMID: 22989750.
175. Selivanova G, Wiman KG. Reactivation of mutant p53: molecular mechanisms and therapeutic potential. Oncogene. 2007;26(15):2243-54. PubMed PMID: 17401433.
176. Li D, Marchenko ND, Moll UM. SAHA shows preferential cytotoxicity in mutant p53 cancer cells by destabilizing mutant p53 through inhibition of the HDAC6-Hsp90 chaperone axis. Cell Death Differ. 2011;18(12):1904-13. Epub 2011/06/04. doi: 10.1038/cdd.2011.71. PubMed PMID: 21637290; PMCID: 3170683.
177. Yan W, Liu S, Xu E, Zhang J, Zhang Y, Chen X, Chen X. Histone deacetylase inhibitors suppress mutant p53 transcription via histone deacetylase 8. Oncogene. 2013;32(5):599-609. doi: 10.1038/onc.2012.81. PubMed PMID: 22391568; PMCID: 3371110.
178. Strano S, Dell'Orso S, Di Agostino S, Fontemaggi G, Sacchi A, Blandino G. Mutant p53: an oncogenic transcription factor. Oncogene. 2007;26(15):2212-9. PubMed PMID: 17401430.
179. Strano S, Blandino G. p73-mediated chemosensitivity: a preferential target of oncogenic mutant p53. Cell cycle (Georgetown, Tex. 2003;2(4):348-9. PubMed PMID: 12851488.
180. Irwin MS, Kondo K, Marin MC, Cheng LS, Hahn WC, Kaelin WG, Jr. Chemosensitivity linked to p73 function. Cancer cell. 2003;3(4):403-10. PubMed PMID: 12726865.
181. Bohuslav J, Chen LF, Kwon H, Mu Y, Greene WC. p53 induces NF-kappaB activation by an IkappaB kinase-independent mechanism involving phosphorylation of p65 by ribosomal S6 kinase 1. The Journal of biological chemistry. 2004;279(25):26115-25. PubMed PMID: 15073170.
182. Piva R, Belardo G, Santoro MG. NF-kappaB: a stress-regulated switch for cell survival. Antioxid Redox Signal. 2006;8(3-4):478-86. PubMed PMID: 16677091.
183. Xiao G, Fong A, Sun SC. Induction of p100 processing by NF-kappaB-inducing kinase involves docking IkappaB kinase alpha (IKKalpha) to p100 and IKKalpha-mediated phosphorylation. The Journal of biological chemistry. 2004;279(29):30099-105. PubMed PMID: 15140882.
184. Nadiminty N, Lou W, Lee SO, Lin X, Trump DL, Gao AC. Stat3 activation of NF-{kappa}B p100 processing involves CBP/p300-mediated acetylation. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(19):7264-9. PubMed PMID: 16651533.
185. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer. 2002;2(4):301-10. PubMed PMID: 12001991.

186. Pham CG, Bubici C, Zazzeroni F, Knabb JR, Papa S, Kuntzen C, Franzoso G. Upregulation of Twist-1 by NF-kappaB blocks cytotoxicity induced by chemotherapeutic drugs. Mol Cell Biol. 2007;27(11):3920-35. PubMed PMID: 17403902.
187. Tamatani T, Azuma M, Ashida Y, Motegi K, Takashima R, Harada K, Kawaguchi S, Sato M. Enhanced radiosensitization and chemosensitization in NF-kappaB-suppressed human oral cancer cells via the inhibition of gamma-irradiation- and 5-FU-induced production of IL-6 and IL-8. Int J Cancer. 2004;108(6):912-21. PubMed PMID: 14712497.
188. Liao G, Sun SC. Regulation of NF-kappaB2/p100 processing by its nuclear shuttling. Oncogene. 2003;22(31):4868-74. PubMed PMID: 12894228.
189. Duan J, Friedman J, Nottingham L, Chen Z, Ara G, Van Waes C. Nuclear factor-kappaB p65 small interfering RNA or proteasome inhibitor bortezomib sensitizes head and neck squamous cell carcinomas to classic histone deacetylase inhibitors and novel histone deacetylase inhibitor PXD101. Molecular cancer therapeutics. 2007;6(1):37-50. PubMed PMID: 17237265.
190. You M, Ku PT, Hrdlickova R, Bose HR, Jr. ch-IAP1, a member of the inhibitor-of-apoptosis protein family, is a mediator of the antiapoptotic activity of the v-Rel oncoprotein. Mol Cell Biol. 1997;17(12):7328-41. PubMed PMID: 9372964.
191. Catz SD, Johnson JL. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. Oncogene. 2001;20(50):7342-51. PubMed PMID: 11704864.
192. Wang Z, Zhang B, Yang L, Ding J, Ding HF. Constitutive production of NF-kappaB2 p52 is not tumorigenic but predisposes mice to inflammatory autoimmune disease by repressing Bim expression. The Journal of biological chemistry. 2008;283(16):10698-706. PubMed PMID: 18281283.
193. Deb SP. Function and dysfunction of the human oncoprotein MDM2. Front Biosci. 2002;7:d235-43. PubMed PMID: 11779693.
194. Sankala H, Vaughan C, Wang J, Deb S, Graves PR. Upregulation of the mitochondrial transport protein, Tim50, by mutant p53 contributes to cell growth and chemoresistance. Archives of biochemistry and biophysics.512(1):52-60. PubMed PMID: 21621504.
195. Wang H, Teh MT, Ji Y, Patel V, Firouzabadian S, Patel AA, Gutkind JS, Yeudall WA. EPS8 upregulates FOXM1 expression, enhancing cell growth and motility. Carcinogenesis.31(6):1132-41. PubMed PMID: 20351091.
196. Ikeda A, Sun X, Li Y, Zhang Y, Eckner R, Doi TS, Takahashi T, Obata Y, Yoshioka K, Yamamoto K. p300/CBP-dependent and -independent transcriptional interference between NF-kappaB RelA and p53. Biochemical and biophysical research communications. 2000;272(2):375-9. PubMed PMID: 10833421.
197. Miyazaki H, Patel V, Wang H, Edmunds RK, Gutkind JS, Yeudall WA. Down-regulation of CXCL5 inhibits squamous carcinogenesis. Cancer Res. 2006;66(8):4279-84. PubMed PMID: 16618752.
198. Ludes-Meyers JH, Subler MA, Shivakumar CV, Munoz RM, Jiang P, Bigger JE, Brown DR, Deb SP, Deb S. Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol Cell Biol. 1996;16(11):6009-19. PubMed PMID: 8887630.
199. Cadwell C, Zambetti GP. The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth. Gene. 2001;277(1-2):15-30. Epub 2001/10/17. PubMed PMID: 11602342.

200. Sigal A, Rotter V. Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome. Cancer Res. 2000;60(24):6788-93. PubMed PMID: 11156366.
201. Singer S, Ehemann V, Brauckhoff A, Keith M, Vreden S, Schirmacher P, Breuhahn K. Protumorigenic overexpression of stathmin/Op18 by gain-of-function mutation in p53 in human hepatocarcinogenesis. Hepatology. 2007;46(3):759-68. PubMed PMID: 17663418.
202. Weisz L, Damalas A, Liontos M, Karakaidos P, Fontemaggi G, Maor-Aloni R, Kalis M, Levrero M, Strano S, Gorgoulis VG, Rotter V, Blandino G, Oren M. Mutant p53 enhances nuclear factor kappaB activation by tumor necrosis factor alpha in cancer cells. Cancer Res. 2007;67(6):2396-401. PubMed PMID: 17363555.
203. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. Cell. 1990;61(2):203-12. PubMed PMID: 2158859.
204. Hafizi S, Dahlback B. Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily. The FEBS journal. 2006;273(23):5231-44. PubMed PMID: 17064312.
205. O'Bryan JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, Espinosa R, 3rd, Le Beau MM, Earp HS, Liu ET. axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. Mol Cell Biol. 1991;11(10):5016-31. PubMed PMID: 1656220.
206. Janssen JW, Schulz AS, Steenvoorden AC, Schmidberger M, Strehl S, Ambros PF, Bartram CR. A novel putative tyrosine kinase receptor with oncogenic potential. Oncogene. 1991;6(11):2113-20. PubMed PMID: 1834974.
207. Li Y, Ye X, Tan C, Hongo JA, Zha J, Liu J, Kallop D, Ludlam MJ, Pei L. Axl as a potential therapeutic target in cancer: role of Axl in tumor growth, metastasis and angiogenesis. Oncogene. 2009;28(39):3442-55. Epub 2009/07/28. doi: 10.1038/onc.2009.212. PubMed PMID: 19633687.
208. Sawabu T, Seno H, Kawashima T, Fukuda A, Uenoyama Y, Kawada M, Kanda N, Sekikawa A, Fukui H, Yanagita M, Yoshibayashi H, Satoh S, Sakai Y, Nakano T, Chiba T. Growth arrest-specific gene 6 and Axl signaling enhances gastric cancer cell survival via Akt pathway. Mol Carcinog. 2007;46(2):155-64. PubMed PMID: 17186543.
209. Rochlitz C, Lohri A, Bacchi M, Schmidt M, Nagel S, Fopp M, Fey MF, Herrmann R, Neubauer A. Axl expression is associated with adverse prognosis and with expression of Bcl-2 and CD34 in de novo acute myeloid leukemia (AML): results from a multicenter trial of the Swiss Group for Clinical Cancer Research (SAKK). Leukemia. 1999;13(9):1352-8. PubMed PMID: 10482985.
210. Wimmel A, Glitz D, Kraus A, Roeder J, Schuermann M. Axl receptor tyrosine kinase expression in human lung cancer cell lines correlates with cellular adhesion. Eur J Cancer. 2001;37(17):2264-74. PubMed PMID: 11677117.
211. Shieh YS, Lai CY, Kao YR, Shiah SG, Chu YW, Lee HS, Wu CW. Expression of axl in lung adenocarcinoma and correlation with tumor progression. Neoplasia. 2005;7(12):1058-64. PubMed PMID: 16354588.
212. Meric F, Lee WP, Sahin A, Zhang H, Kung HJ, Hung MC. Expression profile of tyrosine kinases in breast cancer. Clin Cancer Res. 2002;8(2):361-7. PubMed PMID: 11839650.
213. Wu CW, Li AF, Chi CW, Lai CH, Huang CL, Lo SS, Lui WY, Lin WC. Clinical significance of AXL kinase family in gastric cancer. Anticancer Res. 2002;22(2B):1071-8. PubMed PMID: 12168903.

214. Holland SJ, Powell MJ, Franci C, Chan EW, Friera AM, Atchison RE, McLaughlin J, Swift SE, Pali ES, Yam G, Wong S, Lasaga J, Shen MR, Yu S, Xu W, Hitoshi Y, Bogenberger J, Nor JE, Payan DG, Lorens JB. Multiple roles for the receptor tyrosine kinase axl in tumor formation. Cancer Res. 2005;65(20):9294-303. PubMed PMID: 16230391.
215. Vajkoczy P, Knyazev P, Kunkel A, Capelle HH, Behrndt S, von Tengg-Kobligk H, Kiessling F, Eichelsbacher U, Essig M, Read TA, Erber R, Ullrich A. Dominant-negative inhibition of the Axl receptor tyrosine kinase suppresses brain tumor cell growth and invasion and prolongs survival. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(15):5799-804. PubMed PMID: 16585512.
216. Mitsudomi T, Steinberg SM, Nau MM, Carbone D, D'Amico D, Bodner S, Oie HK, Linnoila RI, Mulshine JL, Minna JD, et al. p53 gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of ras mutations and clinical features. Oncogene. 1992;7(1):171-80. PubMed PMID: 1311061.
217. Sjogren S, Inganas M, Norberg T, Lindgren A, Nordgren H, Holmberg L, Bergh J. The p53 gene in breast cancer: prognostic value of complementary DNA sequencing versus immunohistochemistry. J Natl Cancer Inst. 1996;88(3-4):173-82. PubMed PMID: 8632491.
218. Vaughan CA, Singh S, Windle B, Sankala HM, Graves PR, Andrew Yeudall W, Deb SP, Deb S. p53 mutants induce transcription of NF-kappaB2 in H1299 cells through CBP and STAT binding on the NF-kappaB2 promoter and gain of function activity. Arch Biochem Biophys.518(1):79-88. PubMed PMID: 22198284.
219. Wang D, Baldwin AS, Jr. Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. The Journal of biological chemistry. 1998;273(45):29411-6. PubMed PMID: 9792644.
220. Rivlin N, Brosh R, Oren M, Rotter V. Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. Genes Cancer.2(4):466-74. PubMed PMID: 21779514.
221. Chung BI, Malkowicz SB, Nguyen TB, Libertino JA, McGarvey TW. Expression of the proto-oncogene Axl in renal cell carcinoma. DNA Cell Biol. 2003;22(8):533-40. PubMed PMID: 14565870.
222. Ou WB, Corson JM, Flynn DL, Lu WP, Wise SC, Bueno R, Sugarbaker DJ, Fletcher JA. AXL regulates mesothelioma proliferation and invasiveness. Oncogene.30(14):1643-52. PubMed PMID: 21132014.
223. Nielsen-Preiss SM, Allen MP, Xu M, Linseman DA, Pawlowski JE, Bouchard RJ, Varnum BC, Heidenreich KA, Wierman ME. Adhesion-related kinase induction of migration requires phosphatidylinositol-3-kinase and ras stimulation of rac activity in immortalized gonadotropin-releasing hormone neuronal cells. Endocrinology. 2007;148(6):2806-14. PubMed PMID: 17332061.
224. Sainaghi PP, Castello L, Bergamasco L, Galletti M, Bellosta P, Avanzi GC. Gas6 induces proliferation in prostate carcinoma cell lines expressing the Axl receptor. Journal of cellular physiology. 2005;204(1):36-44. PubMed PMID: 15605394.
225. Scian MJ, Frum R, Deb SP, Deb S. Transactivation and transrepression studies with p53. Methods in molecular biology (Clifton, NJ. 2003;234:93-110. PubMed PMID: 12824527.
226. Muller M, Schleithoff ES, Stremmel W, Melino G, Krammer PH, Schilling T. One, two, three--p53, p63, p73 and chemosensitivity. Drug Resist Updat. 2006;9(6):288-306. PubMed PMID: 17287142.

227. Martynova E, Pozzi S, Basile V, Dolfini D, Zambelli F, Imbriano C, Pavesi G, Mantovani R. Gain-of-function p53 mutants have widespread genomic locations partially overlapping with p63. Oncotarget. 2012;3(2):132-43. Epub 2012/03/01. PubMed PMID: 22361592; PMCID: 3326644.
228. Deb SP, Munoz RM, Brown DR, Subler MA, Deb S. Wild-type human p53 activates the human epidermal growth factor receptor promoter. Oncogene. 1994;9(5):1341-9. PubMed PMID: 8152794.
229. Oda K, Matsuoka Y, Funahashi A, Kitano H. A comprehensive pathway map of epidermal growth factor receptor signaling. Molecular systems biology. 2005;1:2005 0010. Epub 2006/05/27. doi: 10.1038/msb4100014. PubMed PMID: 16729045; PMCID: 1681468.
230. Bethune G, Bethune D, Ridgway N, Xu Z. Epidermal growth factor receptor (EGFR) in lung cancer: an overview and update. Journal of thoracic disease. 2010;2(1):48-51. Epub 2010/03/01. PubMed PMID: 22263017; PMCID: 3256436.
231. Frum R, Ramamoorthy M, Mohanraj L, Deb S, Deb SP. MDM2 controls the timely expression of cyclin A to regulate the cell cycle. Mol Cancer Res. 2009;7(8):1253-67. PubMed PMID: 19671680.
232. Vaughan CA, Singh S, Windle B, Sankala HM, Graves PR, Andrew Yeudall W, Deb SP, Deb S. p53 mutants induce transcription of NF-kappaB2 in H1299 cells through CBP and STAT binding on the NF-kappaB2 promoter and gain of function activity. Archives of biochemistry and biophysics. 2012;518(1):79-88. Epub 2011/12/27. doi: 10.1016/j.abb.2011.12.006. PubMed PMID: 22198284; PMCID: 3272778.
233. Ma H, Nguyen C, Lee KS, Kahn M. Differential roles for the coactivators CBP and p300 on TCF/beta-catenin-mediated survivin gene expression. Oncogene. 2005;24(22):3619-31. doi: 10.1038/sj.onc.1208433. PubMed PMID: 15782138.
234. Chen G, Kronenberger P, Teugels E, Umelo IA, De Greve J. Targeting the epidermal growth factor receptor in non-small cell lung cancer cells: the effect of combining RNA interference with tyrosine kinase inhibitors or cetuximab. BMC medicine. 2012;10:28. Epub 2012/03/23. doi: 10.1186/1741-7015-10-28. PubMed PMID: 22436374; PMCID: 3334713.
235. Ni W, Zhan Y, He H, Maynard E, Balschi JA, Oettgen P. Ets-1 is a critical transcriptional regulator of reactive oxygen species and p47(phox) gene expression in response to angiotensin II. Circulation research. 2007;101(10):985-94. doi: 10.1161/CIRCRESAHA.107.152439. PubMed PMID: 17872466.
236. Barbieri CE, Tang LJ, Brown KA, Pietenpol JA. Loss of p63 leads to increased cell migration and up-regulation of genes involved in invasion and metastasis. Cancer Res. 2006;66(15):7589-97. doi: 10.1158/0008-5472.CAN-06-2020. PubMed PMID: 16885358.
237. Jungert K, Buck A, von Wichert G, Adler G, Konig A, Buchholz M, Gress TM, Ellenrieder V. Sp1 is required for transforming growth factor-beta-induced mesenchymal transition and migration in pancreatic cancer cells. Cancer Res. 2007;67(4):1563-70. doi: 10.1158/0008-5472.CAN-06-1670. PubMed PMID: 17308095.
238. Vaughan C, Mohanraj L, Singh S, Dumur CI, Ramamoorthy M, Garrett CT, Windle B, Yeudall WA, Deb S, Deb SP. Human Oncoprotein MDM2 Up-regulates Expression of NF-kappaB2 Precursor p100 Conferring a Survival Advantage to Lung Cells. Genes & cancer. 2011;2(10):943-55. Epub 2012/06/16. doi: 10.1177/1947601911436008. PubMed PMID: 22701761; PMCID: 3374626.

239. Furlan-Magaril M, Rincon-Arano H, Recillas-Targa F. Sequential chromatin immunoprecipitation protocol: ChIP-reChIP. Methods Mol Biol. 2009;543:253-66. doi: 10.1007/978-1-60327-015-1_17. PubMed PMID: 19378171.
240. Singh S, Ramamoorthy M, Vaughan C, Yeudall WA, Deb S, Palit Deb S. Human oncoprotein MDM2 activates the Akt signaling pathway through an interaction with the repressor element-1 silencing transcription factor conferring a survival advantage to cancer cells. Cell Death Differ. 2013;20(4):558-66. Epub 2012/12/15. doi: 10.1038/cdd.2012.153. PubMed PMID: 23238568; PMCID: 3595481.
241. Brown DR, Deb S, Munoz RM, Subler MA, Deb SP. The tumor suppressor p53 and the oncoprotein simian virus 40 T antigen bind to overlapping domains on the MDM2 protein. Mol Cell Biol. 1993;13(11):6849-57. PubMed PMID: 8413278.
242. Vaughan CSPDSDaBW. Preferred binding of gain-of-function mutant p53 to bidirectional promoters with coordinated binding of ETS1 and GABPA to multiple binding sites. Oncotarget. 2014.
243. Leng P, Brown DR, Shivakumar CV, Deb S, Deb SP. N-terminal 130 amino acids of MDM2 are sufficient to inhibit p53-mediated transcriptional activation. Oncogene. 1995;10(7):1275-82. PubMed PMID: 7731677.
244. Freed-Pastor WA, Mizuno H, Zhao X, Langerod A, Moon SH, Rodriguez-Barrueco R, Barsotti A, Chicas A, Li W, Polotskaia A, Bissell MJ, Osborne TF, Tian B, Lowe SW, Silva JM, Borresen-Dale AL, Levine AJ, Bargonetti J, Prives C. Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. Cell. 2012;148(1-2):244-58. Epub 2012/01/24. doi: 10.1016/j.cell.2011.12.017. PubMed PMID: 22265415; PMCID: 3511889.
245. Koike M, Fujita F, Komori K, Katoh F, Sugimoto T, Sakamoto Y, Matsuda M, Fujita M. Dependence of chemotherapy response on p53 mutation status in a panel of human cancer lines maintained in nude mice. Cancer Sci. 2004;95(6):541-6. PubMed PMID: 15182437.
246. Livengood JA, Scoggin KE, Van Orden K, McBryant SJ, Edayathumangalam RS, Laybourn PJ, Nyborg JK. p53 Transcriptional activity is mediated through the SRC1-interacting domain of CBP/p300. The Journal of biological chemistry. 2002;277(11):9054-61. PubMed PMID: 11782467.
247. Wojciak JM, Martinez-Yamout MA, Dyson HJ, Wright PE. Structural basis for recruitment of CBP/p300 coactivators by STAT1 and STAT2 transactivation domains. Embo J. 2009;28(7):948-58. PubMed PMID: 19214187.
248. Paulson M, Pisharody S, Pan L, Guadagno S, Mui AL, Levy DE. Stat protein transactivation domains recruit p300/CBP through widely divergent sequences. The Journal of biological chemistry. 1999;274(36):25343-9. PubMed PMID: 10464260.
249. Icardi L, De Bosscher K, Tavernier J. The HAT/HDAC interplay: multilevel control of STAT signaling. Cytokine & growth factor reviews. 2012;23(6):283-91. doi: 10.1016/j.cytogfr.2012.08.002. PubMed PMID: 22989617.
250. Wang H, Larris B, Peiris TH, Zhang L, Le Lay J, Gao Y, Greenbaum LE. C/EBPbeta activates E2F-regulated genes in vivo via recruitment of the coactivator CREB-binding protein/P300. The Journal of biological chemistry. 2007;282(34):24679-88. PubMed PMID: 17599912.
251. Freed-Pastor WA, Prives C. Mutant p53: one name, many proteins. Genes & development. 2012;26(12):1268-86. Epub 2012/06/21. doi: 10.1101/gad.190678.112. PubMed PMID: 22713868; PMCID: 3387655.

252. Do PM, Varanasi L, Fan S, Li C, Kubacka I, Newman V, Chauhan K, Daniels SR, Boccetta M, Garrett MR, Li R, Martinez LA. Mutant p53 cooperates with ETS2 to promote etoposide resistance. Genes Dev. 2012;26(8):830-45. Epub 2012/04/18. doi: 10.1101/gad.181685.111. PubMed PMID: 22508727.
253. Foulds CE, Nelson ML, Blaszczak AG, Graves BJ. Ras/mitogen-activated protein kinase signaling activates Ets-1 and Ets-2 by CBP/p300 recruitment. Mol Cell Biol. 2004;24(24):10954-64. Epub 2004/12/02. doi: 10.1128/MCB.24.24.10954-10964.2004. PubMed PMID: 15572696; PMCID: 533975.
254. Yang C, Shapiro LH, Rivera M, Kumar A, Brindle PK. A role for CREB binding protein and p300 transcriptional coactivators in Ets-1 transactivation functions. Mol Cell Biol. 1998;18(4):2218-29. Epub 1998/04/07. PubMed PMID: 9528793; PMCID: 121466.
255. Nelson ML, Kang HS, Lee GM, Blaszczak AG, Lau DK, McIntosh LP, Graves BJ. Ras signaling requires dynamic properties of Ets1 for phosphorylation-enhanced binding to coactivator CBP. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(22):10026-31. Epub 2010/06/11. doi: 10.1073/pnas.0915137107. PubMed PMID: 20534573; PMCID: 2890480.
256. Huang W, Zhao S, Ammanamanchi S, Brattain M, Venkatasubbarao K, Freeman JW. Trichostatin A induces transforming growth factor beta type II receptor promoter activity and acetylation of Sp1 by recruitment of PCAF/p300 to a Sp1.NF-Y complex. The Journal of biological chemistry. 2005;280(11):10047-54. Epub 2005/01/14. doi: 10.1074/jbc.M408680200. PubMed PMID: 15647279.
257. Das C, Lucia MS, Hansen KC, Tyler JK. CBP/p300-mediated acetylation of histone H3 on lysine 56. Nature. 2009;459(7243):113-7. Epub 2009/03/10. doi: 10.1038/nature07861. PubMed PMID: 19270680; PMCID: 2756583.
258. Henry RA, Kuo YM, Andrews AJ. Differences in specificity and selectivity between CBP and p300 acetylation of histone H3 and H3/H4. Biochemistry. 2013;52(34):5746-59. Epub 2013/07/19. doi: 10.1021/bi400684q. PubMed PMID: 23862699; PMCID: 3756530.

Vita

Catherine Vaughan was born September 1, 1986 in Virginia Beach, VA. She graduated from First Colonial High School in 2004. She graduated Cum Laude with her Bachelor of Science in Forensic Science with a concentration in Forensic Biology and a minor in Chemistry from Virginia Commonwealth University in 2008. The last semester of her undergraduate course work she undertook an independent study in the laboratory of Dr. Sumitra Deb in the hope of obtaining laboratory experience. After graduating from VCU she continued working in Dr. Deb's lab as the lab technician before being accepted into the Integrative Life Science Ph.D. program at VCU in 2009.