Mutant p53 cooperates with the SWI/SNF chromatin remodeling complex to mediate VEGFR2 expression in breast cancer cells

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Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2015

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ABSTRACT

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Mutant p53 impacts the expression of numerous genes at the level of transcription to mediate oncogenesis. To investigate how mutant p53 impacts transcription, we studied how mutant p53 regulates vascular endothelial growth factor receptor 2 (VEGFR2), one of its strongest target genes that we identified through global gene expression profiling in mutant p53-expressing MDA-468 breast cancer cells. VEGFR2, the primary functional VEGF receptor and clinical target of bevacizumab, mediates endothelial cell neovascularization by promoting increased cellular proliferation, migration, and pro-survival signaling. In breast tumors, VEGFR2 is often aberrantly expressed on the breast tumor epithelia, which correlates with worse overall survival.

We identify VEGFR2 as a mutant p53 transcriptional target in multiple breast cancer cell lines. Mutant p53-mediated upregulation of VEGFR2 mediates mutant 53 gain of function including increased cellular growth and migration. In humans, breast tumors with *TP53* hotspot mutants have elevated *VEGFR2* levels compared to tumors with loss of function mutations. The same class of tumors has significantly upregulated *HIF1A* and *VEGFA* compared to *TP53* wild-type tumors, indicating that mutant p53-containing breast tumors express a neoangiogenic gene signature that may intensify VEGFR2 autocrine signaling. A clinical trial

suggests that *TP53* mutated breast tumors may specifically respond to anti-VEGF therapy, while *TP53* wild-type tumors may not respond. We suggest that mutant p53-containing breast tumors may be distinctively vulnerable to anti-VEGF therapies.

We investigated how mutant p53 impacts transcription of *VEGFR2* using multiple techniques including scanning ChIP, micrococcal nuclease-PCR, and *in vivo* DNase I footprinting by ligation mediated PCR. Mutant p53 was found to bind near the *VEGFR2* transcriptional start site, causing the promoter to adopt a transcriptionally active conformation. Using SILAC mass spectrometry, we identified subunits of the SWI/SNF chromatin remodeling complex as mutant p53 interactors. Importantly, re-ChIP and immunodepletion ChIP demonstrate that mutant p53 and SWI/SNF co-occupy the VEGFR2 promoter. Depletion of multiple SWI/SNF subunits reduced *VEGFR2* RNA expression, and SWI/SNF is required for maximal mutant p53 promoter occupancy.

Using RNA sequencing, we report that approximately half of all mutant p53 gene alteration requires the SWI/SNF complex. We surmise that mutant p53 impacts transcription of *VEGFR2* as well as myriad other target genes by promoter remodeling through interaction with the SWI/SNF chromatin remodeling complex. Therefore, not only might mutant p53 expressing tumors be uniquely susceptible to anti-VEGF therapies, but restoration of SWI/SNF tumor suppressor function by targeting mutant p53 may have therapeutic potential. Mutant p53 interaction with the SWI/SNF complex may explain how mutant p53 modulates the expression of such a diverse set of genes.

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ACKNOWLEDGEMENTS

This work has been enabled by many scientific mentors that have brought me to this point. Foremost, God has given me the mind, energy, determination, and motivation to pursue work in this field. My parents, grandparents, and childhood friends have encouraged my creativity and independence from the earliest stages. Notably, I would not be in science if not for my AP Biology teacher in high school, Dr. Kim Nichols, who sparked in me an interest in molecular biology. For this specific reason, I enrolled as a Biochemistry and Molecular Biology major in college, for which a requirement was to work on an independent research project. Dr. Mark Compton took me under his wing and taught me my first insights as a scientist. Later, Dr. Michael Terns and Dr. Rebecca Terns mentored my investigations into the CRISPR-Cas system. It was on this project that I developed my confidence as a junior scientist and determined that I would pursue a career in research. It was also the Terns' who suggested that I consider Dr. Carol Prives as a scientific mentor for my Ph.D. research.

Dr. Carol Prives has been the most exceptional mentor. As a scientist and as an individual, Carol exhibits the highest levels of scholarship and citizenship. Without her exceedingly high standards for scientific evidence and rigor, this work would not be what it is. Furthermore, I would not be nearly the scientist that I am. Carol's exceeding breadth of knowledge has pushed me to seek knowledge outside of my immediate field and make connections with this project that otherwise would not have been possible. Most importantly, Carol has allowed me

the freedom to pursue hypotheses (many of which are only partly correct or incorrect) that has furthered my passion for science and my skills as a researcher. This intellectual freedom has been instrumental in enjoying the daily research tasks during the pursuit of this work.

The Prives laboratory is a unique and wonderful place to be a scientist. Ella Freulich is instrumental in setting the tone for how the lab functions and interacts with each other. Ella is a beam of sunshine every single day and a pleasure to have spent the last four years with. Will Freed-Pastor has had an outsize influence on this work has he taught me many of the skills utilized in this work. Oleg Laptenko, Sung-Hwan Moon, Yan Zhu, Masha Poyurovsky, Tony Barsotti, Kausik Regunath, Susumu Rokudai, Stephan Singer, and Vitalay Fomin, among others in the laboratory, have taught me essential concepts or technical skills. I thank Paul Neilsen for selflessly engaging me during a scientific conference.

I thank my wife Cristina Basulescu for endless support during this Ph.D. project, which has definitely shaped our marriage relationship and how we organize our work and home lives. It has been especially difficult to have pursued two years of this project in a long-distance relationship, and words cannot express the gratitude I have for her in enduring. I additionally thank my parents and parents-in-law and extended family for being without me for so much time, and I hope that I can repay each of them for my absence. The manifestation of the Holy Spirit in my heart and the physical presence of Jesus have brought me through each failure that I otherwise would not have recovered.

DEDICATION

This work is dedicated to all those who suffer from disease.

Though disease temporary as we are but dust,

disease affects all facets of our life in

ways that are overwhelmingly negative.

Furthermore, this work is dedicated to those who seek to

understand this physical world.

Chapter 1

INTRODUCTION

TP53 is the most frequently mutated gene found in human cancers (Olivier et al., 2010). Wild-type p53 is a sequence-specific transcription factor that when activated by various stresses such as DNA damage, oncogenic signaling or nutrient depletion, promotes cellular outcomes such as cell arrest, cell death, senescence, metabolic changes and others, depending on the extent and context of the stress (Vousden and Prives, 2009). In human cancer, p53 primarily sustains missense mutations in its conserved DNA binding domain. The small number of residues (~5-6) within this region that are mutated with extraordinarily high frequency are termed hotspot mutations. These mutations can be loosely divided into two categories, the contact mutants (e.g. R273H), which remain well folded but whose mutated residues fail to make specific contact with elements within the DNA binding site and conformational mutants (e.g. R175H) that are partly unfolded leading to loss of zinc coordination and general DNA binding. Evidence from sources as varied as human epidemiology studies, mouse models and cell-based experiments has shown that these hotspot missense mutant forms of p53, which often accumulate to high levels in the cells they inhabit, can acquire neomorphic properties such as increased metastases in mice and increased motility and invasive characteristics in cultured cells (Brosh and Rotter, 2009; Muller and Vousden, 2014). In Li-Fraumeni, patients missense mutation was reported to lead to earlier tumor onset than other forms of p53 loss (Bougeard et al., 2008). p53 hotspot mutant proteins have been reported to associate with chromatin and alter a cell's transcriptional profile, leading to oncogenic cellular changes (Cooks et al., 2013; Di

Agostino et al., 2006; Do et al., 2012; Freed-Pastor et al., 2012; Stambolsky et al., 2010).

p53 Background and Discovery

p53 was initially identified as a cellular oncogene that cooperates with simian virus 40 or H-Ras to transform cells (DeLeo et al., 1979; Eliyahu et al., 1984; Hinds et al., 1989; Lane and Crawford, 1979; Linzer and Levine, 1979; Parada et al., 1984). Histology supported the hypothesis that p53 is an oncogene, as high levels of p53 are observed in transformed cells while low levels of p53 are observed in normal tissues (Bartek et al., 1990a; Cattoretti et al., 1988; Rotter, 1983). However, p53 was soon identified as a tumor suppressor. Consistent with a tumor suppressor, the 17p13.1 chromosomal region containing the *TP53* gene is frequently lost in tumors that contain point mutations in the other allele (Baker et al., 1989; Baker et al., 1990; Nigro et al., 1989). Mutant p53 is a dominant-negative inhibitor of wild-type p53 (Kern et al., 1992), and wild-type p53 confers G1 checkpoint control to cells lacking functional p53 (Yin et al., 1992) in a dose-dependent manner (Chen et al., 1996), which is mediated by p21 (Waldman et al., 1995). Mechanistically, wild-type p53 binds DNA in a sequence-specific manner to mediate its functions while mutant p53 fails to bind to a consensus sequence (Bargonetti et al., 1991; Bargonetti et al., 1993; el-Deiry et al., 1992; Funk et al., 1992; Kern et al., 1991b). Critically, p53 null mice are more tumor-prone than mice with wild-type p53, establishing p53 as a tumor suppressor (Donehower et al., 1992). These data provided a framework to understand Li-Fraumeni syndrome, where germline mutations in *TP53* predispose affected individuals to breast cancer, sarcomas,

lymphomas, and other neoplasms (Malkin et al., 1990; Srivastava et al., 1990; Varley et al., 1997; Wong et al., 2006).

TP53 Mutations in Human Cancers

TP53 mutations are observed in nearly every type of human cancer with a wide range of frequencies ranging from approximately 1% in papillary thyroid cancer to 95% in serous ovarian cancer (Figure 1.1). *TP53* is interesting for a tumor suppressor in that does not frequently sustain deletions (Figure 1.1). Instead, *TP53* primarily sustains various other types of mutations, the most frequent of which are missense mutations in the p53 DNA binding domain (see Figure 1.2 for a specific example with breast cancer). Different datasets may have varying frequencies for *TP53* mutation within the same cancer type. In breast cancer, published *TP53* mutation rates range from 27.2% for breast cancers unstratified by subtype (Banerji et al., 2012) to 53.8% (35/65 samples) in triple negative breast cancer (Shah et al., 2012). The most comprehensive datasets report between 31.7% and 38.2% for two breast cancer TCGA datasets (Figure 1.1; Network, 2012). *TP53* mutation is an independent prognostic indicator (correlating with worse prognosis) in breast cancer (Olivier et al., 2006; Petitjean et al., 2007a), lung cancer (Ahrendt et al., 2003), and with certain hotspot mutations in colon cancer (Samowitz et al., 2002). *TP53* mutations occur at multiple stages in the progression of a tumor, and the stage in which *TP53* mutation occurs may affect the malignancy of the tumor (reviewed in Rivlin et al., 2011).

Essential Selective Advantages of TP53 Mutations in Tumors

TP53 is most frequently mutated in its DNA binding domain as mutations in this domain preclude interaction of p53 with its consensus DNA binding sequence (Kern et al., 1991a). The p53 consensus sequence is a feature in the promoter of canonical p53 target genes such as *MDM2 (Barak et al., 1993; Juven et al., 1993), NOXA (Oda et al., 2000), PUMA (Nakano and Vousden, 2001)*, and *p21* (*CDKN1A*)(el-Deiry et al., 1993). Abrogation of DNA binding by mutation in the p53 DNA binding domain (Kern et al., 1991a) dysregulates the induction of p53-mediated cellular arrest (through p21) and apoptosis (through PUMA and NOXA), and leads to the accumulation of elevated levels of the mutant form of p53 in part due to the impairment of inducing p53's primary negative regulator, the E3 ubiquitin ligase MDM2 (reviewed in Freed-Pastor and Prives, 2012) . Loss of p53 function enables a cell to evade cell arrest mechanisms that would allow adequate repair of damaged DNA and allow a cell to evade apoptosis, which would have eliminated a cell that sustained DNA damage. Hence, abrogation in p53 function is a critical step in oncogenesis (reviewed in Vousden and Prives, 2009). Indeed, even when *TP53* is not directly affected, its negative regulators MDM2 and MDMX are often overexpressed (reviewed in Toledo and Wahl, 2006).

It should be considered that deletion of part or all of the chromosome 17p arm is an understudied mechanism of p53 loss of function. Chromosome 17p deletions (the *TP53* gene resides at chromosome 17p13.1 between base pairs chr17: 7,571,720 bp to 7,590,868 bp of the hg19 assembly) are a frequent occurrence in a variety of cancers, including in around 75% of colorectal cancers (Vogelstein et al., 1988), which may account for a significant additional mechanism of loss of *TP53* from a cell. A cell with a

17p deletion is not necessarily annotated to have a mutation in *TP53,* for instance. In addition to colorectal cancer, 17p loss has been reported in sarcomas (Mulligan et al., 1990), primitive neuroectodermal tumors of childhood (Raffel et al., 1990), bladder cancer (Tsai et al., 1990), breast cancer (Mackay et al., 1988), and lung cancer (Yokota et al., 1987). While 17p loss of the homologous chromosome is known to occur following mutation in *TP53* (Baker et al., 1989; Baker et al., 1990; Nigro et al., 1989), it should be appreciated that chromosome 17p alteration will invariably impact *TP53* status.

TP53 Hotspot Mutations

The spectrum of *TP53* mutations is evidence for an oncogenic role of *TP53* mutations in promoting tumorigenesis. Six codons have been designated as ‘hotspots’ due to increased selection for these mutants: codons 175, 196, 213, 248, 273, and 282 (Hollstein et al., 1991). Each of these codons occurs within the DNA binding domain of p53, the location of the overwhelming majority of p53 mutations, with no significant selection for mutations outside this domain (Petitjean et al., 2007a; Soussi et al., 2005). p53 hotspot mutants are classified as either a DNA contact mutant (eg: codon R248, codon R273) or a conformational mutant (eg: codon R175, codon G245) based on the mechanism of alteration of the DNA binding domain, with conformational mutants being more structurally abnormal, and each class leading to abrogation of sequence-specific DNA binding (Bartek et al., 1990b; Cho et al., 1994; Gannon et al., 1990; Legros et al., 1994). p53 hotspot mutations will be discussed in more detail in later sections.

p53 in Breast Cancer

As noted above, *TP53* mutation in breast cancer occurs with an average frequency among human cancers, with reported mutation rates ranging from between 27.2% for breast cancers unstratified by subtype (Banerji et al., 2012) to 53.8% (35/65 samples) in triple negative breast cancer (Shah et al., 2012), with larger studies reporting *TP53* mutation rates of around 32-38% (See Figure 1.1; Network, 2012). Mutations of p53 in breast cancer result in a point mutation in approximately 80% of cases, with a lower than expected ratio of nonsense, insertion, or deletion mutations (see Figure 1.2; Petitjean et al., 2007b). In breast cancer, somatic mutations occur in similar codon positions as in other tumors with a key difference being an increased frequency of mutations in residue Y220 (reviewed in Walerych et al., 2012). Another difference in the *TP53* mutation spectrum in breast cancer is that codon R213, which is often a missense mutations in other cancers, frequently results in a nonsense mutation in breast cancer. For instance, in the TCGA Provisional dataset (accessed at www.cbioportal.org), mutation in R213 results in a nonsense mutation in 7 out of 7 instances (also compare Figure 1.2 vs. Figure 1.3 for R213 from the IARC dataset). The R213>stop codon mutation has been previously reported in ovarian cancer (Schuyer et al., 1998), so there may be a similarity in the mechanism of R213 nonsense mutations between breast and ovarian cancer.

Overall, R175, Y220, G245, R248, and R273 are the most frequently mutated residues in breast cancer that cause missense mutations and therefore should be considered hotspot mutations (see Figure 1.3; Feki and Irminger-Finger, 2004; Walerych et al., 2012). These genetic alterations are especially important to understand

in breast cancer, where it is clear that *TP53* mutation significantly reduces overall survival (Elledge et al., 1993; Langerod et al., 2007). In breast cancer, specific *TP53* missense mutations are associated with worse outcomes (Alsner et al., 2008; Olivier et al., 2006; Petitjean et al., 2007a), and p53 missense mutants predicted to affect DNA binding confer worse survival than p53 null mutations (Alsner et al., 2008).

Considerations for Mutant p53 Therapeutics in Breast Cancer

p53 Y220C (Basse et al., 2010), in addition to the more well documented p53 R175H mutation (Brown et al., 2011; Bykov et al., 2002; Goldstein et al., 2011; Muller and Vousden, 2014; Olivier et al., 2009), have been prime targets for mutant p53 pharmacologics to 're-fold' conformational mutants of p53 into a wild-type p53 conformation that may be restored in tumor suppressive activities (reviewed in Muller and Vousden, 2014). The Y220C mutation (the most frequent mutation in this residue) is thermodynamically unstable (Bullock et al., 2000) and has been reported to retain some wild-type p53 activities such as binding to p21 promoter sequences and transcactivating a p21-containing promoter sequence at sub-physiological temperatures (Di Como and Prives, 1998). However, Y220C failed to transactivate the p21 reporter construct at physiological temperatures, suggesting that the Y220C mutant may be inactive in most tumor environments (Di Como and Prives, 1998). It is unlikely that DNA-contact mutants (mutations in R248, R273, R280) are candidates for reactivation of wild-type p53 function because the amino acids that interact with the p53 response element are altered. For the p53 contact mutants, other approaches (such as disrupting

mutant p53 interaction with a core transcriptional coactivator) may be feasible in the future.

p53 Domain Structure

This section describes the polypeptide structure of p53 with an emphasis on how the tertiary structure of p53 may be manipulated to investigate mutant p53 function. Structurally, p53 is composed of a bipartite N-terminal transactivation domain, a proline-rich domain, a central DNA-binding domain, an oligomerization domain, and a C-terminal regulatory domain (Figure 1.3; Joerger and Fersht, 2008). p53 is a dimer of dimers, composed of a dimer (formed with interactions within the DNA binding domain of the L2 and L3 loops) that homo-dimerizes through hydrophobic interactions between leucines 344 and 348 in the oligomerization domain to form the functional p53 tetramer (Jeffrey et al., 1995; Joerger and Fersht, 2008). The p53 protein is thus approximately 200 kDa as a tetramer, which allows for four distinct binding sites for binding partners within each tetramer.

The N-terminal transactivation domain is subdivided into two subdomains, TAD1 within the first 40 amino acids and TAD2 within amino acids 41-61 (Chang et al., 1995; Walker and Levine, 1996). TAD1 functionally requires residues 22 (leucine) and 23 (tryptophan) and TAD2 functionally requires residues 53 (tryptophan) and 54 (phenylalanine). These residues can be mutated to polar amino acids (mTAD1 into L22Q/W23S and mTAD2 into W53Q/F54S), leading to abrogation or reduction in p53 transactivation of many target genes (Candau et al., 1997; Lin et al., 1994; Lin et al., 1995; Venot et al., 1999; Yan and Chen, 2010; Zhu et al., 1998). The proline-rich

domain (PRD, amino acids 64 to 92) is likely necessary for mutant p53 conformation as the Pin1 prolyl isomerase is required for optimal mutant p53 function (Girardini et al., 2011), and this domain can be deleted to study PRD-dependent effects (Yan and Chen, 2010). The carboxy-terminal regulatory domain (CTD, approximately residues 363 to 393) has been shown to be necessary for certain mutant p53 target gene activation but dispensable or even inhibitory for mutant p53 pro-proliferation phenotypes, and this domain can be deleted to study CTD-dependent effects (Frazier et al., 1998; Yan and Chen, 2010).

The N- and C-termini of p53 are involved in the majority of described protein-protein interactions with p53 (Vousden and Prives, 2009). Additional specific interaction partners will be described in later sections. The CTD and oligimerization domain (OD, residues 326-356) are required for wild-type p53 interaction with Sp1 and Sp3 (Kamada et al., 2011; Koutsodontis et al., 2005). The CTD contains numerous lysines that are modified to regulate p53, and these residues can be mutated to investigate mutant p53 CTD interactions with other proteins. TAD-independent mutant p53 interactions are thought to depend largely on p63 or p73 interaction, likely through the CTD, while the TAD-dependent functions are thought to depend on transcription factor interaction (Oren and Rotter, 2010). Indeed, specific mutant p53 domains are either required or dispensible to mutant p53 target gene activation, and the mechanisms of this regulation are incompletely defined (Table 1.1). It is imperative to investigate domain-specific p53 interactions in order to understand mechanistically how p53 regulates various genes as well as to integrate new findings in the context of previous literature.

Transcriptional Activation by Wild-Type p53

The following section describes the most well understood mechanisms of how wild-type p53 initiates transcription at genes containing a p53 response element (reviewed in Beckerman and Prives, 2010). It must be stated from the outset that the precise mechanisms in which p53 initiates transcription at its target genes are incompletely understood and undoubtedly more complex than will be discussed in this section. The basic model for activation of a p53 target gene from promoter engagement through transcription initiation occurs in two distinct phases: (1) p53 recognition and association with its DNA response element followed by (2) recruitment of transcriptional modifiers that culminate in the formation of the RNA polymerase II preinitiation complex (Laptenko and Prives, 2006). This section will focus on select p53-mediated transcriptional activation mechanisms that ostensibly occur following p53 binding to DNA.

p53 mediates gene expression changes through complex interaction with multiple chromatin regulators (Laptenko and Prives, 2006). The potential for complex gene activation or regulatory mechanisms is especially complex when the tetrameric structure of p53 is considered. The functional p53 protein composed of four identical monomers allows for four distinct binding sites for binding partners within each tetramer. This allows for a high degree of complexity in the regulation of transcription, as a single tetramer could bind to multiple transcriptional modifiers at its N- or C-termini (or with its DNA binding domain); a single p53 tetramer could theoretically bind to four different transcriptional modifiers at a single binding site in its N-termini, for instance.

p53 preferentially associates with genomic regions with a high degree of nucleosomal occupancy (Lidor Nili et al., 2010). Increased DNase I cleavage (termed hypersensitivity) is a hallmark of active genes, corresponding to an open promoter configuration in which nucleosomes are not present (reviewed in Krebs and Peterson, 2000). DNase I hypersensitivity is a feature of multiple wild-type p53 target gene promoters including *p21* (*CDKN1A*)(Braastad et al., 2003). Interestingly, the p53 binding site within these promoters occurs in regions that are resistant to DNase I (Braastad et al., 2003). These genomic regions where p53 is bound undergo nucleosomal displacement, which is specific to p53 recruitment as the nucleosomal displacement is reversible upon p53 inactivation using a temperature-sensitive p53 mutant (Lidor Nili et al., 2010).

At the *p21* promoter, which is the best studied p53 responsive gene, both distal and proximal p53 response elements contain high levels of nucleosomal occupancy (Laptenko et al., 2011). Upon p53 activation, nucleosomal occupancy is rapidly lost, for which it was predicted that two nucleosomes at the distal (5') response element are displaced upon p53 activation (Laptenko et al., 2011). Nucleosomal displacement is most likely to occur subsequent to p53 DNA binding as it is unlikely that the alternative - that nucleosomes and p53 compete for the same site - occurs, especially considering that p53 can bind to its response element while the response element is engaged by a nucleosome (Laptenko et al., 2011). Interestingly, the ability of p53 to bend DNA within the p53-DNA complex is directly correlated with the stability of the p53-DNA interaction (Nagaich et al., 1997). It is conceivable that the intrinsic sequence of the p53 response element (which dictates the degree of DNA bending upon p53 activation) facilitates

nucleosomal repositioning and impacts subsequent steps in transcriptional activation and resetting of the promoter (promoter turnover)(Laptenko and Prives, 2006; Nagaich et al., 1997). As mutant p53 does not bind to DNA in a stable manner like wild-type p53, these steps of transcriptional activation likely differ.

As summarized above, it is a common feature that p53 target gene promoters are in nucleosomal-enriched sites that undergo nucleosomsal remodeling following p53 recruitment (Laptenko et al., 2011; Lidor Nili et al., 2010). p53 has been reported to cooperate with nucleosomal remodeling complexes including the Mi-2/NuRD complex (Luo et al., 2000) and multiple members of the SWI/SNF chromatin remodeling complex (discussed below; Lee et al., 2002). p53 has not yet been described to function with the INO80, SWR, or ISWI families of chromatin remodeling complexes.

The Mi-2/NuRD complex is interesting because a component of this complex, PID (also known as metastasis-associated protein 2, MTA2), significantly restricts a p53 response by preventing acetylation of p53. The Mi-2/NuRD complex is an atypical chromatin remodeling complex in that it has both histone deacetylase and chromatin remodeling activities in the same complex (Denslow and Wade, 2007). The Mi-2/NuRD complex is composed of the HDAC1 and HDAC2 histone deacetylases and the CHD3 (Mi-2α) and CHD4 (Mi-2β) chromatin remodeling components, among other proteins (Denslow and Wade, 2007). The CHD3 and CHD4 ATPases are chromodomain-containing proteins in the SNF2 family of proteins, which includes the SWI/SNF ATPases BRG1 and BRM (Denslow and Wade, 2007; Eisen et al., 1995; Woodage et al., 1997). The PID component of the Mi-2/NuRD complex binds to the p53 N-terminus between amino acids 1-80, and binding is abolished when the first transactivation

domain of p53 (amino acids 22, 23) is inactivated (Luo et al., 2000). This finding is further interesting because theoretically the Mi-2/NuRD complex could be recruited by p53 to remodel chromatin and then deacetylate p53 to terminate the p53 transcription activation cycle.

p53 is known to direct transcription through interaction with the Mediator complex (Meyer et al., 2010; Zhang et al., 2005). The Mediator complex, initially identified in yeast (Kim et al., 1994) and later identified in humans as a group of thyroid hormone receptor associated-proteins (TRAPs), functions as a gene-specific transcriptional coactivator for RNA polymerase II (Fondell et al., 1996). The pre-initiation complex is composed of RNA polymerase II, the Mediator complex, and the general transcription factors TFII-A, -B, -D, -E, -F, and -H (Esnault et al., 2008; Roeder, 1996). The human Mediator complex interacts directly with TFIID in the process of forming the pre-initiation complex (Johnson et al., 2002). In yeast, Med11 is required to recruit TFIIH and TFIIE to the pre-initiation complex that leads to serine 5 phosphorylation of the RNA Pol II CTD (Esnault et al., 2008). Mediator has also been described to interact directly with the unmodified RNA Pol II CTD, which causes the Mediator complex to adopt a specific CTD-bound conformation (Naar et al., 2002). p53 has been reported to interact with various Mediator components (Gu et al., 1999), including Med17 (TRAP80)(Ito et al., 1999) and Med1 (RB18A)(Drane et al., 1997; Meyer et al., 2010). Med17 interacts with p53 TAD1 (Ito et al., 1999) and Med1 interacts with the p53 CTD (mapped to residues 363-393)(Meyer et al., 2010). Interestingly, increasing titrations of Med1 lead to decreased p53-dependent p21 expression and increased p53-dependent Bax expression (Frade et al., 2000) and increased MDM2 expression (Frade et al., 2002).

Notably, the D5 domain of Med1 has been reported to interact with mutant p53 in Raji lymphoma cells (R213Q, Y234H), although this interaction has not been reviewed by the mutant p53 literature nor subsequently reported on through this point in time (Lottin-Divoux et al., 2005).

Arginine methyltransferaes PRMT1 and CARM1 have been implicated in p53 transcriptional activation (An et al., 2004). PRMT1 and CARM1 bind directly to the p53 N-terminus in a region encompassing TAD1 and cooperate with p300 to stimulate transcription of the p53 target gene GADD45A (An et al., 2004). Histone methylation marks may serve dual roles to recruit co-activators that recognize those sequences and by indirectly enhancing acetylation by blocking histone deacetylases (Nishioka et al., 2002) including the NuRD complex (Zegerman et al., 2002). PRMT1 has been described to modify histones in a manner that permits them to be subsequently modified to transcriptionally active modification modes (Huang et al., 2005), and PRMT1 is known to cooperate with CARM1 to mediate gene expression (Hassa et al., 2008).

The N-terminus of p53 (involving the transactivation domain residues 22 and 23) interacts with p300 (Gu et al., 1997). p300 is a histone acetyltransferase that serves to regulate p53 through direct acetylation (Avantaggiati et al., 1997; Gu and Roeder, 1997; Lill et al., 1997). Acetylation of the p53 CTD correlates with increased acetylation of histones H3/H4 and increases the interaction of p53 with p300 as well as with the TAF10 subunit of TFIID (Barlev et al., 2001). Interestingly, at the p21 promoter it has been reported that p300 does not function in transcriptional co-activation through acetylation of p53 (Espinosa and Emerson, 2001). Rather, p53 was found to recruit p300 in order to acetylate nucleosomal histones to mediate transcriptional activation

(Espinosa and Emerson, 2001; see also An et al., 2004). Histone acetylation marks are known activating marks for other transcriptional components including SWI/SNF (Agalioti et al., 2002), which functions in an ATP-dependent manner to reorganize chromatin to allow the binding of transcription factors (Kwon et al., 1994).

It can be surmised that at the p21 locus p53 binds to its response element, recruits p300 which acetylates key histone residues, which stimulates SWI/SNF recruitment and subsequent nucleosomal repositioning to facilitate the recruitment of other transcriptional components that culminate in the formation of the RNA pol II pre-initiation complex. While this is a simplified model, these key ideas - specifically, that wild-type p53 binds to its response element, recruits chromatin modifying proteins that modify both p53 and neighboring histones and chromatin-associated proteins to stimulate subsequent transcriptional processes such as additional co-activator recruitment - will serve as a guide to understand mutant p53 gain of function whereby one of the most critical actions of mutant p53 may be the recruitment of transcriptional regulators to specific promoters to impact transcription.

Mutant p53 Gain of Function

The initial reports that p53 is an oncogene are not entirely misleading as it is now firmly established that there is a pro-oncogenic gain of function role for mutant p53. Li-Fraumeni patients with missense mutations are associated with a 9-year earlier tumor onset than patients with other mechanisms of p53 haploinsufficiency (Bougeard et al., 2008). Mice engineered with haploinsufficient mutant p53 ($p53^{+/mut}$) have accelerated tumor growth, increased tumor count, altered tumor spectrum, and increased

metastases than mice with true haploinsufficiency ($p53^{+/-}$)(Caulin et al., 2007; Dittmer et al., 1993; Lang et al., 2004; Liu et al., 2000; Olive et al., 2004). Critically, $p53^{mut/-}$ mice spontaneously acquire a variety of additional carcinomas in addition to the normal tumors associated with $p53^{-/-}$ mice, defining an *in vivo* gain of function phenotype for mutant p53 (Olive et al., 2004).

It is generally appreciated that the majority of mutant p53 gain of function effects derive from the ability of mutant p53 to affect transcription of a variety of genes. Indeed, mutant p53 transcriptional effects have been shown to lead to increased cell proliferation, decreased apoptosis, increased migration, increased invasion through Matrigel, increased tumor inflammation, and increased metastases (Adorno et al., 2009; Bossi et al., 2006; Bossi et al., 2008; Freed-Pastor et al., 2012; Haupt et al., 2009; Hsiao et al., 1994; Lim et al., 2009; Muller et al., 2009; Muller et al., 2013; Preuss et al., 2000; Scian et al., 2005; Strano et al., 2002; Werner et al., 1996; Yan and Chen, 2009; Yan et al., 2008). These studies will be described below.

In mouse models, mutant p53 is associated with increased rates of metastasis (Adorno et al., 2009; Heinlein et al., 2008; Hsiao et al., 1994; Pohl et al., 1988; Weissmueller et al., 2014). This effect was initially identified in 1988 (Pohl et al., 1988) and was defined to be missense mutant specific in 1994 by utilizing xenotransplants of leukemia cells expressing different missense p53 mutations (Hsiao et al., 1994). Leukemia cells (Be-13 cells) expressing p53 R175H, R248Q, and R213Q were able to disseminate to distant sites and induce further hematological disease, while the same cells expressing Y234H and R273C were unable to metastasize (Hsiao et al., 1994). The missense mutations that resulted in disseminated disease correlated with

decreased survival, and these mice underwent rapid death following the mutant p53-mediated metastases (Hsiao et al., 1994). In a mouse model of invasive breast cancer, the mouse equivalent of p53 R273H (R270H) has also been found to increase the rate of lung metastasis (Heinlein et al., 2008).

Two more recent studies describe how mutant p53 impedes p63 (Adorno et al., 2009) or p73 function to promote metastasis (Weissmueller et al., 2014). Contact mutant p53 R280K (endogenously expressed in MDA-231 breast cancer cells) were studied in xenotransplants. 21 out of 22 mice with unaltered levels of mutant p53 had lymph node positivity, while 12 out of 22 mice exhibited lymph node positivity when mutant p53 was depleted (Adorno et al., 2009). This correlated with the number of micrometastases to the lung, as depletion of mutant p53 resulted in 75-90% (depending on the clone) reduction in micrometastases (Adorno et al., 2009). Furthermore, when R175H was overexpressed in the presence of depleted R280K, the number of lung micrometastases was rescued to normal levels (Adorno et al., 2009). This study further describes how Smad3 (a component of TGFβ signaling) cooperates with mutant p53 to oppose p63 action (which impedes metastatic spread to the lung)(Adorno et al., 2009). A second study defined how a p73/NF-Y complex repressed PDGFRβ expression in a mouse model of pancreatic cancer (Weissmueller et al., 2014). Pancreatic cancer cell lines were derived from mice engineered with the mouse equivalent of p53 R175H (R172H). These cell lines have been previously defined and also express constitutively active K-Ras under tissue specific control (Hingorani et al., 2005). These cells were analyzed in the presence and absence of p53 R172H. Following orthotopic injection into the pancreas, metastasis to the lung and liver were significantly reduced when mutant

p53 was depleted (Weissmueller et al., 2014). Mutant p53 interaction with p73 was found to inhibit p73 interaction with NF-YB, allowing NF-Y to activate PDGFRβ expression that is critical to the metastatic phenotype of mutant p53 in these cells (Weissmueller et al., 2014). Hence, it is well established that mutant p53 promotes metastasis in mouse models, which is consistent with the observations that mutant p53 correlates with worse survival in human cancers (Alsner et al., 2008; Elledge et al., 1993; Langerod et al., 2007; Olivier et al., 2006; Petitjean et al., 2007a)

Hotspot p53 mutants have been reported to contribute to inflammation-associated colorectal cancer by cooperation with NF-κB to affect inflammation-associated genes (Cooks et al., 2013). This observation was especially interesting because mutant p53 is documented to affect the expression of genes in the NF-κB pathway that lead to increased cell growth and survival (Scian et al., 2005), not to say much about the multifaceted role of inflammation in cancer (reviewed in Grivennikov et al., 2010). Hotspot mutations of p53 are also well documented to lead to increased cell proliferation, (Bossi et al., 2006; Bossi et al., 2008; Freed-Pastor et al., 2012; Haupt et al., 2009; Preuss et al., 2000; Scian et al., 2004; Strano et al., 2002; Yan and Chen, 2009; Yan et al., 2008), resistance to apoptosis (Bossi et al., 2008; Lim et al., 2009) which can be mediated through mutant p53 interaction with Ets-2 (Do et al., 2012), increased migration (Adorno et al., 2009; Weissmueller et al., 2014), and increased cellular invasion through Matrigel (Muller et al., 2009; Muller et al., 2013).

These studies identify a variety of mechanisms through which mutant p53 promotes oncogenesis. As each tumor is unique in its development, it should be considered at this point that mutant p53 may be a promiscuous transcription factor that

is utilized by the tumor cell - based on its specific mutations, dominant signaling pathways, and interaction with the microenvironment - in a manner that is selectively advantageous. The next section will summarize what is known about mutant p53-mediated transcription with a focus on specific mutant p53 interacting partners and transcriptional targets.

Transcriptional Activation by Mutant p53

It remains to be understood how mutant p53 mechanistically affects transcription. Mutant p53 does not directly bind to a consensus DNA sequence (Bargonetti et al., 1991; Bargonetti et al., 1993; el-Deiry et al., 1992). Rather, it is likely that mutant p53 through its N-terminal transactivation domains or C-terminal regulatory domain associates with other transcription factors, histone-modifying machinery, or the transcription initiation complex to promote transcription (summarized in Table 1.2). Note that the majority of transcription factors that interact with mutant p53 also interact with wild-type p53.

Mutant p53 has been demonstrated to interact with the following transcription factors: p53 homologues p63 and p73, Sp1, Smad2, Smad3, NF-Y, E2F1, Ets-1, Ets-2, and the Med1 component of the Mediator complex (see Table 1.2; Adorno et al., 2009; Bargonetti et al., 1997; Bensaad et al., 2003; Chicas et al., 2000; Davison et al., 1999; Di Agostino et al., 2008; Di Agostino et al., 2006; Do et al., 2012; Fontemaggi et al., 2009; Frazier et al., 1998; Gaiddon et al., 2001; Lee et al., 2000; Lottin-Divoux et al., 2005; Marin et al., 2000; Sampath et al., 2001; Strano et al., 2002; Yan et al., 2008). It is presumed that mutant p53 depends on these interactions (and others not yet

identified) to mediate the transcription of numerous genes to mediate its gain of function effects. A list of mutant p53-regulated genes is provided in Table 1.3.

The list of mutant p53-regulated genes, most of which have demonstrated functional purpose, is extensive and includes ABCB1 (MDR)(Bush and Li, 2002; Chin et al., 1992; Lin et al., 1995; Sampath et al., 2001; Strauss and Haas, 1995), the GRO1 chemokine (Yan and Chen, 2009), PCNA (Deb et al., 1992), the ID2 transcription regulator (Yan et al., 2008), the ID4 transcription regulator (Fontemaggi et al., 2009), the hsMAD1 mitotic spindle checkpoint protein (Iwanaga and Jeang, 2002), 15-lipoxygenase (Kelavkar and Badr, 1999), the galectin-3 anti-apoptotic protein (Lavra et al., 2009), insulin-like growth factor II (Lee et al., 2000), insulin-like growth factor receptor I (Werner et al., 1996), epidermal growth factor receptor (Ludes-Meyers et al., 1996), c-Fos (Preuss et al., 2000), NF-kappaB2 (Cooks et al., 2013; Scian et al., 2005; Weisz et al., 2007), c-Myc (Frazier et al., 1998), the EGR transcription factor (Weisz et al., 2004), mitogen-activated protein kinase kinase 3 (Gurtner et al., 2010) asparagine synthetase (Scian et al., 2004), human telomerase reverse transcriptase (Scian et al., 2004), the stathmin microtubule-destabilizing protein (Singer et al., 2007), matrix metalloproteinase 13 (Sun et al., 2000), genes of the mevalonate pathway (Freed-Pastor et al., 2012), as well as numerous others (Table 1.3).

Mutant p53 regulation of these genes varies based on the specific p53 mutant and the cell line. Notably, many of these genes were studied by overexpressing mutant p53 in p53-null cell backgrounds (indicated in the table as Overexpression) and often with reporter assays (Table 1.3). Reporter assays are inadequate to study mutant p53-dependent transcription because they lack the full complexity of chromatin and mutant

p53 does not bind to a DNA response element. Overexpressing mutant p53 in p53-null cell backgrounds is adequate to study mutant p53 gain of function effects because there is no impact of wild-type p53 yet imperfect because the cell lines underwent selective changes without regard to the selective advantages conferred by mutant p53. Such selective changes - the tumor evolving to harness and depend on mutant p53 for its pro-proliferative or other capacities - impact how mutant p53 functions in a particular tumor. Therefore, we suggest that cell culture studies involving mutant p53 be performed using cell lines that express single point mutations in one allele of p53 and have underwent loss of heterozygosity (see Freed-Pastor et al., 2012 for examples).

Tumor-specific genetic alterations, chromatin landscape, and the availability of specific transcription factors affect mutant p53 function (Adorno et al., 2009; Dell'Orso et al., 2011; Haupt et al., 2009; Kim and Deppert, 2003, 2004; Li et al., 2008; Strano et al., 2007). It follows that one p53 mutant may be observed to behave differently than another p53 mutant based on changes in conformation, binding partners, cellular localization, and transactivation capability. Indeed, mutant p53 gain of function depends on cell and tumor context. Further note that there is significant evidence linking Ras-pathway activation to enhanced mutant p53 gain of function (Solomon et al., 2012). Note that post-translational modifications, nucleosome state, quarter-site orientation and spacer length of the p53 response element, and cofactors affect wild-type p53 gene regulation (Riley et al., 2008). Post-translational modifications, nucleosome state, and cofactors likely impact mutant p53 transcription as well.

These observations (in addition to the fact that mutant p53 does not bind to the p53 response element) may explain why wild-type p53 and mutant p53, despite binding

to many of the same transcription factors (Table 1.2) differentially affect gene expression. Mutant p53 can even lead to diametric outcomes of gene expression, even though mutant p53 can still cooperate with wild-type p53 co-activators, such as the histone acetyltransferase p300 (Avantaggiati et al., 1997; Di Agostino et al., 2006). For instance, mutant p53, when located at genes that wild-type p53 activates such as p21, GADD45, PERP, and PTEN, leads to their repression (Vikhanskaya et al., 2007). It is unclear why mutant p53 and wild-type p53 lead to diametric gene expression outcomes when they can interact with similar subsets of transcription factors and co-activators. One possibility is that wild-type p53 has interactions with the same transcription factors that mutant p53 binds, but when overexpressed or activated, wild-type p53 retains preference to its response element and therefore induces an appropriately timed, step-wise transcriptional response while mutant p53 prevents a transcriptional response (or even actively suppresses a response) by being constitutively located at the gene, by failing to initiate a specific activation sequence, or by failing to recruit the appropriate transcription factors (such as by recruiting transcriptional co-repressors).

Mutant p53 likely mediates transcription by co-opting sets of transcription factors to initiate gene activation at the transcription factor’s location. Co-activators recruited by the transcription factor or mutant p53 then stimulate gene expression. The extent that mutant p53 co-opts individual transcription factors for target gene activation is unclear and likely dependent on the specific mutation in p53 and the active cell signaling pathways leading to subsets of active transcription factors in the cell. It is also possible that mutant p53, following recruitment by a transcription factor or chromatin modulator, recruits additional factors that can stimulate the function of the initial recruiting factor

(discussed in Chapter 3). It is possible that there are mechanisms for mutant p53 to change the state of chromatin, and these mechanisms may rely on mutant p53 recruitment to DNA through transcription factor binding followed by a change in chromatin architecture by known and unknown chromatin-modifying machinery that are recruited by the mutant p53-TF complex.

Transcription factors may not need to be active to be recruited by mutant p53, although mutant p53 may need to be modified in a specific manner. Mutant p53 engagement may lead to conformational change of an inactive transcription factor to a conformation that can bind DNA. In this mechanism, the presence of mutant p53 in a cell leads to transcription factor engagement by mutant p53. If these transcription factors were inactive and cytoplasmic, mutant p53 presence would shift their localization to the nucleus, forming a complex with mutant p53, the engaged transcription factor, and a co-activator such as p300. Indeed, cell context, perhaps through mutant p53 modifications, can alter mutant p53-coactivator binding (Di Agostino et al., 2006). Promoters known to be engaged by NF-Y and mutant p53 shift from containing the repressive HDAC1 to the activating p300 upon DNA damage by doxorubicin (Di Agostino et al., 2006). Additionally, serine-6 and serine-9 phosphorylations are required for mutant p53-Smad binding (Adorno et al., 2009; Cordenonsi et al., 2007). Mutant p53 contact mutants can cooperate through NF-κB signaling to increase mutant p53 target gene activation (Solomon et al., 2012). It should also be considered that mutant p53 may refine transcription factor binding specificities, a process termed latent specificity (a cofactor-induced change in DNA recognition)(Slattery et al., 2011).

It must be emphasized that the mechanism of mutant p53-transcription factor and mutant p53-coactivator interaction is not understood, and it is these interactions culminating in transcriptional changes that likely represent the majority of mutant p53 gain of function effects. To understand the mechanism of mutant p53 gain of function transcriptional effects, the mechanisms for mutant p53-transcription factor and mutant p53-coactivator (or mutant p53-chromatin regulator) interaction, culminating in gene expression changes, must be delineated. This will allow for investigation into the extent that different transcription factors function with mutant p53 by itself or coordinately with each other and mutant p53 to lead to gene expression changes. Moreover, novel co-activators and co-repressors must be identified that could account for mutant p53 transcriptional outcomes (see Chapter 3). Future drug discovery targets rely entirely on the previous point- if a factor is found that is required for mutant p53 gene transactivation, such as a novel co-activator, then a drug could be developed that would be highly specific for the mutant p53-coactivator complex that would only exist in cells expressing mutant p53 gain of function mutants.

Mutant p53 Regulation of Receptor Tyrosine Kinases

Mutant p53 has been reported to stimulate expression of multiple receptor tyrosine kinases and other signaling components. It is well described that receptor tyrosine kinases promote pro-proliferative signaling (Lemmon and Schlessinger, 2010). Common signaling nodes can be engaged by multiple receptor tyrosine kinases, even though the output is different, eg: the same signaling pathway can shift from promoting a differentiated state (common to normal cells) to a pro-proliferative state (Lemmon and

Schlessinger, 2010; Marshall, 1995). It is conceivable that mutant p53 may affect cell signaling pathways to promote de-differentiation of tumor cells. Mutant p53 has been reported to stimulate additional receptor tyrosine kinases, including EGFR (Ludes-Meyers et al., 1996), IGF1R (Werner et al., 1996), MET (Muller et al., 2013), and PDGFRβ (Weissmueller et al., 2014), all of which promote pro-proliferative signaling. This activation, in the case of EGFR and MET, is dependent on Rab-coupling protein, which increases recycling of these receptor tyrosine kinases to enhance their signaling outputs (Muller et al., 2009; Muller et al., 2013). It is worth considering that as a tumor forms, acquisition of a hotspot mutation in *TP53* may facilitate transcriptional plasticity, whereby tumor cells increase capacity for gene expression changes and therefore undergo selection for the greatest pro-proliferative transcriptional program for the particular tumor context. This hypothesis explains, for instance, why such a wide array of genes and pathways has been reported to mediate mutant p53 gain of function.

VEGFR2 Signaling

The receptor tyrosine kinase *VEGFR2* (*KDR*/*FLK1*) is of great clinical importance because it is the functional target of the humanized monoclonal antibody bevacizumab (Avastin®), which inhibits vascular endothelial growth factor A (VEGF) activation of VEGFR2 (Presta et al., 1997). Bevacizumab is approved for adjuvant use in the treatment of metastatic renal cell carcinoma, non-small cell lung cancer, glioblastoma multiforme, and metastatic colorectal cancer but in November 2011 lost FDA approval for the treatment of breast cancer. Despite the efficacy of bevacizumab in subsets of breast cancer patients, the lack of well-defined patient selection criteria meant that the

number needed to treat was far too high when cost and the limited side effect profile were considered (Bear et al., 2012; Cobleigh et al., 2003; Link et al., 2007; Miller et al., 2007; Miller et al., 2005; Schneider et al., 2008; von Minckwitz et al., 2012; Wedam et al., 2006). There exists a critical demand for new knowledge to improve patient selection criteria for response to anti-VEGF pathway treatment, for which there are multiple treatments currently in phase II or III clinical trials (Saharinen et al., 2011).

VEGFR2 is a receptor tyrosine kinase that under normal physiological conditions is only expressed on endothelial cells, which form the lining of blood vessels (Holmes et al., 2007; Millauer et al., 1993; Quinn et al., 1993; Shalaby et al., 1997; Terman et al., 1992). Upon VEGF stimulation, VEGFR2 homo-dimerizes and trans-phosphorylates to initiate pro-migratory signaling to initiate endothelial cell chemotaxis toward the VEGF-producing tissue and, once seeded, pro-proliferative signaling to promote neovascularization from existing blood vessels near the site (Holmes et al., 2007; Millauer et al., 1994; Zhang et al., 2002). Intriguingly, VEGFR2 is induced to varying extents on the tumor epithelium - the malignant cells - in the majority of epithelial tumor types, including breast, colon, lung, and kidney cancers (Guo et al., 2010; Nakopoulou et al., 2002; Speirs and Atkin, 1999; Wedam et al., 2006). VEGFR2 is postulated to lead to the same pro-migratory and pro-proliferative effects in tumor cells that occur in endothelial cells (Guo et al., 2010). Because malignant breast tumors invariably express VEGF, VEGFR2 expression on tumor epithelia led to the hypothesis of an autocrine loop, whereby the tumor epithelia expresses the ligand for its own receptor (Ghosh et al., 2008; Kranz et al., 1999; Ryden et al., 2003; Ryden et al., 2005; Weigand et al., 2005). It follows that if certain tumors are addicted to the VEGF-VEGFR2 autocrine

loop, then anti-VEGF therapy will be more effective in tumors that express VEGFR2 because the therapy will antagonize the tumor by direct repression of pro-proliferative signaling on the tumor cells in addition to atrophy of the vascular supply (Weigand et al., 2005).

Remarkably, VEGFR2 signaling affects endothelial cells in manner that could be pro-oncogenic if expressed on tumor cells by increasing proliferation, migration, and survival signaling (Azam et al., 2010; Grunewald et al., 2006; Holmes et al., 2007; Takahashi et al., 2001; Takahashi et al., 1995; Tugues et al., 2011). Blockade of VEGF-VEGFR2 signaling by reducing VEGF expression, blocking VEGF-VEGFR2 interaction, inhibiting VEGFR2 with small molecules, expression of dominant negative VEGFR2 mutants, or siRNA depletion of VEGFR2 each lead to tumor inhibition by antagonism of endothelial cell neovascularization (Gerber et al., 2000; Holash et al., 2002; Kuo et al., 2001; Millauer et al., 1996; Millauer et al., 1994; Oku et al., 1998; Prewett et al., 1999; Saleh et al., 1996a; Saleh et al., 1996b; Strawn et al., 1996; Wedge et al., 2000; Wood et al., 2000). In cell culture, which allows the study of autocrine VEGF-VEGFR2 signaling because there are no confounding vascular effects, breast cancer cells upon impaired VEGF signaling experience reduced cell proliferation, increased apoptosis, reduced migration, and reduced invasion through Matrigel (Bachelder et al., 2001; Beliveau et al., 2010; Ge et al., 2009a; Ge et al., 2009b; Lee et al., 2003; Liang et al., 2006; Pidgeon et al., 2001; Price et al., 2001; Scherbakov et al., 2006; Timoshenko et al., 2007; Weigand et al., 2005).

We postulate that VEGFR2 is a candidate receptor tyrosine kinase that can mediate mutant p53 gain of function because many mutant p53 gain of function effects

(discussed previously) overlap with known outcomes of VEGFR2 signaling. It is reasonable to hypothesize that if mutant p53 upregulates VEGFR2, then VEGFR2 signaling accounts for a proportion of observed mutant p53 gain of function effects. The VEGFR2 promoter is known to be regulated by Sp1 (Meister et al., 1999), E2F1 (Pillai et al., 2010), and Ets-1 (Sato et al., 2000), and the VEGFR2 promoter also contains NF-kappaB (Patterson et al., 1995) and p53-family member sequences motifs (Guo et al., 2010). Each of these elements has a reported relationship with mutant p53 (discussed previously) that may account for potential regulation of mutant p53 at the VEGFR2 promoter. Interestingly, VEGFR2 has been reported to translocate to the nucleus to regulate its own expression by functioning as its own transcription factor, similar in mechanism to the EGF Receptor, implying the existence of a positive feedback loop (Domingues et al., 2011; Lin et al., 2001). It is interesting to consider that a cell could reinforce pathways until they are dominant through positive feedback mechanisms. It is feasible that a cell with mutant p53 has multiple competing cell signaling pathways that culminate in a dominant signaling pathway.

p53 and Angiogenesis

p53 exerts tumor suppressive functions through multiple mechanisms in the parent cell, but p53 is also tumor suppressive in the context of the tissue. An incipient tumor invariably requires the elaboration of VEGF to initiate the angiogenic switch to promote growth beyond several millimeters (Bergers and Benjamin, 2003). Wild-type p53 prevents angiogenesis through multiple mechanisms: transcriptional suppression of VEGF expression (Mukhopadhyay et al., 1995; Pal et al., 2001; Zhang et al., 2000),

induction of thrombospondin-1 (Tsp1) and brain-specific angiogenesis inhibitor 1 (Dameron et al., 1994a, b; Grossfeld et al., 1997; Nishimori et al., 1997), increased degradation of hypoxia inducible factor 1-α (Ravi et al., 2000), increased expression of anti-angiogenic collagen fragments (Assadian et al., 2012; Bian and Sun, 1997; Teodoro et al., 2006), downregulation of COX-2 (Subbaramaiah et al., 1999), upregulation of ephrin receptor A2 (Dohn et al., 2001), and suppression of bFGF-binding protein expression (Hammond and Giaccia, 2002; Sherif et al., 2001; Van Meir et al., 1994). p53 may also limit angiogenesis through upregulation of miR34a (Chang et al., 2007). Furthermore, mutant p53 has been shown to cooperate with protein kinase C to stimulate VEGF expression (Kieser et al., 1994).

In breast tumor cells, in which *TP53* mutation is often (but not always) an early genetic lesion, VEGF is invariably overproduced, leading to neovascularization of the incipient tumor (Borresen-Dale, 2003; Done et al., 2001; Ghosh et al., 2008; Jerry et al., 1993; Kranz et al., 1999; Ryden et al., 2003; Zhou et al., 2009). Indeed, p53 status impacts response to anti-angiogenic therapy (Yu et al., 2002). This may be because of a reduced dependence on the vascular supply in p53 null tumors, which undergo less apoptosis than wild-type p53 expressing tumors in hypoxic conditions (Yu et al., 2002). Mutant p53 and VEGF expression independently predict worse outcome in breast cancer (Linderholm et al., 2000; Linderholm et al., 1998), and the combined expression of both proteins allows for significant prognostic value as the expression of both correlate with poor outcomes (Linderholm et al., 2000; Linderholm et al., 2001).

In Chapter 2, the hypothesis that certain hotspot p53 mutants are selected during the progression of breast cancer in part due to the selective advantages conferred by

pro-proliferative, pro-migratory VEGFR2 autocrine signaling is explored. We speculate that hotspot mutation of p53 coincides with the angiogenic switch in this class of tumors (tumors with p53 hotspot mutations) because mutation of p53 will simultaneously de-repress VEGF expression and stimulate VEGFR2 expression leading to increased tumor growth and thus additional elaboration of VEGF, forming a feed-forward system that further stimulates neovascularization and tumor proliferation. Chapter 3 explores how mutant p53 regulates the *VEGFR2* promoter, which we find is mediated through interaction with the SWI/SNF chromatin remodeling complex.

The Mammalian SWI/SNF Chromatin Remodeling Complex

The mammalian SWI/SNF complex associates genome-wide with transcription regulatory elements (Euskirchen et al., 2011) to regulate nucleosome occupancy (Tolstorukov et al., 2013). This complex is composed of either BRG1 or BRM ATPases, a set of core proteins, and other context-specific components (Narlikar et al., 2002; Wilson and Roberts, 2011). SWI/SNF complexes are subdivided into PBAF and BAF complexes based on the presence of BAF250A or BAF250B (BAF complex, contains either BRG1 or BRM ATPase) or BAF180 (PBAF complex contains only BRG1 ATPase), although this distinction may not be absolute (Euskirchen et al., 2012; Ryme et al., 2009; Wilson and Roberts, 2011).

The SWI/SNF Complex Functions as a Tumor Suppressor

Inactivating mutations in several SWI/SNF components are found at high frequency in a variety of cancers, including breast cancer, implicating SWI/SNF in tumor

suppression (Reisman et al., 2009; Weissman and Knudsen, 2009; Wilson and Roberts, 2011). The frequency of SWI/SNF mutations in various cancers is depicted in Figure 1.4. Note that in the largest breast cancer datasets, SWI/SNF genes undergo mutation at a similar rate to *TP53* (Figure 1.1 and Figure 1.4; Figure 1.4 demonstrates the total percentage of tumors with any SWI/SNF mutation). Unlike *TP53* and atypical of a tumor suppressor, SWI/SNF components are often found amplified in some tumors (although this is not well described in the literature). It may be informative to explore the genetic backgrounds of tumor-specific SWI/SNF components with amplification versus loss of function mutations to see if specific alterations correlate with mutational status of other genes such as *TP53*. The sum of *TP53* and SWI/SNF subunit mutations in various cancers is depicted in Figure 1.5. Note that the addition of *TP53* mutations to the SWI/SNF mutant tumors depicted in Figure 1.4 demonstrates a significant proportion of tumors that are *TP53* mutated and wild-type for SWI/SNF (Figure 1.5). Note that for most tumors, there is a greater likelihood that there is a *TP53* mutation or a SWI/SNF mutation (or both) than to be wild-type for *TP53* and SWI/SNF (Figure 1.5).

In a small study of 12 patients with a small cell carcinoma of the ovary (hypercalcemic type) 12 patients had biallelic BRG1 mutations (Jelinic et al., 2014). Eleven of these patients for which *TP53* status is known are wild-type for *TP53* (www.cbioportal.org; Jelinic et al., 2014). This observation is consistent with a much larger study where it was found that SWI/SNF mutations and *TP53* mutations have a tendency toward mutual exclusivity in some cancer types including colorectal, clear cell ovarian, gastric, hepatocellular, and medulloblastoma cancers (Kadoch et al., 2013). This same tendency exists in breast cancer and suggests that loss of SWI/SNF function

may phenocopy p53 loss to mediate oncogenesis (Kadoch et al., 2013). This study specifically excluded amplifications in the analysis of SWI/SNF mutations (Kadoch et al., 2013).

SWI/SNF proteins are tumor suppressive in some specific contexts. For instance, BRG1 and SNF5 have also been described to mediate p53-dependent transcription of p21 (Lee et al., 2002), BRG1 is necessary for efficient RB-mediated cell cycle arrest (Bartlett et al., 2011; Strobeck et al., 2000), and BRG1 cooperates with ATM to promote the DNA damage response (Kwon et al., 2014). The Parsons laboratory has also defined a role for the PBAF subunit BAF180 in mediating p21 expression (causing G1 arrest) in breast tumor cells in response to radiation or TGFβ signaling (Xia et al., 2008). The roles for SWI/SNF as a tumor suppressor are well described (Reisman et al., 2009; Weissman and Knudsen, 2009; Wilson and Roberts, 2011). Notably, BRG1 heterozygous mice have increased frequency of mammary tumors (Bultman et al., 2008). Moreover, targeted BRG1 homozygous deletion predisposes to ethyl carbamate-induced lung tumors (Glaros et al., 2008).

It is interesting to consider that the tumor suppressive functions of SWI/SNF may be counterbalanced by the dependence of a cancer cell to utilize SWI/SNF function for its own pro-survival purposes. It is conceivable that alteration of specific SWI/SNF subunits or interacting partners may obviate SWI/SNF tumor suppressor function and allow the complex to function more as an oncogene. This may explain, for instance, why SWI/SNF components are frequently amplified in multiple tumor types (see Figure 1.4). Interestingly, in tumors with mutated SWI/SNF residues, other functional SWI/SNF components can retain oncogenic potential for the cell. This has been described for

BRG1 and BRM (the ATPases of the SWI/SNF complex). When BRG1 is mutated, intact SWI/SNF complex is still present in tumor cells (Wilson et al., 2014). This is thought to be because BRM may substitute for BRG1 (Wilson et al., 2014). This hypothesis led to the discovery using an shRNA screen that in BRG1 mutant tumors, BRM is the most important genetic vulnerability that can be targeted (Hoffman et al., 2014; Wilson et al., 2014). The authors describe the concept of cancer-selective paralog dependency, whereby loss of one genetic paralog (BRG1) reveals dependence to the paralogous gene (BRM)(Hoffman et al., 2014).

SWI/SNF Functions with p53

The genetic and physical interactions between SWI/SNF components and p53 have been well described. Multiple components of the SWI/SNF complex have been identified as wild-type p53 binding partners including BRG1 (Lee et al., 2002; Naidu et al., 2009), SNF5 (Lee et al., 2002), BAF60A and BAF155 (Oh et al., 2008), ARID1A (Guan et al., 2011), and BRD7 (Burrows et al., 2010). BAF60A interaction with p53 has been mapped to the oligomerization domain, which suggests that p53 must be in its tetrameric form for interaction with SWI/SNF (Oh et al., 2008). BRG1 depletion has been reported to activate p53 signaling (Naidu et al., 2009), while SNF5 depletion has been reported to lead to loss of p53 expression (Xu et al., 2010). A proline-rich region of BRG1 is necessary for interaction with p53 (Naidu et al., 2009). CBP (which is closely related to p300) has been reported to dissociate from BRG1 upon DNA damage, correlating with decreased CBP and BRG1 levels at the p21 promoter and increased p300 and p53 levels at the p21 promoter (Naidu et al., 2009). As previously mentioned,

the p21 promoter undergoes nucleosomal remodeling upon p53 binding (Laptenko et al., 2011) and SWI/SNF complex components including BAF180 (Xia et al., 2008), SNF5 (Lee et al., 2002), and BRG1 (Lee et al., 2002) are required for p21 expression, so the relationship with p53 and SWI/SNF components is complex.

As mutant p53 and wild-type p53 often mediate opposing effects on their interacting partners, in theory mutant p53 could dysregulate normal SWI/SNF complex function that wild-type 53 requires for transcriptional activities (Lee et al., 2002; Xu et al., 2007) by affecting its activity, interaction with other proteins, or chromosomal positioning. Mutant p53 has been proposed to facilitate transcriptional plasticity (Quante et al., 2012), and functional interaction of mutant p53 with a chromatin remodeling complex like SWI/SNF that has broad genomic distribution (Euskirchen et al., 2011) may explain the ability of mutant p53 to mediate gene expression at multiple loci. Because nucleosomal positioning is a critical factor in gene regulation, promoting or inhibiting transcription by regulating access to DNA-binding proteins (Wilson and Roberts, 2011), mutant p53 could theoretically co-opt SWI/SNF activity to mediate both gene activation and repression.

Three-dimensional (3D) Cell Culture

This section describes the use of three-dimensional culture to study cell culture models of breast cancer. Three-dimensional (3D) cell culture more faithfully recapitulates the cell-cell and cell-extracellular matrix (ECM) interactions that exist in a tumor (Kenny et al., 2007). In 3D culture, breast cancer cells are grown on a laminin-rich extracellular matrix (Matrigel®) to form defined structures based on the extent of

dysplasia of the parent cell (Kenny et al., 2007). 3D culture is an improved method to study signaling pathways, which are affected by the ECM through multiple mechanisms (Beliveau et al., 2010; Boudreau et al., 1995; Guo et al., 2006; Hansen and Bissell, 2000; Koch et al., 2011; Liu et al., 2004; Muller et al., 2009; Schatzmann et al., 2003; Wang et al., 1998; Weaver et al., 2002; Weaver et al., 1997; Zachary and Gliki, 2001; Zhang et al., 2009). These cell-matrix interactions have striking effects on global gene expression, with the implication that 3D culture provides more reliable gene expression and phenotypic data than 2D culture (Bissell, 2007; Bissell et al., 1982; Carrio et al., 2005; Fournier et al., 2009; Fournier and Martin, 2006; Fournier et al., 2006; Freed-Pastor et al., 2012; Petersen et al., 1992; Roskelley et al., 1994; Wang et al., 2002; Weaver et al., 1997; Zutter et al., 1995). Moreover, phenotypic reversion can be modeled in 3D, whereby a single change or group of changes to a malignant breast cancer cell may cause reversion to a normal-appearing, non-malignant acinus (Carrio et al., 2005; Freed-Pastor et al., 2012; Wang et al., 2002; Weaver et al., 1997; Zutter et al., 1995). In 3D culture, mutant p53 is required for the optimal growth and invasive properties of breast cancer cells, and reduction of mutant p53 results in significant loss of invasive properties in MDA-MB-231 cells and phenotypic reversion to normal-appearing acini in MDA-MB-468 cells (Freed-Pastor et al., 2012). Defining the molecular mechanisms for mutant p53 gain of function in a physiologically relevant system is critical for the ascertainment of data that is transferable to additional cell and tumor contexts.

Concluding Remarks

Oncology is headed toward highly adaptable treatment regimens based on the particular genetic alterations of a tumor. Defining the contributions of mutant p53 to breast cancer tumorigenicity is a critical step toward identifying specific tumor alterations that can be therapeutically harnessed. It is our hope that this work will lead to insight into the molecular biology of cancer specifically pertaining to gene regulation in cancer cells. We hope to identify novel drug targets that can be targeted to mutant p53-containing tumors. We also hope to identify signaling pathways that may cooperate with mutant p53 to enhance tumorigenicity. Defining the transcriptional changes mediated by mutant p53 in breast tumors is fundamental to the classification and treatment of breast tumors harboring mutant p53 and provides fundamental understanding to the mechanism of mutant p53 target gene activation. Ultimately we hope this work furthers the goal to define common vulnerabilities in mutant p53-expressing tumors that are common to multiple types of p53 alterations (so treatment options are simplified) that significantly enhance survival.

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FIGURE LEGENDS

Figure 1.1 *TP53* Mutations in Human Cancers

Histogram for *TP53* mutation frequency in human cancers organized by frequency of alteration per study. The type of *TP53* mutational status is indicated by color coding. Multiple alterations (listed in grey) indicates the presence of a copy number aberration (eg: amplification of 17p with homozygous loss of *TP53*) in conjunction with a mutation in the other allele (eg: point mutation) or the amplification of a mutant allele in that tumor sample. Breast cancer studies are indicated by arrows. Two of The Cancer Genome Atlas (TCGA) studies are detailed including the TCGA Provisional Breast Invasive Carcinoma Dataset and the *Nature* 2012 Breast Invasive Carcinoma Dataset (Network, 2012). Data accessed and reproduced from the cBioPortal (http://www.cbioportal.org) in December, 2014.

Figure 1.2 *TP53* Somatic Mutations in Breast Cancer

(A) Histogram for *TP53* somatic mutations in breast cancer organized by affected codon from the IARC *TP53* Database R17 Release. Mutations with greater than 1.75% mutation frequency are enumerated.

(B) Circle chart representing *TP53* somatic mutation categories in breast cancer from the entire IARC *TP53* Database R17 Release. The ‘Other’ category includes in-frame deletions or insertions. The NA category refers to *TP53* mutations in which the outcome on the protein is unknown (eg: large insertions).

(C) Circle chart representing *TP53* point mutation categories in breast cancer from the IARC *TP53* Database R17 Release.

Figure 1.3 *TP53* Missense Mutations in Breast Cancer

Histogram for *TP53* missense mutations in breast cancer from the IARC TP53 Database R17 Release. Missense mutations with greater than 2.25% mutation frequency are enumerated. The p53 secondary structure is defined as follows: Transactivation domain (TAD, composed of transactivation subdomains TAD1 and TAD2), Proline-Rich Domain (PRD), DNA-Binding Domain, Oligomerization (Tetramerization) Domain (OD), and Carboxy-Terminal Domain (CTD). The p53 protein domains are demonstrated by amino acid position as previously described (Joerger and Fersht, 2008) with the codon missense mutation frequency demonstrated above.

Figure 1.4 *SWI/SNF* Mutations in Human Cancers

Histogram for SWI/SNF mutation frequency in human cancers organized by frequency of alteration per study. The type of SWI/SNF mutational status is indicated by color coding. Multiple alterations (listed in grey) typically indicates the presence of two or more SWI/SNF mutations in that tumor sample. Breast cancer studies are indicated by arrows. Two of The Cancer Genome Atlas (TCGA) studies are detailed including the TCGA Provisional Breast Invasive Carcinoma Dataset and the *Nature* 2012 Breast Invasive Carcinoma Dataset (Network, 2012). The SWI/SNF input list included the following genes: *ARID1A, ARID1B, ARID2, PBRM1, SMARCA2, SMARCA4, SMARCB1, SMARCC1, SMARCC2, SMARCD1, SMARCD2, SMARCD3, SMARCE1,*

ACTL6A, PHF10, DPF1, DPF3, and DPF2. Data accessed and reproduced from the cBioPortal (http://www.cbioportal.org) in December, 2014.

Figure 1.5 *TP53* and *SWI/SNF* Mutations in Human Cancers

Histogram for TP53 and SWI*/SNF combined* mutation frequency in human cancers organized by frequency of alteration per study. The type of SWI/SNF mutational status is indicated by color coding. Multiple alterations (listed in grey) typically indicates the presence of two or more SWI/SNF mutations or a *TP53* and a SWI/SNF mutation in that tumor sample. Breast cancer studies are indicated by arrows. Two of The Cancer Genome Atlas (TCGA) studies are detailed including the TCGA Provisional Breast Invasive Carcinoma Dataset and the *Nature* 2012 Breast Invasive Carcinoma Dataset (Network, 2012). The SWI/SNF input list included the following genes: *TP53, ARID1A, ARID1B, ARID2, PBRM1, SMARCA2, SMARCA4, SMARCB1, SMARCC1, SMARCC2, SMARCD1, SMARCD2, SMARCD3, SMARCE1, ACTL6A, PHF10, DPF1, DPF3, and DPF2.* Data accessed and reproduced from the cBioPortal (http://www.cbioportal.org) in December, 2014.

Table 1.1 Studies Exploring Mutant p53 Domain-Specific Effects on Gene Expression

The table summarizes known studies that have investigated domain-specific effects of mutant p53 on transcription. The cell lines in which the stated gene was found to be regulated, the p53 mutants that were utilized, the experiment system that was employed, and the associated references are listed.

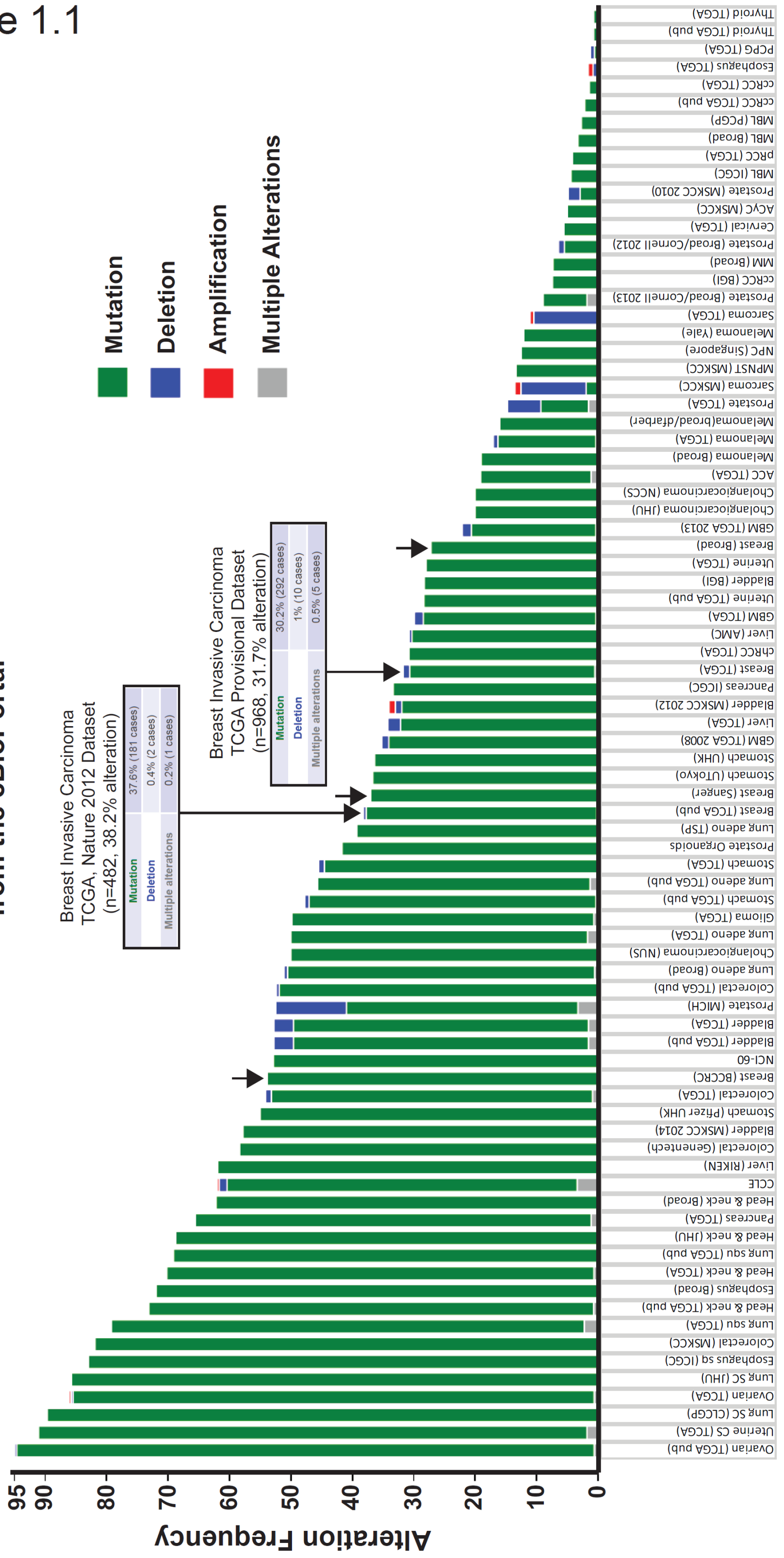
Table 1.2 Transcription Factors that Interact with Mutant p53

The table summarizes known transcription factors or chromatin modifers for which there is a reported interaction with mutant p53. Whether or not wild-type p53 is known to interaction with the same factor is listed. Known domain-specific TF interactions with wild-type p53 may predict domain interactions with mutant p53. Note that NF-κB may interact with mutant p53 (Solomon, Buganim et al. 2012). The table is an extension of a similar table prepared by Freed-Pastor and Prives (Freed-Pastor and Prives, 2012)

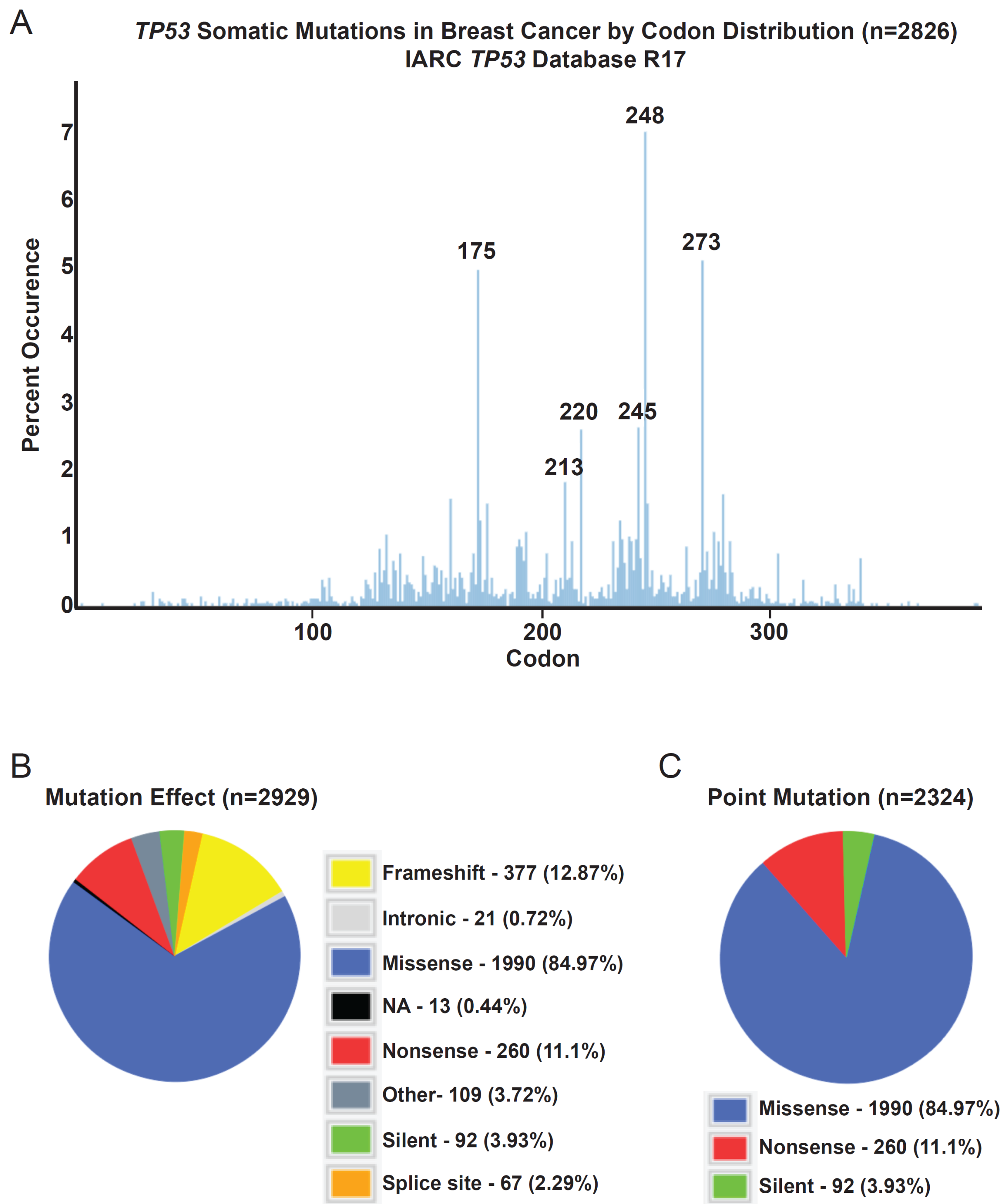
Table 1.3 Known Mutant p53 Target Genes

The table summarizes known mutant p53-regulated genes (primarily resulting in increased expression), the cell lines in which the gene was found to be regulated, the p53 mutants that were utilized, the experiment system that was employed, and the associated references. Note mutant p53 represses other genes, such as CD95 (Fas Receptor)(Gurova et al., 2003; Zalcenstein et al., 2003), ATF3 (a CREB TF family protein)(Buganim et al., 2006), TGF-Beta Receptor 2 (Kalo et al., 2007), Caspase 3 (Wong et al., 2007), Id2 (Yan et al., 2008), and wild-type p53 target genes p21, GADD45, PERP, and PTEN (Vikhanskaya et al., 2007).

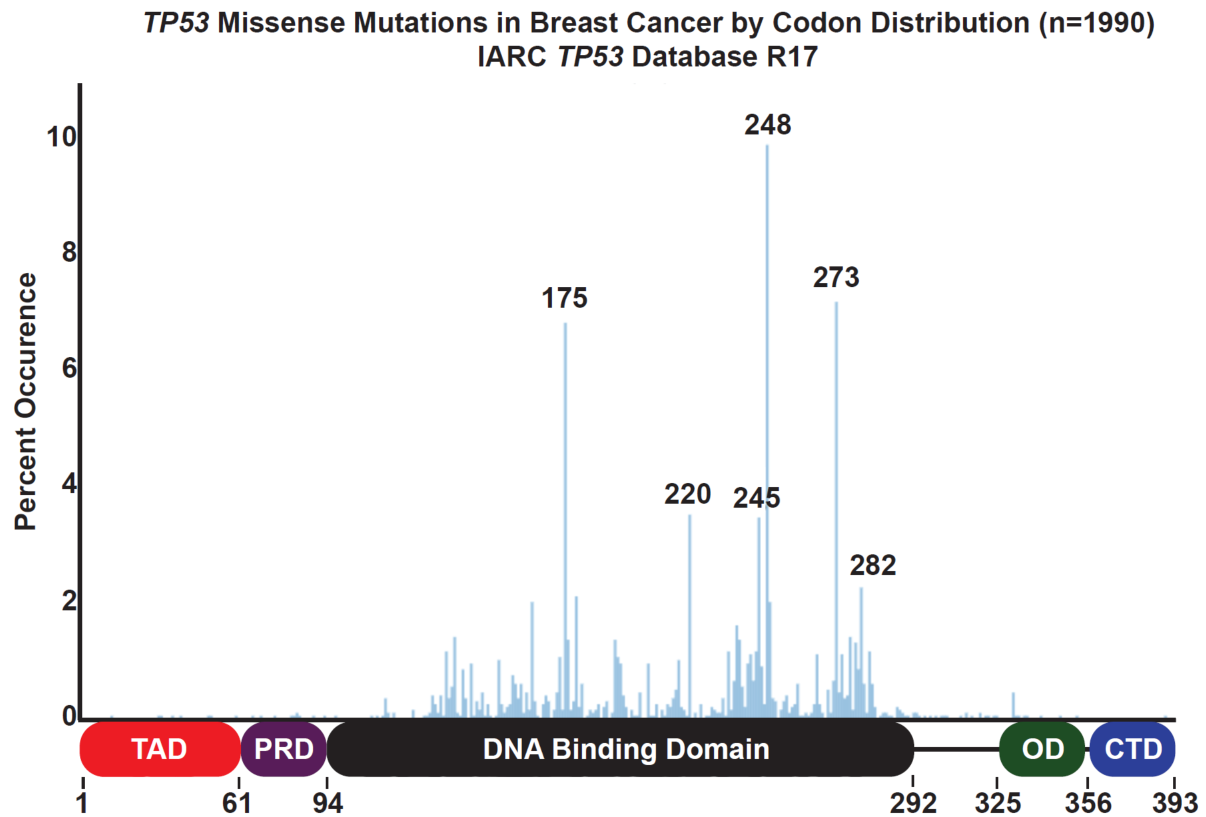
Pfister et al., Figure 1.1



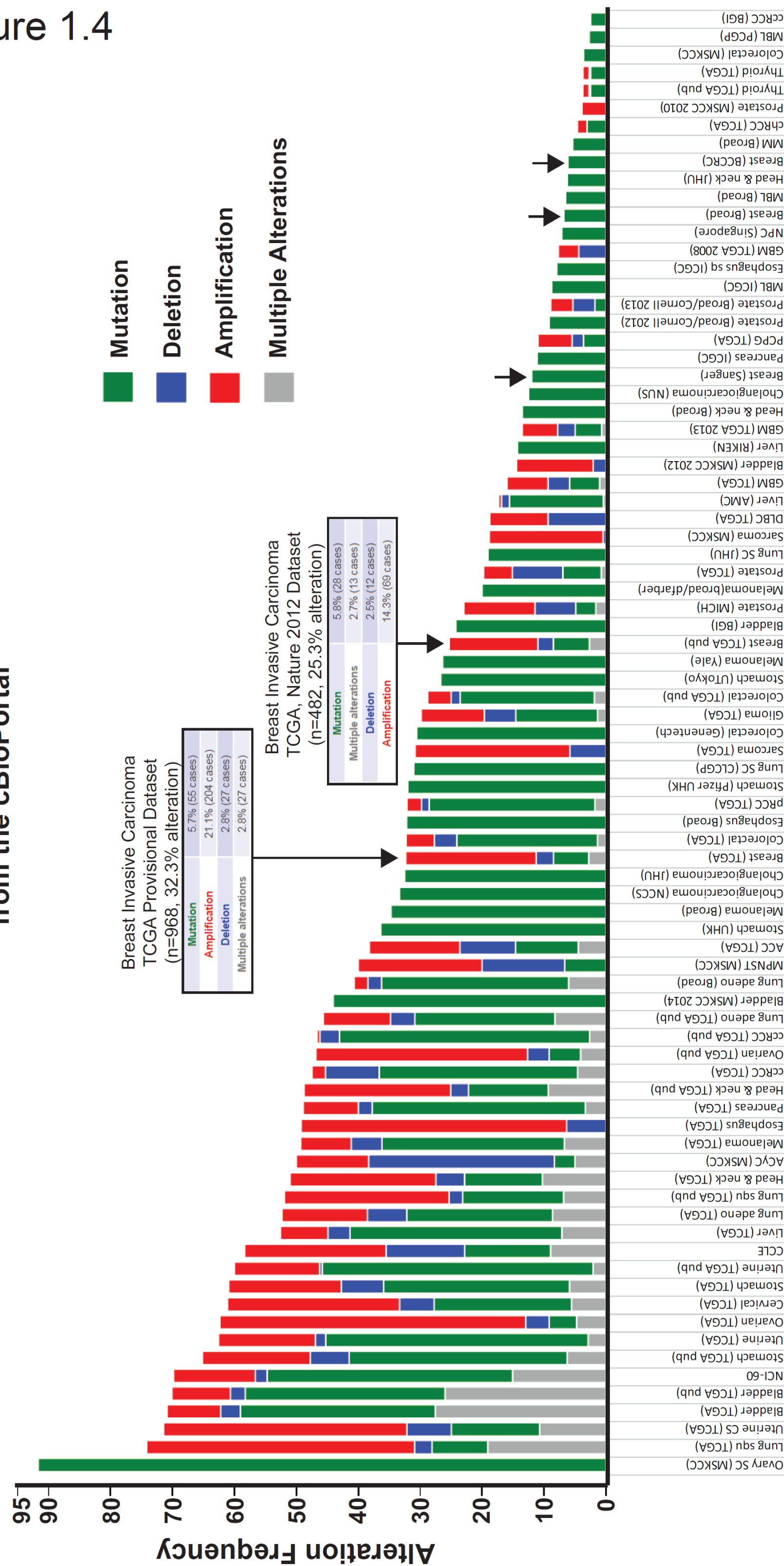
Pfister et al., Figure 1.2



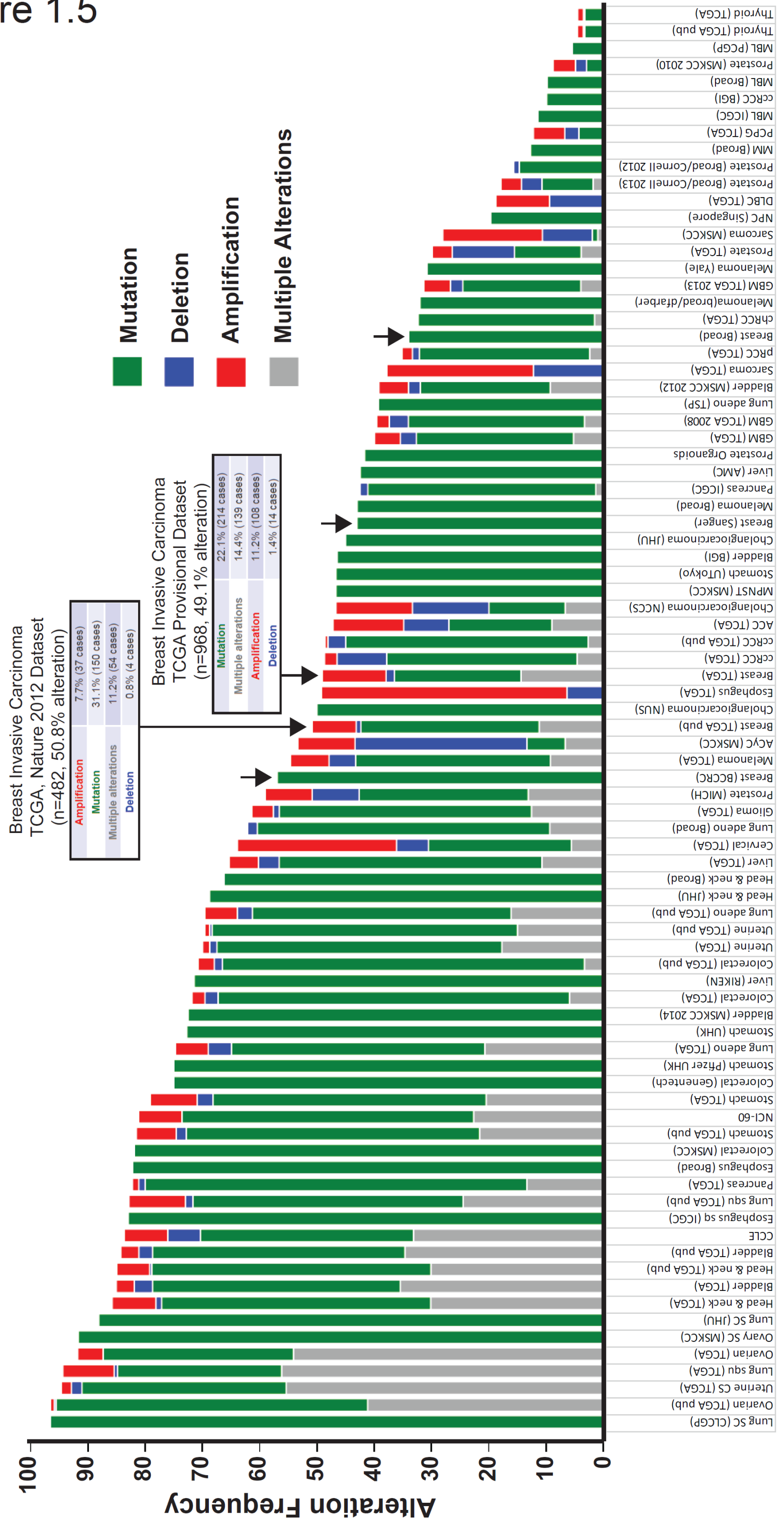
Pfister et al., Figure 1.3



Pfister et al., Figure 1.4



Pfister et al., Figure 1.5



Pfister et al., Table 1.1

GENE	CELL LINE	MUTANT	METHOD	STUDY
MDR1	NIH3T3, Saos-2, Caco-2, BHK	175H but not wild-type p53, 281G, requires TAD residues 14/19.Requires ETS-1 site; Del22/23 blocks Ets binding, Del360CTD still have Ets binding to 281G, and also Ets binding to 143A, 175H, 248W, 273H; 213Q and 234H no effect on MDR1; 281G mTAD1 ineffective	Overexpression- CAT reporter assay, Reporter assay	(Candau et al., 1997; Chin et al., 1992; Lin et al., 1995; Sampath et al., 2001; Strauss and Haas, 1995)
dUTPase	SK-OV-3 and 10(1)	175H, 248W (273H weak/failed, mTAD1 175H failed).	Overexpression , qPCR/northern	(Pugacheva et al., 2002)
TIM50 (ets-1, CREB ChIP)	1299, SKBR3, MDA-MB-468, Saos-2	175H, 273H, 281G, mTAD1 281G ineffective	Overexpression, siRNA, reporter assay, ChIP	(Sankala et al., 2011)
NF-kB2	H1299, 21PT, Saos-2	175H, 273H,281G, but not mTAD1-281G (mTAD = partial). 175H= increased NF-kB activity	Gene Expression Array w ectopic expression, qPCR, Reporter assay, EMSA	(Scian et al., 2005)
EBAG9, ITGA6, E2F5, MCM6, C-SYN	H1299	281G, but not mTAD1-281G	Gene Expression Array w ectopic expression, qPCR,	(Scian et al., 2005)
VDR gene (protein), VDR promoters RNA (IGFBP3, CYP24A1)	SKBR3, H1299, SW480, MDA-MB-231	175H (VDR motif over-represented), 273H, mTAD1-175H no effect on reporter assay	ChIP-on-chip, overexpression, southwestern blot, reporter assay	(Scian et al., 2005)
EGR1	H1299, PC3, SKBR3, HeLa	175H, 248W, 273H, 281G, but not mTAD1-175H or 179E or wtp53	Overexpression,Gene Expression Array, ChIP, Reporter assay, VEGF-induction by EGR1	(Weisz et al., 2004)
CXCL1 (GRO1)	SW480, MIA-PaCa-2, HCT116	273H/309S, 248W, 175H but not wtp53, mTAD1, mTAD2, PRD required in 245S and 248W, CTD inhibitory 248W, no effect 245S	siRNA, ectopic expression, ChIP,	(Yan and Chen, 2009, 2010)
Id2 (inhibition by mutant p53)	SW480, MIA-PaCa-2, HCT116	273H/309S, 248W, 175H but not wtp53, TAD1, TAD2, PRD required in 245S and 248W, CTD not required & perhaps inhibitory	siRNA, ectopic expression, ChIP,	(Yan and Chen, 2010; Yan et al., 2008)
c-Myc (mTAD1 & CTD required)	Cx3Ras (rat), 10(1) mouse cells, Saos-2,SK-OV-3 and 10 (1)	143A, 281G, 175H, 273H, 248; mTAD1 & CTD = intermediate phenotype with 281G; 175H, 248W, 273H activated c-myc but mTAD1 does not	Overexpression, Reporter assay, qPCR/Norther	(Frazier et al., 1998; Pugacheva et al., 2002)
c-Myc, apoptosis suppression	M1/2 myeloid cells	143A, mTAD1 required for c-myc and apoptosis suppression	Overexpression	(Matas et al., 2001)
induction of invasion/metastasis or p63/p73 inactivation	H1299	175H, 273H, TAD not required for transactivation	Overexpression	(Adorno et al., 2009; Oren and Rotter, 2010)
Spindle Checkpoint Control	Li-Fraumeni Fibroblasts	281G, TAD not required for transactivation	Overexpression	(Gualberto et al., 1998)
Apoptosis suppression, G2 arrest suppression	M1/2 myeloid cells	135V, CTD required for apoptosis suppression	Overexpression	(Sigal et al., 2001)
CXCL1	WI-38, Ras expressing	175H, 179R, TAD1 not required	Overexpression	(Solomon et al., 2012)
IL-1 beta	WI-38, Ras expressing	175H, 179R, TAD1 not required	Overexpression	(Solomon et al., 2012)
MMP3	WI-38, Ras expressing	175H, 179R, TAD1 not required	Overexpression	(Solomon et al., 2012)
TGF-Beta Receptor 2	H1299	175H, mTAD1 required for TGF-Beta Receptor 2 repression	Overexpression, Reporter Assay	(Kalo et al., 2007)

Pfister et al., Table 1.2

PROTEIN	INTERACTION WITH WILD-TYPE p53	MUTANT INTERACTION	STUDY
Ets-1	Yes	V143A, D281G, CTD may be required	(Do et al., 2012; Kim and Deppert, 2007; Kim et al., 2003; Sampath et al., 2001; Strano et al., 2007)
Ets-2	Yes	R175H (interaction requires part of mutant p53 containing the oligomerization domain), 248W	(Do et al., 2012)
Sp1	Yes	V134A, R175H, R249S, R273H, CTD/OD required for wtp53 interaction	(Bargonetti et al., 1997; Chicas et al., 2000; Gualberto and Baldwin, 1995; Hwang et al., 2011; Koutsodontis et al., 2005; Lee et al., 2000; Torgeman et al., 2001)
NF-Y	Yes	R175H, R273H, R273C, CTD required for binding to wtp53	(Di Agostino et al., 2006; Imbriano et al., 2005; Liu et al., 2011)
VDR	Yes	R175H, interaction does not occur in 1-292 amino acid mutant	(Stambolsky et al., 2010)
SMADs (2/3, maybe 4)	Unclear	175H, 273H, TAD likely required	(Adorno et al., 2009; Wilkinson et al., 2008)
E2F1	Yes	E2F1 binds wtp53 C-terminus, 175H and perhaps 280K recruit E2F1 to CDE consensus sequence	(Fogal et al., 2005; Fontemaggi et al., 2009)
TBP	Yes	mTAD1&2 required for wtp53	(Chang et al., 1995; Lee et al., 2000; Ragimov et al., 1993; Seto et al., 1992)
p63	No	R175H, Y220C, R248W, R273H (not D281G), interaction may not require TAD	(Adorno et al., 2009; Davison et al., 1999; Gaiddon et al., 2001; Strano et al., 2002)
p73	No	R175H, Y220C, V143A, R248W (not R273H), interaction may not require TAD	(Bensaad et al., 2003; Davison et al., 1999; Di Agostino et al., 2008; Di Como et al., 1999; Gaiddon et al., 2001; Marin et al., 2000; Oren and Rotter, 2010)
Med1	Yes	R213Q and/or Y234H (cell line contains both)	(Lottin-Divoux et al., 2005); (Drane et al., 1997; Meyer et al., 2010)
p300	Yes	R175H (note p53 mutants R175H, R273H were found to interact with NF-YA and NF-YB as well)	(Avantaggiati et al., 1997; Di Agostino et al., 2006)

Pfister et al., Table 1.3

GENE	CELL LINE	MUTANT	METHOD	STUDY
RhoGDI alpha	H1299, SKBR3, HT29	175H, 175H, 273H	Overexpression, qPCR, siRNA	(Bossi et al., 2008)
RANGAP1	H1299, SKBR3, HT29	175H, 175H, 273H	Overexpression, qPCR, siRNA	(Bossi et al., 2008)
RAB6KIFL	H1299, SKBR3, HT29	175H, 175H, 273H	Overexpression, qPCR, siRNA	(Bossi et al., 2008)
Seladin1 (DHCR24)	H1299, SKBR3, HT29, MDA-MB-468	175H, 175H, 273H	Overexpression, qPCR, siRNA	(Bossi et al., 2008; Freed-Pastor et al., 2012)
MAP2K3 (no TATA)	H1299, SKBR3, HT29 / also MDA-MB-468, MDA-MB-231	175H, 175H, 273H/ 280K, reg by NF-κB, NFY	Overexpression, qPCR, siRNA/ Overexpression (175/273), reporter assay, siRNA, ChIP	(Bossi et al., 2008; Gurtner et al., 2010)
IGFR1	H1299, SKBR3, HT29, Saos-2, RD, HeLa	175H, 175H, 273H,143A, but wtp53 suppresses	Overexpression, qPCR, siRNA, Reporter assay	(Bossi et al., 2008; Werner et al., 1996)
Paxillin Beta	SKBR3, HT29	175H, 273H	qPCR, siRNA	(Bossi et al., 2008)
BCL2L1	SKBR3, HT29	175H, 273H	qPCR, siRNA	(Bossi et al., 2008)
MDR1	NIH3T3, Saos-2, Caco-2, BHK	175H but not wild-type p53 (antagonistic to 175H mutant).281G, requires TAD residues 14/19.Requires ETS-1 site; Del22/23 block Ets binding, Del360CTD still have Ets binding to 281G, and also Ets binding to 143A, 175H, 248W, 273H; 213Q and 234H no effect on MDR1; 281G mTAD1 ineffective	Overexpression- CAT reporter assay, Reporter assay	(Candau et al., 1997; Chin et al., 1992; Lin et al., 1995; Sampath et al., 2001; Strauss and Haas, 1995)
PCNA	HeLa, Saos-2	V143A, R175H, R248W, R273H, D281G but not wild-type p53	Overexpression- CAT reporter assay	(Deb et al., 1992)
CCNA2	SKBR3, HT29, SW480, H1299	175H, 273H, 273H/309S, induction by adriamycin inhibited	WB, ChIP, Reporter Assay	(Di Agostino et al., 2006)
CCNB1	SKBR3, HT29, SW480	175H, 273H, 273H/309S, induction by adriamycin inhibited	WB, ChIP	(Di Agostino et al., 2006)
CCNB2	SKBR3, HT29, SW480, H1299-281G	175H, 273H, 273H/309S, 281G, induction by adriamycin inhibited	WB, ChIP, Overexpression, Reporter Assay	(Di Agostino et al., 2006)
CDK1	SKBR3, HT29, SW480	175H, 273H, 273H/309S, induction by adriamycin inhibited	WB, ChIP	(Di Agostino et al., 2006)
CDC25C	SKBR3, HT29, SW480, H1299-281G	175H, 273H, 273H/309S, 281G, induction by adriamycin inhibited	WB, ChIP, Overexpression, Reporter Assay	(Di Agostino et al., 2006)
ID4	H1299, SKBR3	175H, 273H, Sp1 and NF-kB implicated	Overexpression, gene array, ChIP, EMSA	(Fontemaggi et al., 2009)
c-Myc	Cx3Ras(rat), (10)1 mouse, Saos-2,	143A, 281G, 175H, 273H, 248(W?); mTAD1 & CTD = intermediate phenotype with 281G	Overexpression, Reporter assay	(Frazier et al., 1998)
ACAT2, HMGCS1, HMGCR, PMVK, MVD, IDI1, FDPS, SQLE, LSS, CYP51A1, SC4MOL, DHCR7	MDA-MB-468	273H	siRNA, qPCR, +/-ChIP	(Freed-Pastor et al., 2012)
MVK, FDFT1, TM7SF2, NSDH	MDA-MB-468, MDA-MB-231	273H, 280K	siRNA, qPCR, +/-ChIP	(Freed-Pastor et al., 2012)

TGF-Beta Receptor 2	H1299	175H, mTAD1 required for TGF-Beta Receptor 2 repression	Overexpression, Reporter Assay	(Kalo et al., 2007)
EPB41L4B, BUB1, MIS18A (C21orf45), NCAPH, CENPA, FAM64A, DEPDC1, CCNE2	MDA-MB-231, MDA-MB-468	280K, 273H	siRNA, qPCR, ChIP	(Girardini et al., 2011)
CPSF6, WDR67	MDA-MB-231	280K	siRNA, qPCR, ChIP	(Girardini et al., 2011)
hsMAD1	HeLa, HCT116	281G but not 143A	Overexpression, Reporter assay	(Iwanaga and Jeang, 2002)
Galectin-3	Saos-2, SW-1736, ARO	273H	Overexpression	(Lavra et al., 2009)
EGFR	Saos-2	V143A, R175H, R248W, R273H, D281G and also wild-type p53	Overeexpression, Reporter assay	(Ludes-Meyers et al., 1996)
Fos	Saos-2	C174Y (fails to transactivate MDR1)	Reporter assay	(Preuss et al., 2000)
dUTPase (DUT) (TAD1 required)	SK-OV-3 and 10 (1)	175H, 248W (273H weak/failed, mTAD1 175H failed). 175H, 248W, 273H activated c-Myc but mTAD1 does not	Overexpression , qPCR/northern	(Pugacheva et al., 2002)
TIM50 (Ets-1, CREB target)	H1299, SKBR3, MDA-MB-468, Saos-2	175H, 273H, 281G	Overexpression, siRNA, reporter assay (mTAD1 281G = maybe no activation), ChIP	(Sankala et al., 2011)
Asparagine synthetase	H1299, 10 (3), Saos-2	143A, 157F, 163C, 175H, 179Y, 194R, 273H, 281G, 282W	Overexpression, Reporter assay, ChIP-273H	(Scian et al., 2004)
hTERT	H1299, 10 (3), Saos-2	143A, 157F, 163C, 175H, 179Y, 194R,273H, 281G, not 282W	Overexpression, Reporter assay, ChIP-273H	(Scian et al., 2004)
NF-kB2	H1299, 21PT, Saos-2	175H, 273H,281G, but not mTAD1-281G (some mTAD = partial). 175H= increased NF-κB activity	Gene Expression Array w ectopic expression, qPCR, Reporter assay, EMSA	(Scian et al., 2005)
EBAG9, ITGA6, E2F5, MCM6, C-SYN	H1299	281G, but not mTAD1-281G	Gene Expression Array w ectopic expression, qPCR,	(Scian et al., 2005)
Stathmin	Huh-7, HepG2, U138-MG	213Q, 220C but not wtp53	Knockdown, WB	(Singer et al., 2007)
IGFBP3 and CYP24A1 (VDR target genes)	SKBR3, H1299, SW480, MDA-MB-231	175H (VDR motif over-represented), 273H, mTAD1-175H no effect on reporter assay	ChIP-on-chip, overexpression, southwestern blot, reporter assay	(Stambolsky et al., 2010)
hMMP-13	Saos-2	175H, 281G	Overexpression, WB, reporter assay	(Sun et al., 2000)
EGR1	H1299, PC3, SKBR3, HeLa	175H, 248W, 273H, 281G, but not mTAD1-175H or 179E or wtp53	Overexpression,Gene Expression Array, ChIP, Reporter assay, VEGF-induction by EGR1	(Weisz et al., 2004)
CXCL1 (GRO1)	SW480, MIA-PaCa-2, HCT116	273H/309S, 248W, 175H but not wtp53, mTAD1, mTAD2, PRD required in 245S and 248W, CTD inhibitory 248W, no effect 245S	siRNA, ectopic expression, ChIP,	(Yan and Chen, 2009, 2010)
Id2 (inhibition by mutant p53)	SW480, MIA-PaCa-2, HCT116	273H/309S, 248W, 175H but not wtp53, mTAD1, mTAD2, PRD required in 245S and 248W, CTD not required	siRNA, ectopic expression, ChIP,	(Yan and Chen, 2010; Yan et al., 2008)

Chapter 2

Mutant p53 Gain of Function in Breast Cancer Cells is Mediated by Cell Autonomous Expression of *VEGFR2*

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SUMMARY

Mutant p53 impacts the expression of numerous genes at the level of transcription to mediate oncogenesis. We identified vascular endothelial growth factor receptor 2 (*VEGFR2*), the primary functional VEGF receptor that mediates endothelial cell vascularization, as a mutant p53 transcriptional target in multiple breast cancer cell lines. By making use of 3D cell culture and other techniques, we demonstrate that mutant p53-mediated upregulation of VEGFR2 mediates mutant p53 gain of function by enhancing cellular growth and migration. We find that breast tumors with p53 hotspot mutants have elevated *VEGFR2* levels compared to tumors lacking p53 and elevated *VEGFA* and *HIF1A* levels compared to wild-type p53-expressing tumors. Importantly, a clinical trial suggests that *TP53* mutated breast tumors may specifically respond to anti-VEGFR2 therapy, while *TP53* wild-type tumors may not respond. These data suggest that mutant p53-containing breast tumors may be distinctively vulnerable to anti-VEGF therapies.

INTRODUCTION

TP53 is the most frequently mutated gene found in human cancers (Olivier et al., 2010). Wild-type p53 is a sequence-specific transcription factor that when activated by various stresses such as DNA damage, oncogenic signaling or nutrient depletion, promotes cellular outcomes such as cell arrest, cell death, senescence, metabolic changes and others, depending on the extent and context of the stress (Vousden and Prives, 2009). In human cancer p53 primarily sustains missense mutations in its conserved DNA binding domain. The small number of residues (~5-6) within this region that are mutated with extraordinarily high frequency are termed hotspot mutations. These mutations can be loosely divided into two categories, the contact mutants (e.g. R273H), which remain well folded but whose mutated residues fail to make specific contact with elements within the DNA binding site and conformational mutants (e.g. R175H) that are partly unfolded leading to loss of zinc coordination and general DNA binding. Evidence from sources as varied as human epidemiology studies, mouse models and cell-based experiments has shown that these hotspot missense mutant forms of p53, which often accumulate to high levels in the cells they inhabit, can acquire neomorphic properties such as increased metastases in mice and increased motility and invasive characteristics in cultured cells (Brosh and Rotter, 2009; Muller and Vousden, 2014). In Li-Fraumeni patients, missense mutation was reported to lead to earlier tumor onset than other forms of p53 loss (Bougeard et al., 2008). p53 hotspot mutant proteins have been reported to associate with chromatin and alter a cell's transcriptional profile, leading to oncogenic cellular changes (Cooks et al., 2013; Di

Agostino et al., 2006; Do et al., 2012; Freed-Pastor et al., 2012; Stambolsky et al., 2010).

By examining an array-based data set comparing MDA-468 cells with normal vs. reduced levels of mutant p53 we discovered that mutant p53 activates the mevalonate pathway to promote invasive properties of breast cancer cells (Freed-Pastor et al., 2012). When we reanalyzed the global gene expression analysis from these data, vascular endothelial growth receptor 2 (*VEGFR2*/*KDR*/*FLK1*) was identified as a gene strongly induced by mutant p53. VEGFR2 is a receptor tyrosine kinase that is activated upon VEGF ligand binding and, under normal physiological conditions, mediates angiogenesis (Ferrara, 2004). VEGFR2 is the key receptor for endothelial cell neovascularization and mediates increased cellular proliferation, migration, and pro-survival signaling (Ferrara, 2004).

In addition to the breast tumor vasculature, VEGFR2 is often aberrantly expressed on the breast tumor epithelia (Ryden et al., 2003). Increased VEGF or VEGFR2 expression on breast tumor cells each correlate with decreased survival (Ghosh et al., 2008). The VEGFR2 ligand VEGF is the clinical target of anti-VEGF therapies including bevacizumab, which in 2011 lost FDA approval for metastatic breast cancer, revocation of which may have been due to inability to distinguish the candidates who would respond to treatment. Interestingly, wild-type p53 is a canonical repressor of the VEGF pathway through multiple mechanisms including transcriptional repression of *VEGFA* (Mukhopadhyay et al., 1995) and MDM2-induced degradation of HIF1A (Ravi et al., 2000). Loss of wild-type p53 function promotes the angiogenic switch by derepressing HIF1A and VEGFA, thereby promoting tumor neovascularization (Ravi et

al., 2000). We propose that hotspot mutation in p53 provides additional oncogenic potential to breast cancer cells compared to simple loss of p53 function due to the induction of VEGFR2 expression.

RESULTS

Mutant p53 Promotes *VEGFR2* Expression in Breast Cancer Cell Lines

Using a 3D tissue culture system, global gene expression profiling was performed in MDA-468 breast cancer cells that contain a doxycycline-inducible short hairpin RNA (shRNA) to the 3'-untranslated region of the p53 messenger RNA (MDA-468.shp53 cells) (Freed-Pastor et al., 2012). MDA-468 cells express only the R273H p53 hotspot mutant from the endogenous *TP53* locus. Upon re-analysis of the gene expression profiling datasets from our earlier study, *VEGFR2* was identified as the number 8 overall most upregulated gene by mutant p53 and in the top percentile of upregulated genes (Table 2.S1). *VEGFR2* was chosen for further study because it is a clinically important gene that is known to mediate tumor neovascularization and in breast cancer cells can mediate pro-oncogenic signaling through autocrine activation (Guo et al., 2010).

Using quantitative RT-PCR (qRT-PCR), we confirmed that *VEGFR2* RNA levels are strongly correlated with mutant p53 protein levels in MDA-468.shp53 cells in 3D culture conditions (Figure 2.1A). We also observed decreased *VEGFR2* expression in 2D culture conditions (Figure 2.S1A), although to a lesser extent than was observed in 3D culture (75% depletion to 90% depletion of *VEGFR2* transcript in 2D and 3D cultures, respectively). Mutant p53 regulated expression of *VEGFR2* at the level of

transcription, as we detected reduced expression of intronic *VEGFR2* transcript to the same extent as total *VEGFR2* RNA (Figure 2.S1B). Stepwise depletion of mutant p53 led to increasing reductions in VEGFR2 levels (Figure 2.S1C). Reduction of *VEGFR2* RNA corresponded to depletion of VEGFR2 protein isoforms, which differ in migration pattern based on varying post-translational modifications (Figures 2.1A, 2.S1A, 2.S1C)(Bruns et al., 2010). Mutant p53 regulated *VEGFR2* in two additional breast cancer cell lines that express endogenous p53 hotspot mutants. Using MDA-231 cells (p53 R280K), we found that p53 depletion by two different siRNAs (Figure 2.1B) or using doxycycline to induce p53 shRNA in MDA-231.shp53 cells (see Figure 2.3B) resulted in significant reduction in *VEGFR2* expression. Depletion of mutant p53 by two different siRNAs in SK-BR-3 cells (p53 R175H) grown in 2D cultures also led to reduction in *VEGFR2* transcript (Figure 2.S1D). Thus, mutant p53 is a regulator of *VEGFR2* expression in multiple breast cancer cell lines endogenously expressing both conformational and contact p53 hotspot mutations.

To determine whether different p53 hotspot mutants activate expression of *VEGFR2*, we engineered MDA-468.shp53 cells to express hotspot p53 mutants R175H, G245S, and R248W that lack the targeting region of the inducible p53 shRNA in these cells (Freed-Pastor et al., 2012). In this situation endogenous p53 R273H was depleted upon addition of doxycyline, so the great majority of the remaining p53 isoform in each cell was the respective ectopic hotspot mutant (Figure 2.1C). We found that p53 R175H fully rescued and p53 G245S partially rescued the ability of the depleted endogenous mutant p53 to transactivate the *VEGFR2* promoter as compared to *VEGFR2* expression levels in untreated MDA-468.shp53 cells. By contrast, expression of p53 R248W, also a

hotspot mutant, failed to increase *VEGFR2* expression (Figure 2.1C). With the caveat that these ectopically expression proteins were overexpressed when compared to the endogenously expressed p53, these data indicate that at least three different hotspot mutants can activate *VEGFR2* expression, including a contact mutant (endogenous p53 R273H) and conformational mutants (p53 R175H and p53 G245S) in the MDA-468 cell line. These data also suggest that different p53 hotspot mutants possess intrinsically different capacities to activate *VEGFR2* transcription.

Mutant p53 Status Correlates with Increased *VEGFR2* in Human Breast Cancer Samples

A fundamental question is whether mutant p53 impacts *VEGFR2* expression in human breast tumors. To address this, we sorted the Breast Invasive Carcinoma TCGA Provisional dataset into *TP53* mutation classes including wild-type, hotspot missense mutation, non-hotspot missense mutation, and truncation mutation, which includes nonsense, frameshift, in-frame deletion, and in-frame insertion mutations that are predicted to alter wild-type p53 activities such as ability to repress VEGF; Table 2.S2). To extend our query to other angiogenesis related genes known to be affected by p53 status, we analyzed normalized RNA-Seq expression values for *VEGFR2*, *VEGFA*, and *HIF1A*. Five hotspot mutants of p53 observed in breast cancer (Walerych et al., 2012) were selected prior to analysis and are present in 49 out of 969 tumors in the dataset (R175, Y220, G245, R248, R273; Table 2.S3). Comparing expression levels of *VEGFR2* to tumors that contain truncation mutations in p53, hotspot mutant tumors express significantly elevated levels of *VEGFR2* ($p < 0.05$, Figure 2.1D). Furthermore,

compared to wild-type p53, hotspot mutants of p53 express elevated levels of *VEGFA* and *HIF1A*, which are potent pro-angiogenic factors that potentiate *VEGFR2* activation (Figure 2.1E-F)(Ferrara, 2004). All classes of inactivating p53 mutations (hotspot, non-hotspot missense and truncation mutants) correlated with significantly increased levels of *VEGFA* and *HIF1A* ($p < .05$ in each case), suggesting that upregulation of *VEGFA* and *HIF1A* is due to de-repression of wild-type p53 rather than activation by mutant p53 (Figure 2.1E-F). We speculate that hotspot mutant p53-containing breast tumors are unique in being able to regulate a pattern of pro-angiogenic gene expression that may preferentially potentiate *VEGFR2* autocrine signaling compared to tumors with wild-type p53 or other forms of loss of p53 function.

Cell-Autonomous *VEGFR2* Expression Mediates Mutant p53 Gain of Function

Mutant p53 has been reported to promote cell growth and invasiveness in 3D culture models of breast cancer (Freed-Pastor et al., 2012; Muller et al., 2009). To investigate whether *VEGFR2* mediates downstream effects of mutant p53, including increased cellular growth and invasive characteristics, we pharmacologically inhibited VEGFR2 with semaxanib (SU5416), a potent inhibitor of VEGFR2 autophosphorylation with an IC50 of 1.23 μM (Fong et al., 1999). In 3D cultures, inhibition of VEGFR2 with semaxanib prevented growth of MDA-231 and MDA-468 breast cancer cells but not of MCF10A immortal breast cells or MCF7 breast cancer cells that express wild-type p53 (Figure 2.S2A-D).

To further define VEGFR2 as an oncogene that can mediate mutant p53 gain of function, we depleted VEGFR2 with two different siRNAs and monitored cell growth in

3D culture. MDA-231 and MDA-468 cells were significantly inhibited in 3D growth upon depletion of VEGFR2 with siRNA, recapitulating the effect of depletion of mutant p53 (Figure 2.2A-B with corresponding immunoblots in Figure 2.S2E-F). Furthermore, we observed that the MDA-231 cells, which in 3D cultures form stellate-appearing clusters, had mostly lost their characteristic invasive-appearing processes (Figure 2.2A)(Kenny et al., 2007). These data indicate that, with respect to 3D culture gross morphology, loss of VEGFR2 phenocopies loss of mutant p53 and suggest that VEGFR2 is required for efficient growth of mutant p53-containing breast cancer cells. They also suggest that cell-autonomous VEGFR2 signaling is required for cell growth in cell lines that contain mutant p53.

To determine whether VEGFR2 expression can rescue loss of mutant p53, MDA-231.shp53 cells expressing a doxycycline-inducible short hairpin RNA to p53 (Freed-Pastor et al., 2012) were engineered to stably express VEGFR2 or a phosphorylation-defective VEGFR2 mutant (VEGFR2-Y1059F)(Jinnin et al., 2008). As expected, loss of mutant p53 led to dramatic reduction in size of the invasive, stellate-shaped clusters of MDA-231.shp53 cells (top panels of Figure 2.3A). Remarkably, when VEGFR2 was expressed in cells with reduced endogenous mutant p53, the growth properties and morphological characteristics of the cell clusters were restored ($p < 0.001$, Figure 2.3A-C). Further, cells expressing phosphorylation-defective VEGFR2-Y1059F failed to rescue the loss of mutant p53, indicating that the rescue with VEGFR2 is due to pro-oncogenic signaling properties mediated by this receptor tyrosine kinase (Figure 2.3A-C). Furthermore, using a wound closure assay in MDA-231 cells our data indicated that VEGFR2 and mutant p53 are each required for cellular migration (Figure 2.3D, Figure

2.S3A). Note that MDA-468 cells do not migrate efficiently and are not amenable to such measurements. These data implicate VEGFR2 as a proto-oncogene in breast cancer cells that, when transactivated by mutant p53, functions as an oncogene that can mediate mutant p53 gain of function effects that are consistent with characteristic growth and invasive properties of tumor cells.

Mutant p53 Breast Tumors Preferentially Respond to Bevacizumab

To determine whether mutant p53-expressing breast tumors preferentially respond to anti-VEGF therapy, we analyzed the response in tumors with wild-type *TP53* vs. mutated *TP53* from the NeoAva study (Figure 2.3E-F; see Methods for further description). Interestingly, across all patients, response ratios were higher in patients with *TP53* mutated tumors (Figure 2.S3B-D). Most relevantly, among patients who received chemotherapy+bevacizumab, the pathological complete response (pCR) was 33.3% vs. 17.1% in *TP53* mutated vs. wild-type tumors (Figure 2.S3C). A higher pCR rate in *TP53* mutated tumors compared to wild-type tumors (27.7% vs. 4.5%) was also observed among patients receiving chemotherapy alone, so a benefit of bevacizumab cannot be concluded based on pCR in this patient cohort (Figure 2.S3C). When treatment response was analyzed as a continuous variable, however, a greater reduction in tumor volume was observed when bevacizumab was combined with chemotherapy in *TP53* mutated tumors compared to tumors with wild-type *TP53* (Figure 2.3E-F). The p-value of this observation, $p = 0.28$, suggests that a larger sample size is necessary to confirm a therapeutic effect of bevacizumab on p53 mutated breast tumors. Nevertheless, these data suggest that, as predicted by the cell culture data,

inclusion of anti-VEGF therapy in p53 mutated breast tumors might lead to enhanced anti-tumor response.

DISCUSSION

VEGFR2 is a candidate proto-oncogene (Ding et al., 2008) that is correlated with decreased survival in breast cancer patients (Ghosh et al., 2008). Here we identified *VEGFR2* as a transcriptional target of mutant p53 in breast cancer cells (Figure 2.1). In human tumors, hotspot mutation in *TP53* correlates with increased *VEGFR2* expression and elevated *HIF1A* and *VEGFA* levels, which are repressed in tumors with wild-type p53 (Figure 2.1D-F). Wild-type p53 is known to inhibit the VEGF pathway by multiple mechanisms including repression of *VEGF* expression and reduced HIF1A, so mutation in *TP53* leading to loss of such activities will promote VEGF pathway signaling (Bergers and Benjamin, 2003). In our experiments mutant p53-stimulated *VEGFR2* expression is necessary and sufficient for increased growth and migration of cultured breast cancer cell lines due to cell-autonomous VEGFR2 signaling. That tumors containing mutant p53 are likely to be more susceptible to anti-angiogenic therapy is supported by clinical data shown in Figure 2.3E-F.

Interestingly, mutant p53 has been reported to stimulate additional receptor tyrosine kinases including EGFR (Ludes-Meyers et al., 1996), IGF1R (Werner et al., 1996), MET (Muller et al., 2013), and PDGFRB (Weissmueller et al., 2014), all of which, along with VEGFR2, promote pro-proliferative signaling. As a tumor forms, acquisition of a hotspot mutation in *TP53* may facilitate transcriptional plasticity, whereby tumor cells increase capacity for gene expression changes and therefore undergo selection for

the greatest pro-proliferative transcriptional program for the particular tumor context. This hypothesis explains, for instance, why such a wide array of genes and pathways has been reported to mediate mutant p53 gain of function.

Mutant p53 is associated with decreased overall survival in breast cancer (Langerod et al., 2007), which is most likely due to increased rate of metastases, a known phenotype in mutant p53 mouse models (Adorno et al., 2009; Lang et al., 2004; Olive et al., 2004; Weissmueller et al., 2014). *TP53* mutation facilitates the angiogenic switch by de-repressing HIF1A and VEGFA expression (Mukhopadhyay et al., 1995; Ravi et al., 2000), promoting expression of pro-angiogenic factors that enhance tumor angiogenesis, growth, and metastatic potential (Folkman, 2002). Our data suggest that p53 hotspot mutants may be selected over loss of function p53 mutants during the progression of breast cancer in part due to the advantages conferred by cell-autonomous VEGFR2 signaling.

We point out that the mevalonate pathway previously shown to be regulated by mutant p53 (Freed-Pastor et al., 2012) and VEGFR2 pathways are not mutually exclusive. VEGFR2 requires multiple products of the mevalonate pathway to function including plasma membrane components as well as post-translational lipid modifications to signaling mediators (Guo et al., 2010; Mo and Elson, 2004). Indeed, multiple pathways may be altered by mutant p53 within an individual tumor, or even due to mutual interactions among tumor cells in the microenvironment, to promote pro-proliferative capacities.

We have reported on a mutant p53 transcriptional target that could lead to clinical interventions. VEGFR2 is upregulated by mutant p53 and functions as an oncogene that

can independently mediate the pro-proliferative and pro-migratory effects of mutant p53 (Figures 2.1-3). *TP53* loss of function mutations correlate with increased angiogenic potential in breast tumors (Figure 2.1E-F), while *TP53* hotspot mutations correlate with increased *VEGFR2* levels (Figure 2.1D). We suggest that classifying breast tumors by *TP53* mutational status could improve response rates to anti-VEGF therapy (Figure 2.3E-F). Because VEGFR2 functions as an oncogene in mutant p53-expressing cells, we postulate that breast tumors expressing hotspot mutants of p53 will be especially sensitive to anti-VEGF therapy due to the combined effect of inhibiting mutant p53-induced pro-proliferative *VEGFR2* signaling compounded with antagonistic effects on tumor vasculature. Oncology is headed toward highly adaptable treatment regimens based on the particular genetic alterations of a tumor. Defining the contributions of mutant p53 and VEGFR2 to breast cancer tumorigenicity are likely to be critical steps toward identifying specific tumor alterations that can be therapeutically harnessed. Future work should define whether patients with mutant p53-expressing breast tumors demonstrate improved survival with anti-VEGF treatment.

EXPERIMENTAL PROCEDURES

Reagents

Plasmids

pLNCX-Flag-p53-R175H, -G245S, -R248W and doxycycline-inducible shp53 plasmids were generated as previously described (Freed-Pastor et al., 2012). pcDNA3.1-*VEGFR2* and pcDNA3.1-*VEGFR2*-Y1059F were a kind gifts from Dr. Lena

Claesson-Welsh and Dr. Bjorn Olsen, respectively (Jinnin et al., 2008). Constructs were verified by sequencing using primers listed in Table 2.S4.

siRNAs

For siRNA knockdown experiments, Silencer® Select siRNAs were purchased from Life Technologies and are the following: siRNA to *TP53* (s605 and s606) and *VEGFR2* (s7822 and s7823). Silencer® Select Negative Control #1 siRNA (Life Technologies) was used as control siRNA. DharmaFECT 1 (Thermo Scientific) was used as the transfection reagent for all siRNA knockdown experiments. siRNA sequences are listed in Table 2.S4.

Antibodies

p53 was detected using a combination of mAb 1801/mAb DO-1 (both in-house purified from hybridoma supernatants) or with polyclonal FL393 (sc-6243, Santa Cruz Biotechnology). Anti-Actin (A2066) antibodies were purchased from Sigma. Anti-VEGFR2 (55B11) rabbit mAb was purchased from Cell Signaling Technology.

Drugs

The following drugs were purchased from Sigma Aldrich: SU5416 (semaxanib, S8442), Hydrocortisone (H4001), Insulin (I1882), Doxycycline (D9891), DMSO (D5879), and Mitomycin C (Sigma M4287). EGF was purchased from Peprotech (AF-100-15). For drug treatment experiments doxycyline was dissolved in H_2O and utilized at a final concentration of 10 μg/mL, which was determined to generate maximal depletion of endogenous mutant p53. SU5416 (semaxanib) was dissolved in DMSO and added to cell cultures 48 hours post-plating at the listed experimental concentrations. DMSO was used as a vehicle control in untreated cells.

Cell Cultures

Cell Lines and Generation of Stable Cell Lines

MDA-468, MDA-231, SK-BR-3, and MCF7 cells were maintained in DMEM + 10% Fetal Bovine Serum (FBS, Gemini Bio-Products). MCF10A cells were maintained in DMEM/F12 supplemented with 5% horse serum (Life Technologies), 10 μg/ml Insulin, 0.5 μg/ml Hydrocortisone and 20 ng/ml Epidermal Growth Factor (EGF). All cells were maintained at 37°C in 5% CO_2. Unless otherwise stated we refer to these growth conditions as two-dimensional (2D) cultures to distinguish them from three-dimensional (3D) culture conditions described below.

Clonal MDA-468.shp53 and clonal MDA-231.shp53 cells, as well as MDA-468.shp53-175H, -245S, -248W derivative cell lines are previously described (Freed-Pastor et al., 2012). Stable MDA-231.shp53 cell lines were developed to overexpress control vector (pcDNA3.1-GFP), pcDNA3.1-*VEGFR2*, and pcDNA3.1-*VEGFR2*-Y1059F by transfection of linearized pcDNA3.1 vector. Stable clones were selected with G418 (Gemini Bio-Products). To induce shRNA expression, cells were treated with 10 μg/ml doxycycline from day 0 for time periods indicated in the figure legends. When overexpressing *VEGFR2* or mutant p53 derivatives, MDA-468.shp53 and MDA-231.shp53 cells lines were maintained in doxycycline to deplete endogenous mutant p53. For siRNA knockdown experiments, cells were seeded 24 hours prior to transfection.

3D Cultures

The 3D cell culture protocol was performed as previously described (Debnath et al., 2003). For routine imaging, 8-well chamber slides were lined with 45 μL of growth

factor reduced Matrigel (356231, BD Biosciences). Cells were seeded at 5,000 cells/well in assay medium (DMEM/F12 + 2% Horse Serum + 10 μg/mL Insulin + 0.5 μg/mL Hydrocortisone + 2% Matrigel), with 5 ng/ml EGF supplemented to MCF10A cultures. For RNA, protein, or chromatin analyses from 3D cultures, 35 mm plates were lined with 475 μl Matrigel and cells were seeded at a density of 175,000 to 225,000 cells/plate in assay medium + 2% Matrigel. Cells were re-fed with assay medium on day 4 and imaged or collected for analysis on day 8. When siRNA was utilized, cells grown in 2D conditions were transfected with 50 nM of siRNA and 24 hours later cells were plated in 3D culture conditions. Cells were harvested using Cell Recovery Solution (BD Biosciences). Where indicated drug concentrations in 3D cultures were maintained when refreshing media. Differential interference contrast images were acquired by live imaging at 10X magnification using a LSM 700 confocal microscope with ZEN 2011 software (ZEISS). Multiple fields of each imaged were obtained and representative images were chosen for presentation. Where needed the Colony Blob Count Tool (Baecker, 2012) program was utilized within ImageJ (Schneider et al., 2012) to calculate the area of cells grown in 3D culture conditions. Areas of each independent replicate were quantitated using settings to control for background lighting. Incorrect program measurements, determined by counting an area of greater than one cell cluster as an individual colony or by counting an area in which no cell cluster exists were manually excluded.

Migration Assay

Cell culture inserts (Ibidi #80209) were place in 35 mm tissue culture dishes to form an approximately 500 μm cell-free gap. Approximately 25,000 MDA-231 cells that

were transfected with 50 nM of siRNA to deplete mutant p53 or VEGFR2 24 hours earlier were trypsinized, quantitated by MOXI Z automated cell counter (ORFLO Technologies), and added to each side of the cell culture insert gap. Approximately 36 hours after the cells were seeded (60 hours with siRNA), the cell culture insert was removed with sterile forceps. Fresh media was added that was supplemented with 5 μg/mL mitomycin C (Sigma M4287) to prevent cell proliferation. Cell were imaged at 0 and 48 hours, which approximated wound closure for the control sample, using differential interference contrast images acquired by live imaging at 10X magnification using a LSM 700 confocal microscope with ZEN 2011 software (Carl Zeiss AG). Total migration was calculated by measuring with Adobe Photoshop ruler tool the total distance migrated by each side of the wound relative to 0 hours for three images per biological replicate.

RNA Expression

Quantitative RT-PCR

For most experiments, RNA was isolated from cells using the Qiagen RNeasy Mini Kit. Complementary DNA was generated using the Qiagen Quantitect reverse transcription kit using 1 μg of input RNA as measured by NanoDrop Spectrophotometer (Thermo Scientific). Real-time PCR was carried out on an ABI StepOne Plus machine using SYBR green dye. Transcript levels were assayed in triplicate and normalized to *RPL32* mRNA expression. Relative changes in cDNA levels were calculated using the Comparative-Ct Method ($\Delta\Delta C_T$ method). All qRT-PCR primers were designed with Primer Express (Applied Biosystems) from genomic DNA sequence from the UCSC

Human Genome Browser hg19 assembly. Primer targeting was confirmed with the UCSC Human Genome Browser *in silico* PCR tool. All primer sequences were validated for amplification efficiency by comparison to a genomic DNA standard curve and amplify single targets as determined by melting curve analysis. Primer sequences are listed in Table 2.S4. All primers were purchased from Life Technologies.

Breast Cancer Patient Datasets

Breast Tumor Analysis from TCGA Provisional Breast Cancer Dataset

The Cancer Genome Atlas (TCGA) datasets (Network, 2012) were downloaded directly from the TCGA data portal (February 2014). The Breast Invasive Carcinoma (BRCA) TCGA Provisional dataset was used for analysis. The datasets were imported into Matlab and data analysis was performed using Matlab scripts (Sobie, 2011). First, the somatic mutations dataset was analyzed to determine tumor samples that had mutations in *TP53*. We stratified the tumor samples based on their *TP53* mutational status. The tumor samples that were sequenced for somatic mutations but did not report any mutations in the *TP53* locus are assumed to be wild-type for *TP53*. This dataset included information on the type of mutations in *TP53* such as missense, nonsense, in-frame deletion, in-frame insertion, frameshift and silent mutations. The nonsense, frameshift, in-frame deletion, and in-frame insertion mutations generally produce a truncated, nonfunctional transcript and by this justification were pooled into one group and labeled as truncation mutations. For the purposes of our analysis, missense mutations in residues R175, Y220, G245, R248, and R273 were classified *a priori* as hotspot mutations, as these are the most frequently mutated residues in breast cancer

(Table 2.S3)(Feki and Irminger-Finger, 2004; Walerych et al., 2012). All other missense mutations were classified as non-hotspot missense mutations. Tumor samples with silent mutations were not considered for the purpose of our analysis. Thus, all tumor samples were stratified on the basis of *TP53* mutational status. Then, the RNA-sequence V2 (RNA-SeqV2) dataset was downloaded and analyzed to determine the expression levels of genes of interest. In the TCGA portal, the RNA-SeqV2 dataset includes the normalized gene expression of all genes as estimated by upper quartile normalization procedure using the RSEM software package. RNA expression values were analyzed as upper quartile normalized RNA-Seq by Expectation-Maximization (RSEM) of reads. This data was imported into Matlab and used for analysis. The median gene expression was calculated for each gene of interest following tumor sample stratification based on *TP53* status and plotted using the box plots function. The statistical significance of the findings was determined by Welch's t-test (Jeanmougin et al., 2010). In the case of *VEGFR2* gene, we hypothesized that the gene expression (as determined by RNA sequencing) of tumor samples with hotspot mutations in *TP53* would be higher than other samples. Hence, the one tailed t-test was used in this case. We then extended our analysis to other genes that are also involved in the angiogenic pathway. In this case, we used the two-tailed t-test and corrected for multiple testing by using the false discovery rate procedure (FDR) of Benjamini and Hochberg to obtain the adjusted p-values (Hochberg and Benjamini, 1990). The box plots in the figure were plotted in Matlab and are standard box plots with the notch to show the confidence intervals of the median of gene expression. For the sake of visual clarity, the outliers are not displayed on the plot. In the plots, the asterisk (*) symbol denotes statistical

significance (p-value < 0.05). The accuracy of the analytical procedure was verified by corroborating multiple samples to the results obtained from the cBioPortal website (Gao et al., 2013).

The NeoAva Study

Patients with HER2 negative mammary carcinomas (> 2.5 cm; stage T2, T3 or T4) previously untreated for the current disease were included in the NeoAva study. The study was approved by the institutional protocol review board, the regional ethics committee, the Norwegian Medicines Agency and was carried out in accordance with the Declaration of Helsinki and ICH guidelines for Good Clinical Practice. The study was registered in the ClinicalTrials.gov database with the identifier NCT00773695. The patients were recruited into the study at 3 sites in Norway (The Norwegian Radium Hospital, Ullevål University Hospital and St. Olav's hospital). Written informed consents were obtained from all the patients prior to inclusion. While 132 patients received neoadjuvant chemotherapy, 12 patients (not reported here) were allocated to an endocrine treatment arm. The patients were further randomized to receive or not to receive bevacizumab in addition to chemotherapy. Pathological complete response (pCR) was the primary endpoint and was defined as complete eradication of all invasive and non-invasive forms of cancer from breast and lymph nodes. Percentage of tumor shrinkage was determined by taking ratio of the size of the tumor at surgery to the size of the tumor at inclusion (termed 'response ratio'), giving a continuous scale of response to treatment.

TP53 mutational status was assessed by sequencing the entire coding region (exons 2–11), including splice junctions using BigDye Direct Cycle Sequencing Kit (Life

Technologies). The samples were run on a 3730 DNA Analyzer (Life Technologies), a capillary electrophoresis-based automated DNA sequencer. *TP53* mutational status was successfully obtained for 124 of the total 132 patients in the chemotherapy cohort. Response ratio data is missing and thus not included for 7 out of 124 samples with *TP53* status available due to unavailability of post-treatment tumor measurements. P-values are derived from the Kruskal-Wallis test.

ACKNOWLEDGEMENTS

We thank Ella Freulich for excellent technical assistance and Lynn Biderman, Yan Zhu, Masha Poyurovsky as well as other members of the Prives laboratory for helpful discussions. This work was supported by NIH grant CA87497 and the MSTP program at Columbia University Medical School.

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FIGURE LEGENDS

Figure 2.1 Mutant p53 Promotes *VEGFR2* Expression in Breast Cancer Cells

(A) MDA-468.shp53 cells were grown in 3D culture conditions for 8 days with (+ DOX) and without (-DOX) doxycycline to induce an shRNA targeting mutant p53. Total *VEGFR2* transcript was assayed by qRT-PCR and normalized to -DOX condition. **$p < 0.001$ by one tailed t-test. Below is the related immunoblot showing levels of the indicated proteins.

(B) MDA-231 cells were grown in 3D culture conditions and assayed for *VEGFR2* expression following depletion of mutant p53 with two different siRNAs as described in Methods. Expression is normalized to control siRNA. *$p < 0.05$, **$p < 0.01$ by one tailed t-test. Immunoblot at right shows indicated protein levels with control or p53 siRNAs.

(C) MDA-468.shp53 cells were selected to stably express mutant p53 hotspot mutants R175H, R245S, or R248W that lack the short hairpin sequence used target endogenous mutant p53 R273H. A control cell line containing empty vector or the cells expressing the indicated p53 hotspot mutants were grown in 3D culture in the presence of doxycycline to deplete the endogenous mutant p53 R273H. Total *VEGFR2* messenger RNA was analyzed by qRT-PCR and normalized to -DOX control condition. Corresponding immunoblot of p53 proteins with actin loading control is shown below. In panels **A-C** error bars represent standard error. In each experiment, at least three biological replicates were performed. Endogenous VEGFR2 was detected with anti-

VEGFR2 antibody, and mutant p53 was identified with a mixture of mABs 1801 and DO-1.

(D-F) TCGA breast cancer RNA-Seq V2 dataset analysis stratified by *TP53* mutational status (wild-type, truncation mutation, hotspot missense mutation, or non-hotspot missense mutation as indicated). RNA expression of **(D)** *VEGFR2* **(E)** *VEGFA* and **(F)** *HIF1A* is presented as a boxplot, where the box contains the interquartile range. The central line represents the median gene expression. Median expression values are delineated for the truncation mutant category in (**D**) and for *TP53* wild-type category in (**E-F**). RNA expression values were analyzed as upper quartile normalized RNA-Seq by Expectation-Maximization (RSEM) of reads. *$p < 0.05$ by Welch's one-tailed t-test in (**D**).

Figure 2.2 *VEGFR2* Inhibition Phenocopies Loss of Mutant p53

(A) MDA-231 cells and **(B)** MDA-468 cells were transfected with two independent siRNAs to deplete mutant p53 or VEGFR2 and then grown in 3D culture conditions for 8 days. Representative differential interference contrast images were acquired at 10X magnification on live imaging. Relative cell areas of an average of at least 95 colonies per condition for 3 independent replicates was calculated and shown in the corresponding bar graphs. Error bars represent standard deviation. Scale bar, 100 µm. *$p < 0.01$ by one-tailed t-test.

Figure 2.3 Mutant p53 Gain of Function is Mediated by VEGFR2 and May Predict Response to Bevacizumab

(A) MDA-231.shp53 cells were engineered to express control vector, VEGFR2, or VEGFR2 tyrosine phosphorylation mutant Y1059F as described in Methods and then grown in 3D culture conditions for up to 8 days. Where indicated, cells were grown in the presence of doxycycline (DOX, low Mut p53) to deplete endogenous mutant p53. DIC images were acquired at 10X magnification on live imaging. Scale bar, 100 μm.

(B) Immunoblot of indicated proteins from panel A. The black line adjoins non-adjacent lanes from the same immunoblot.

(C) Relative cell area of an average of at least 85 colonies per condition among 4 independent replicates was analyzed. Error bars represent standard deviation. $^*p < 0.001$ calculated by one-tailed t-test.

(D) For wound migration analysis, MDA-231 cells were transfected with control siRNA and two independent siRNAs each to deplete mutant p53 or VEGFR2 and then seeded to confluency in a tissue culture plates containing inserts. Representative differential interference contrast images (Supplemental Figure 2.3A) were acquired immediately upon removal of the insert (0 hours) and 48 hours later. Relative migration was calculated by dividing the total distance migrated of each sample to the total migration in the siControl sample. At least three images were quantitated per sample. The data is an

average of four biological replicates. Error bars represent standard deviation. *p < 0.01, **p < 0.001 by two-sided t-test.

(E-F) Response ratio showing reduction in tumor volume in (**E**) *TP53* wild-type tumors and (**F**) *TP53* mutated tumors treated with chemotherapy alone (Chemo) or chemotherapy plus bevacizumab (Chemo + Bev). Each data point represents one patient's response to the indicated treatment which was calculated as the tumor volume of residual tumor divided by the initial tumor volume. Data are plotted as a boxplot and the sample size is indicated by 'n'. P-value is derived from the Kruskal-Wallis test. Median values of the chemotherapy-only cohorts are delineated.

Figure 2.S1 Mutant p53 Promotes *VEGFR2* Expression in Breast Cancer Cells, (Related to Figure 2.1)

(A) MDA-468.shp53 cells were grown in 2D culture condition for 5 days with and without doxycycline (DOX). Total *VEGFR2* transcript was assayed by qRT-PCR and normalized to -DOX condition. Immunoblot at right shows VEGFR2 and mutant p53 protein levels.

(B) MDA-468.shp53 cells were grown in 3D culture for 8 days with and without doxycycline (DOX). *VEGFR2* transcript from intron 1 was assayed by qRT-PCR and normalized to -DOX condition.

(C) Immunoblot from MDA-468.shp53 cells grown in 3D culture for 8 days with 0, 5, and 10 µg/mL doxycycline (DOX) to deplete mutant p53.

(D) SK-BR-3 cells were grown in 2D culture and assayed for *VEGFR2* expression following depletion of mutant p53 with two different siRNAs. Expression is normalized to control siRNA. In each experiment, at least three biological replicates were performed, and the same cell lysates for the extracted RNA were used for immunoblots. Error bars represent standard error. *$p < 0.01$, **$p < 0.001$ by one-tailed t-test.

Figure 2.S2 VEGFR2 Inhibition Phenocopies Loss of Mutant p53, (Related to Figure 2.2)

MDA-468.shp53 **(A)**, MDA-231 **(B)**, MCF10A **(C)** and MCF7 **(D)** cells were grown in 3D culture conditions. After 2 days of growth, DMSO vehicle or 5 µM of semaxanib were supplemented to the media. Cells were refed with fresh media and DMSO or semaxanib at day 4. Cells were imaged at day 8. Representative differential interference contrast images were acquired at 10X magnification on live imaging. Scale bar, 100 µm.

(E) Immunoblot corresponds to cells shown in Figure 2.2A. MDA-231 cells were transfected with two independent siRNAs to mutant p53 or *VEGFR2* and then grown in 3D culture conditions for up to 8 days. VEGFR2, mutant p53, and actin loading controls are demonstrated.

(F) Immunoblot corresponds to cells shown in Figure 2.2B. MDA-468 cells were transfected with two independent siRNAs to mutant p53 or *VEGFR2* and then grown in 3D culture for up to 8 days. VEGFR2, mutant p53, and actin loading controls are demonstrated.

Figure 2.S3 Mutant p53 Gain of Function is Mediated by *VEGFR2* and Mutant p53 Tumors Respond Better to Cancer Therapy than Wild-Type p53 Tumors, (Related to Figure 2.3)

(A) MDA-231 cells were transfected with control siRNA and two independent siRNAs each to deplete mutant p53 or VEGFR2. After trypsinization, approximately 25,000 cells were seeded into culture dishes with Ibidi cell culture-inserts for wound migration, which leaves an approximately 500 μm space where no cells are seeded. 60 hours post-transfection, cells were confluent, and the tissue culture insert was removed. Representative differential interference contrast images were acquired at 10X magnification on live imaging immediately upon removal of the tissue culture insert (0 hours) and at 48 hours. Scale bar, 200 μm. Images correspond to Figure 2.3D.

(B) NeoAva clinical trial results stratified by *TP53* status. 79 breast cancer patients with *TP53* wild-type tumors and 38 breast cancer patients with *TP53* mutated tumors were imaged to establish tumor size prior to treatment. Patients were stratified to receive chemotherapy alone or chemotherapy plus bevacizumab. Following treatment, tumor size was analyzed. Each datapoint represents one patient’s response to the indicated treatment plotted as the remaining tumor volume divided by the initial tumor volume (which is the response ratio). Data are plotted as a boxplot. The sample size (n) and median response are indicated. P-value was derived from the Kruskal-Wallis test.

(C) Table summarizing the total number of tumors that had pathological Complete Response (pCR). Six patients with wild-type p53-containing tumors and one patient with

a mutant p53-containing tumor that received chemotherapy did not have tumor measurements before therapy and were excluded from analysis in (**B**) and Figure 2.3E-F; these patients are included in (**C**) because pCR status is known.

(D) Average change in tumor volume (response ratio) was plotted by *TP53* status (blue, wild-type *TP53*; red, mutant *TP53*) for patients in the NeoAva study. Response is shown as a continuous variable (ranging from 0-2.34).

Table 2.S1 Gene Expression Profiling Identifies VEGFR2 as a Potential Mutant p53 Regulated Gene

Using a 3D tissue culture system, global gene expression profiling was performed in MDA-468.shp53 breast cancer cells that contain a doxycycline-inducible short hairpin RNA (shRNA) to *TP53* (Freed-Pastor et al., 2012). Three independent experiments were averaged, and the top 10 genes that were downregulated upon mutant p53 depletion (and thus are genes mutant p53 may upregulate) at 5% significance are listed with the log2 expression values. *IGFBP5*, *Ceruloplasmin* (*CP*), and *Mammaglobin-A* (*SCGB2A2*) were verified as mutant p53 target genes and investigated in Chapter 3 (see Figure 3.S3E).

Table 2.S2 *TP53* Mutation Categories in the Breast Invasive Carcinoma TCGA Provisional Dataset

TP53 mutation classes were categorized from the Breast Invasive Carcinoma TCGA Provisional dataset. 969 breast tumors that had exome or genome sequencing and

RNA-sequencing data were included in the analysis. *TP53* mutations were characterized as wild-type, hotspot missense, non-hotspot missense, or truncation mutations (which includes in-frame deletion, in-frame insertion, frameshift, and nonsense mutations). The frequency of each type of *TP53* mutation is listed.

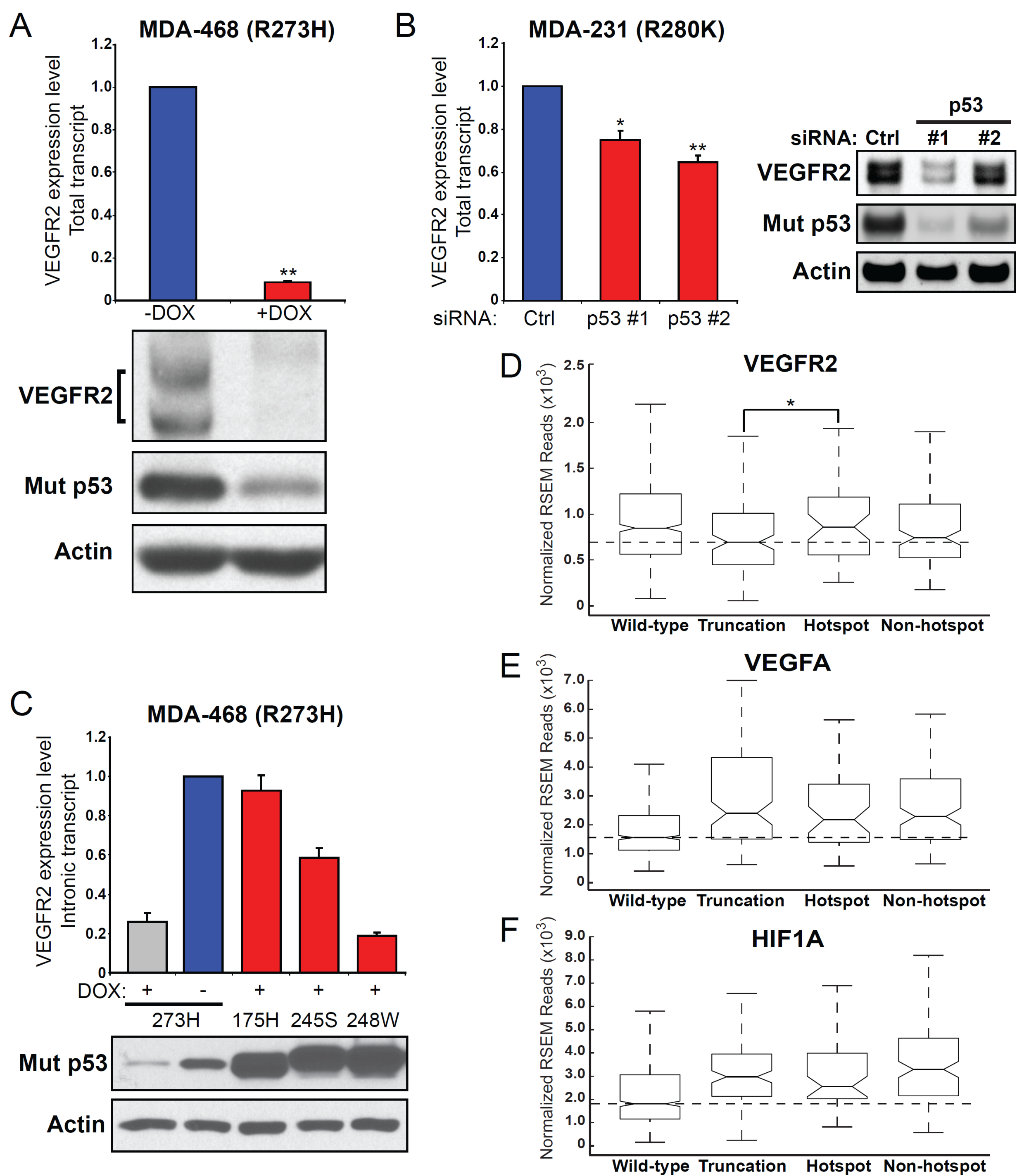
Table 2.S3 *TP53* Missense Mutation Categories in the Breast Invasive Carcinoma TCGA Provisional Dataset

TP53 mutations was categorized from the Breast Invasive Carcinoma TCGA Provisional dataset. The frequency of missense mutation in *TP53* codons are listed for every occurrence greater than 5 times in the dataset (middle column). Codon 245 is provided separately as it is a hotspot mutant (Feki and Irminger-Finger, 2004; Walerych et al., 2012). Not every sample had RNA-sequencing data, so the frequency of missense mutations with RNA-sequencing data is provided in the rightmost column. Missense mutations in codons R175, Y220, G245, R248, and R273 were classified *a priori* for analysis as hotspot mutations, as these are reported to be the most frequently mutated residues in breast cancer (Feki and Irminger-Finger, 2004; Walerych et al., 2012). These codons are underlined in the top part of the table and shown separately in the bottom section of the table. The sum total of non-hotspot missense and hotspot missense mutations with RNA-seq data is 126 and 49, respectively (Table 2.S2).

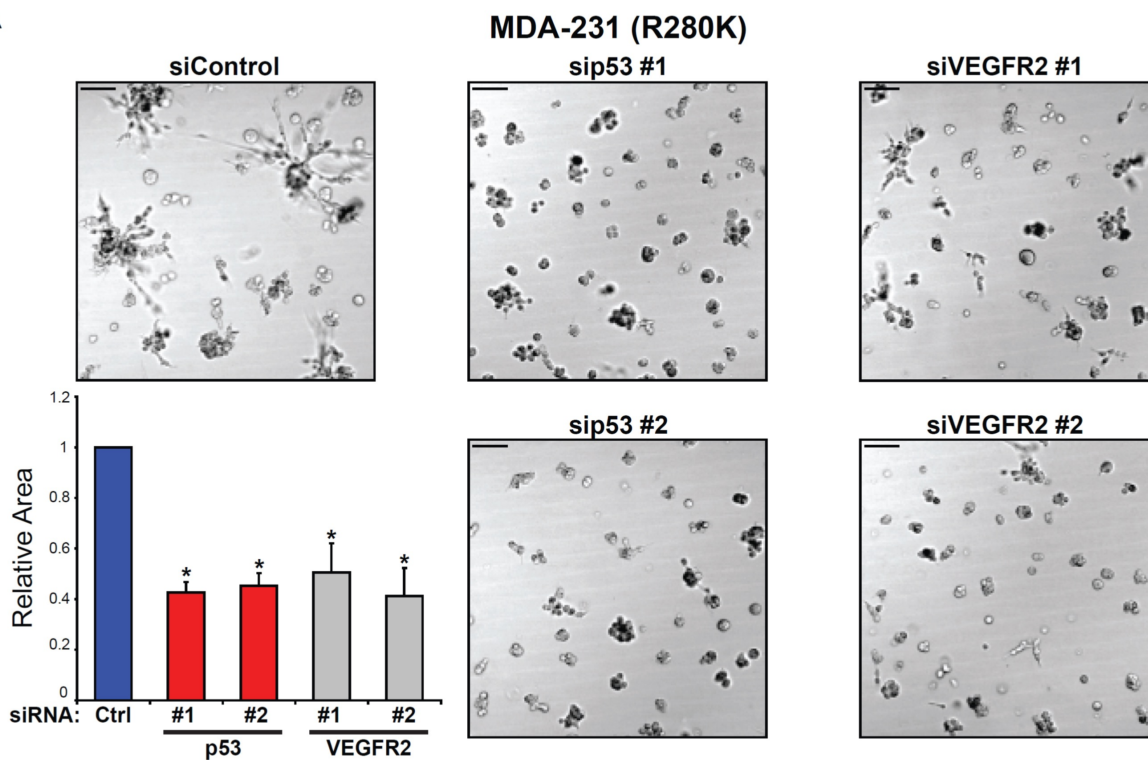
Table 2.S4 Primer, Oligonucleotide, and siRNA List

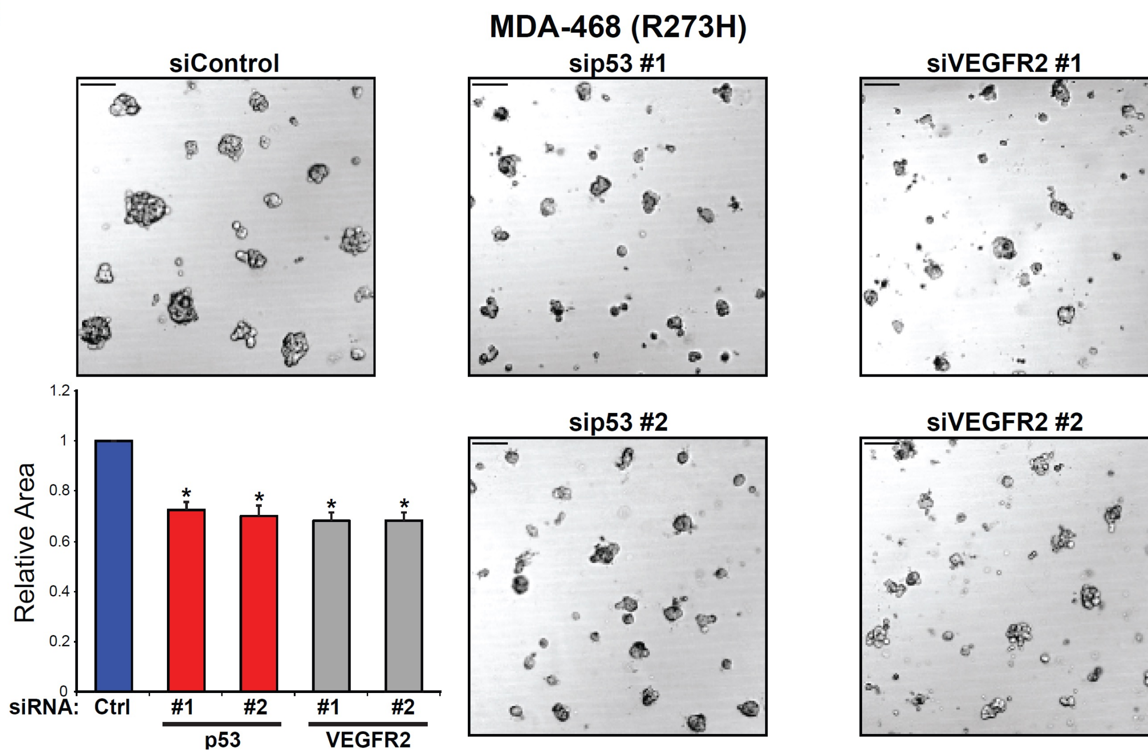
Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) primers, plasmid sequencing primers, and siRNA sequences are shown.

Pfister et al., Figure 2.1

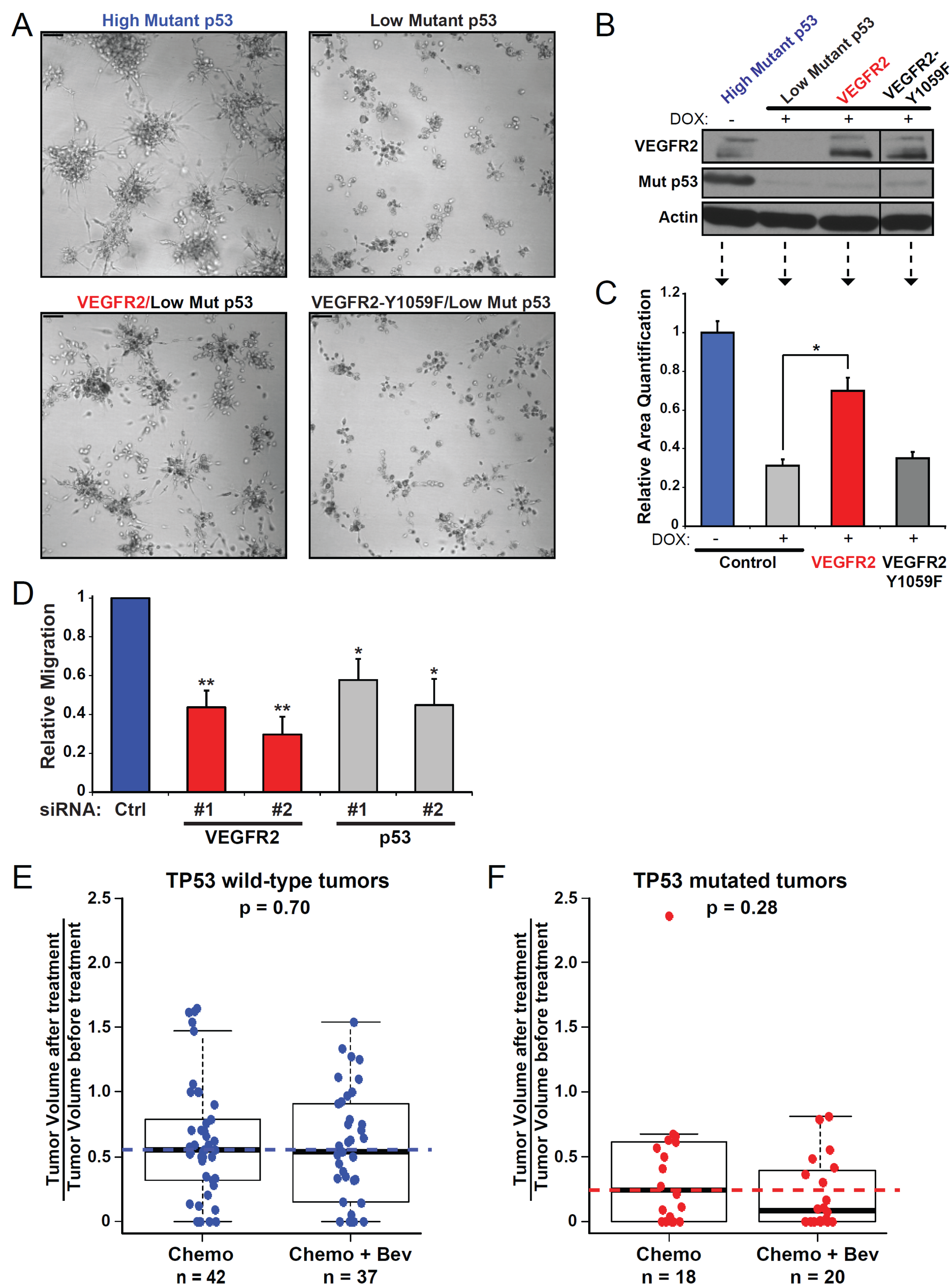


Pfister et al., Figure 2.2

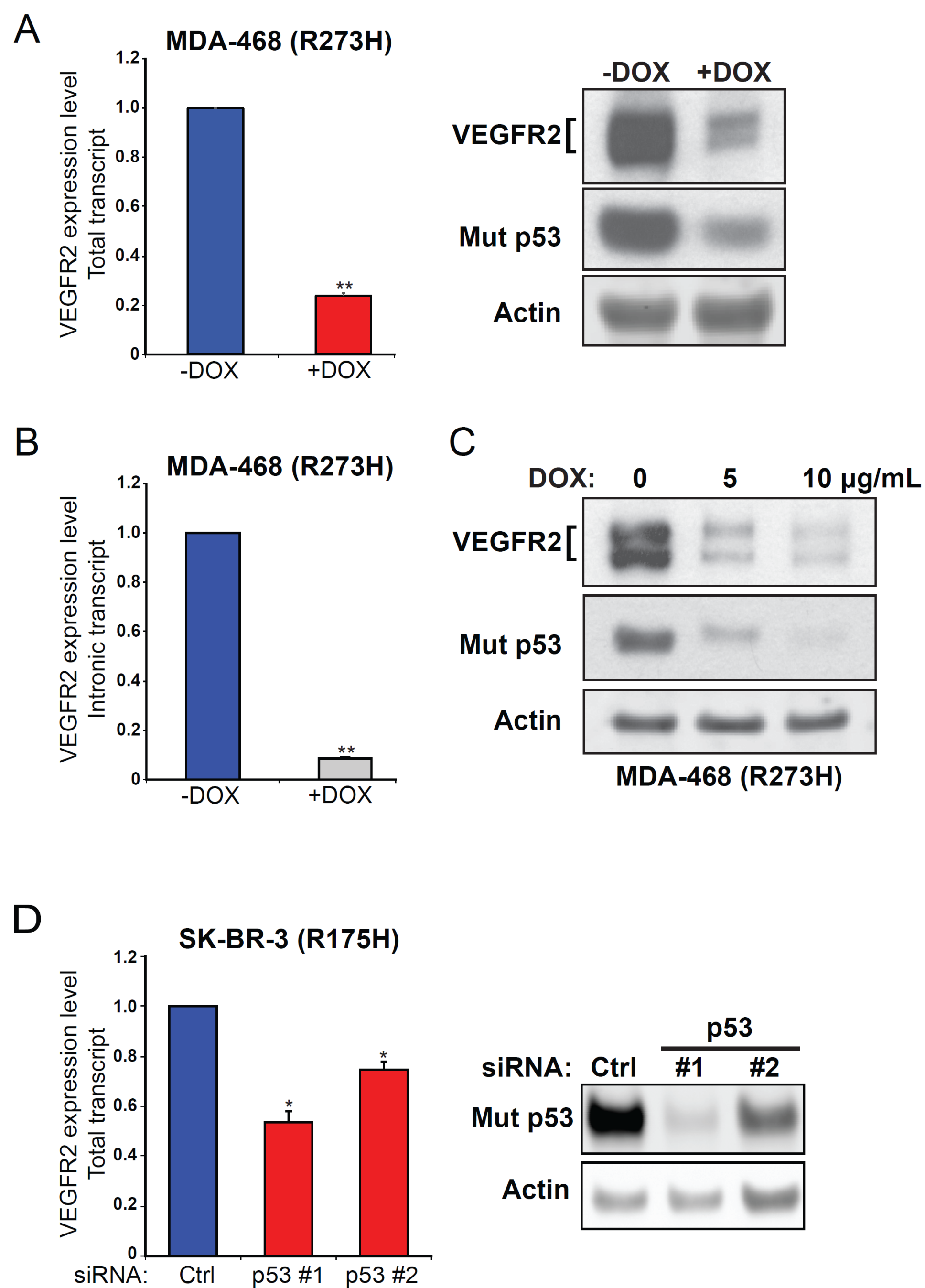




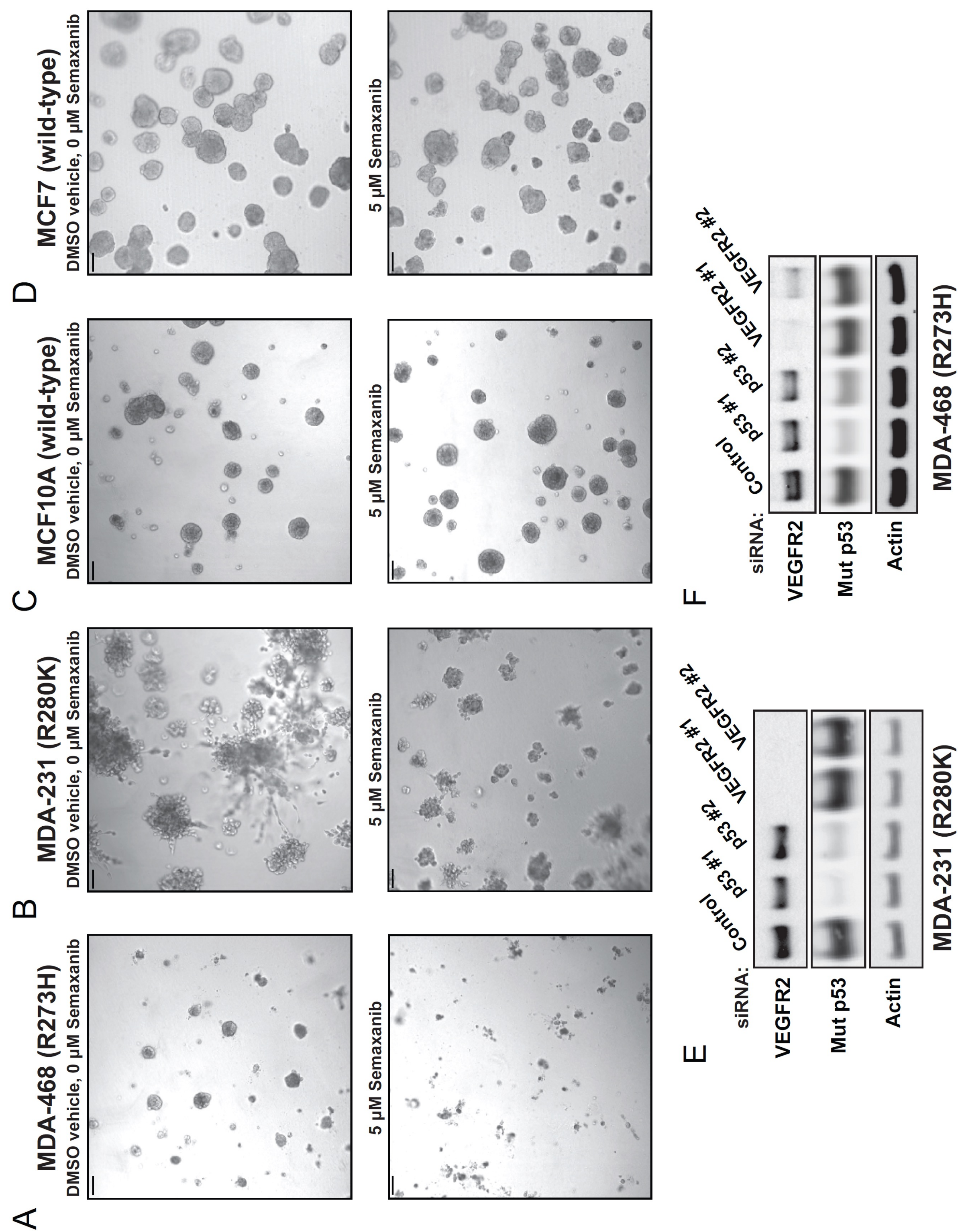
Pfister et al., Figure 2.3



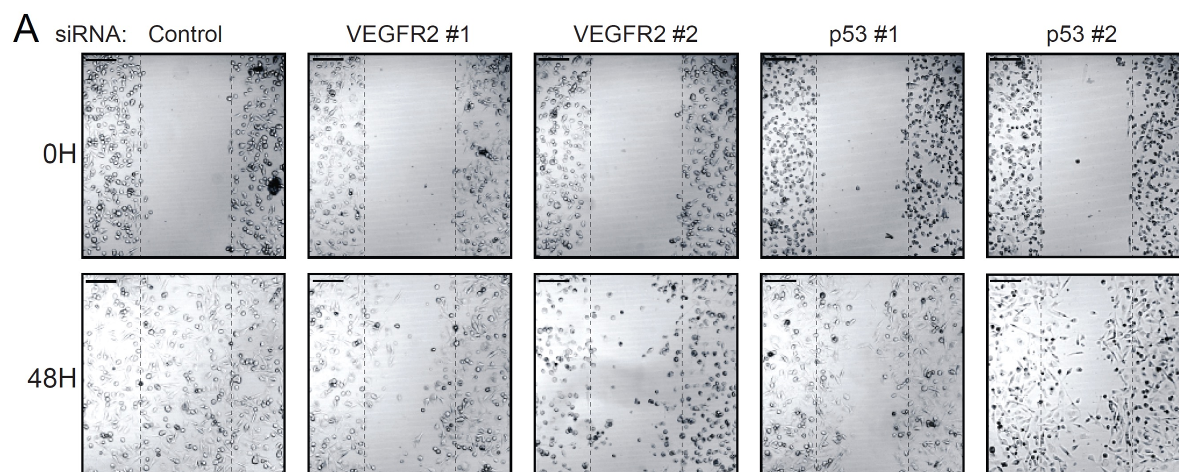
Pfister et al., Figure 2.S1

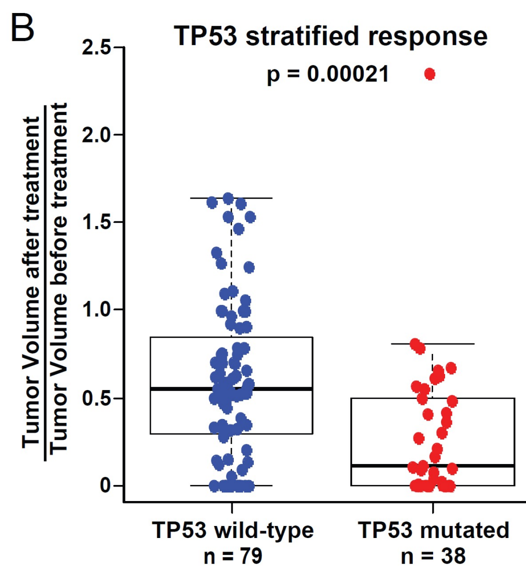


Pfister et al., Figure 2.S2



Pfister et al., Figure 2.S3

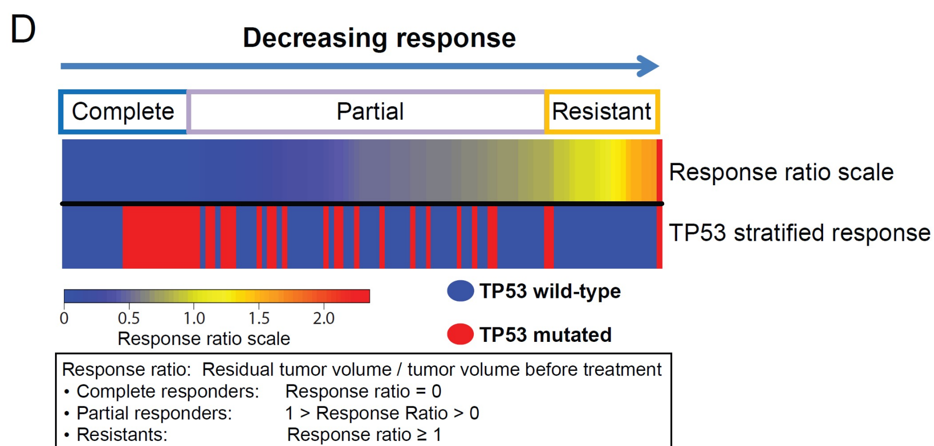




C

Distribution of Complete Responders

Complete responders	TP53 wt (ntot = 85)	TP53 mut (ntot = 39)
Chemo only (n = 62)	2 (of 44) = 4.5%	5 (of 18) = 27.7%
Chemo + Bev (n = 62)	7 (of 41) = 17.1%	7 (of 21) = 33.3%



Pfister et al., Table 2.S1

Rank	Log2 Change	Gene
1	3.739	IGFBP5
2	2.806	HIST1H2BM
3	2.791	EFEMP1
4	2.765	Ceruloplasmin
5	2.707	Mammaglobin-A
6	2.625	TMPRSS11E
7	2.538	RNU5E
8	2.511	VEGFR2
9	2.414	SAA1
10	2.410	S100A8

Pfister et al., Table 2.S2

TP53 Classification	Frequency
Wild-type	672
Hotspot mutation	49
Non-Hotspot missense mutation	126
Truncation mutation	122
Total	969

Pfister et al., Table 2.S3

TP53 Missense Mutation	Frequency	Samples with RNA-Seq
175	21	18
273	17	15
193	10	9
248	9	6
220	8	7
176	6	5
132	5	5
179	5	5
194	5	5
195	5	5
286	5	4
245	3	3

TP53 Hotspot Mutant	Frequency	Samples with RNA-Seq
175	21	18
273	17	15
248	9	6
220	8	7
245	3	3

Pfister et al., Table 2.S4

	qRT-PCR Primers:	
Forward	*RPL32*	TTCCTGGTCCACAACGTCAAG
Reverse	*RPL32*	TGTGAGCGATCTCGGCAC
Forward	*VEGFR2* exonic primer	CCTCCCCCGCATCACAT
Reverse	*VEGFR2* exonic primer	GCTCGTTGGCGCACTCTT
Forward	*VEGFR2* intronic primer	TCCTTTTCTAGGACTCTGGTTTGC
Reverse	*VEGFR2* intronic primer	CGGCATCTCAGGACATGCT

	Plasmid Sequencing Primers	
Forward	LNCX Forward Sequencing Primer	AGCTCGTTTAGTGAACCGTCAG
Reverse	LNCX Reverse Sequencing Primer	ACCTACAGGTGGGGTCTTTCATTC
Forward	pcDNA3.1 T7 Forward Sequencing Primer	AATTAATACGACTCACTATAGGG
Forward	VEGFR2 Sequencing Primer-Walk-1	TTCTGTTAGTGACCAACATGG
Forward	VEGFR2 Sequencing Primer-Walk-2	TGAGCACCTTAACTATAGATGG
Forward	VEGFR2 Sequencing Primer-Walk-3	ACTCAAACGCTGACATGTACG
Forward	VEGFR2 Sequencing Primer-Walk-4	CAAGAACTTGGATACTCTTTGG
Forward	VEGFR2 Sequencing Primer-Walk-5	TGATTGCCATGTTCTTCTGG
Forward	VEGFR2 Sequencing Primer-Walk-6	AAGGGAAAGACTACGTTGG
Forward	VEGFR2 Sequencing Primer-Walk-7	TCAGAGTTGGTGGAACATTTG

	siRNA sequences:	Life Technologies Silencer Select® siRNA Reference	Gene
Sense	GUAAUCUACUGGGACGGAATT	s605	*TP53*
Antisense	UUCCGUCCCAGUAGAUUACCA	s605	*TP53*
Sense	GAAAUUUGCGUGUGGAGUATT	s606	*TP53*
Antisense	UACUCCACACGCAAAUUUCCT	s606	*TP53*
Sense	CAUGUUCUCUAAUAGCACATT	s7822	*VEGFR2*
Antisense	UGUGCUAUUAGAGAACAUGGT	s7822	*VEGFR2*
Sense	CCAUCGUCAUGGAUCCAGATT	s7823	*VEGFR2*
Antisense	UCUGGAUCCAUGACGAUGGAC	s7823	*VEGFR2*

Chapter 3

Mutant p53 Cooperates with the SWI/SNF Chromatin Remodeling Complex to Mediate Global Transcriptional Changes

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SUMMARY

Mutant p53 impacts the expression of numerous genes at the level of transcription to mediate oncogenesis. We previously identified vascular endothelial growth factor receptor 2 (*VEGFR2*), the primary functional VEGF receptor that mediates endothelial cell vascularization, as a mutant p53 transcriptional target in multiple breast cancer cell lines (Chapter 2). Up-regulation of *VEGFR2* mediates the role of mutant p53 in increasing cellular growth and migration in 2D and 3D culture conditions (Chapter 2). We extend these findings in this study by investigating how mutant p53 impacts transcription of *VEGFR2* using multiple techniques including scanning ChIP, micrococcal nuclease-PCR, and *in vivo* DNase I footprinting by ligation-mediated PCR. Mutant p53 was found to bind near the *VEGFR2* transcriptional start site, causing the promoter to adopt a transcriptionally active conformation. Relatedly, mutant p53 interacts with the SWI/SNF chromatin remodeling complex which is required for remodeling the *VEGFR2* promoter. Our results indicate that approximately half of all mutant p53 regulated genes are mediated by SWI/SNF. We suggest that mutant p53 co-opts SWI/SNF function to mediate gene expression changes across a wide variety of genes that allow mutant p53-expressing cells to generate transcriptional plasticity that serves as a selective advantage to tumor cells.

INTRODUCTION

TP53 is the most frequently mutated gene found in human cancers (Olivier et al., 2010). Wild-type p53 is a sequence-specific transcription factor that when activated by various stresses such as DNA damage, oncogenic signaling or nutrient depletion, promotes cellular outcomes such as cell arrest, cell death, senescence, metabolic changes and others, depending on the extent and context of the stress (Vousden and Prives, 2009). In human cancer p53 primarily sustains missense mutations in its conserved DNA binding domain. The small number of residues (~5-6) within this region that are mutated with extraordinarily high frequency are termed hotspot mutations. These mutations can be loosely divided into two categories, the contact mutants (e.g. R273H), which remain well folded but whose mutated residues fail to make specific contact with elements within the DNA binding site and conformational mutants (e.g. R175H) that are partly unfolded leading to loss of zinc coordination and general DNA binding. Evidence from sources as varied as human epidemiology studies, mouse models and cell-based experiments has shown that these hotspot missense mutant forms of p53, which often accumulate to high levels in the cells they inhabit, can acquire neomorphic properties such as increased metastases in mice and increased motility and invasive characteristics in cultured cells (Brosh and Rotter, 2009; Muller and Vousden, 2014). In Li-Fraumeni patients, missense mutation was reported to lead to earlier tumor onset than other forms of p53 loss (Bougeard et al., 2008). p53 hotspot mutant proteins have been reported to associate with chromatin and alter a cell's transcriptional profile, leading to oncogenic

cellular changes (Cooks et al., 2013; Di Agostino et al., 2006; Do et al., 2012; Freed-Pastor et al., 2012; Stambolsky et al., 2010).

We previously identified VEGFR2 as a mutant p53 transcriptional target that mediates mutant p53-dependent functions including increased growth and migration in breast cancer cells (Chapter 2). Here, we additionally report that mutant p53 regulates the chromatin architecture of the *VEGFR2* promoter by mediating nucleosomal displacement through the SWI/SNF chromatin remodeling complex. The SWI/SNF complex associates genome-wide with transcription regulatory elements (Euskirchen et al., 2011) to regulate nucleosome occupancy (Tolstorukov et al., 2013). This complex is composed of either BRG1 or BRM ATPases, a set of core proteins, and other context-specific components (Narlikar et al., 2002; Wilson and Roberts, 2011). SWI/SNF complexes are subdivided into PBAF and BAF complexes based on the presence of BAF250A or BAF250B (BAF complex, contains either BRG1 or BRM ATPase) or BAF180 (PBAF complex contains only BRG1 ATPase), although this distinction may not be absolute (Euskirchen et al., 2012; Ryme et al., 2009; Wilson and Roberts, 2011). Importantly, inactivating mutations in several SWI/SNF components are found at high frequency in a variety of cancers, including breast cancer, implicating SWI/SNF in tumor suppression (Reisman et al., 2009; Wilson and Roberts, 2011).

We found that the mammalian SWI/SNF complex, previously reported as a wild-type p53 interactor and regulator of wild-type p53 gene expression at the *CDKN1A* (*p21*/*WAF1*) locus (Lee et al., 2002), is a novel mutant p53 interactor that is required for mutant p53 transcriptional effects at the *VEGFR2* promoter as well as at

multiple other mutant p53-regulated genes. We hypothesize that mutant p53 co-opts SWI/SNF complex function to mediate its gain-of-function transcriptional effects. A model is proposed whereby mutant p53 expression imparts transcriptional plasticity to a tumor that is mediated through interaction with the SWI/SNF chromatin remodeling complex.

RESULTS

Mutant p53 Mediates Chromatin Remodeling at the *VEGFR2* Promoter

Because *VEGFR2* expression was one of the genes most strongly activated by mutant p53 in MDA-468.shp53 cells, we sought to define how mutant p53 regulates the *VEGFR2* promoter using this clonal cell line. We utilized quantitative promoter scanning chromatin immunoprecipitation (ChIP) along 4 kb of the *VEGFR2* promoter using 9 primer sets. Mutant p53 was associated with the *VEGFR2* promoter, with peak binding at the proximal promoter (primer set -150 bp, Figure 3.1A). Peak binding was ~5-fold higher than background levels (normalized to percent input) at the -150 site and 3-fold above the lowest mutant p53 signal, located at the -2350 site (Figure 3.1A). The binding signal was specific, in that it was consistently significantly decreased when mutant p53 was depleted (Figure 3.1A and Figure S3.1A-C). Mutant p53 could be detected along at least 1.5 kb of the *VEGFR2* promoter, even though peak binding was in the vicinity of the transcriptional start site. This broad binding pattern is consistent with the view that mutant p53 is not likely to bind to a specific DNA sequence. Rather, the p53 binding distribution observed is consistent with a more diffuse association with the proximal *VEGFR2* promoter, a

region predicted to be associated with a complex array of transcription factors, chromatin regulators, transcriptional machinery, nucleosomes, and other factors.

As wild-type p53 mediates gene expression changes through complex interaction with multiple chromatin regulators (Laptenko and Prives, 2006), many of which are known mutant p53 binding partners (Freed-Pastor and Prives, 2012), and because wild-type p53 binding to DNA leads to nucleosome displacement (Laptenko et al., 2011; Lidor Nili et al., 2010), we sought to characterize mutant p53-dependent changes in chromatin architecture. The *VEGFR2* promoter is a GC-rich, TATA-less promoter that is tightly regulated and under tissue-specific control (Patterson et al., 1997). We postulated that mutant p53 could either mediate a step preceding promoter activation in which mutant p53 would initiate or facilitate changes in chromatin architecture such as by promoting nucleosomal displacement, or mutant p53 could be recruited to active promoters and augment transcription subsequent to and without affecting promoter remodeling.

To distinguish between these two possibilities we characterized the chromatin architecture of the *VEGFR2* promoter using micrococcal nuclease (MNase) digestion followed by promoter-scanning qPCR (MNase-PCR). MNase eliminates DNA that is not allosterically protected from digestion by association with DNA-binding proteins and, specifically, core nucleosomes (Noll and Kornberg, 1977). MNase-PCR primers were designed to span the *VEGFR2* promoter from -390 bp to +56 bp relative to the transcriptional start site with an average amplicon length of 66 bp and average overlap of 2.5 bp (Table 3.S1). Unfortunately primers could not be developed for the region defined by amplicon 5, spanning 63 bp between amplicons 4 and 6, as this

region has high GC content and homology to other genomic regions. The average amplicon was 66 bp, so each amplicon is less than half the length of DNA associated with a core nucleosome (which coordinates ~147 bp of DNA). Thus, observed changes were much more defined than would be by ChIP that typically has resolution of approximately 300-500 bp (Laptenko et al., 2011). Using crosslinked chromatin, MNase digestion was performed on isolated nuclear fractions with full or reduced expression of mutant p53. MNase-treated chromatin were separated via agarose gel electrophoresis, and mononucleosomal-length DNA was excised, purified, and PCR amplified using the described MNase primer sets (Figure 3.1B). Indeed, in the presence of mutant p53, there was increased chromatin digestion by MNase that was localized to the proximal promoter region between amplicons 3-6, corresponding to -261 bp to -10 bp from the transcriptional start site, but not amplicons 1, 2, or 7 that correspond to flanking regions, suggestive of a relaxed, transcriptionally permissible, open-chromatin state in the region where mutant p53 was localized (Figure 3.1B). Note that amplicon 4 uses the same primer set as the -150 bp site where peak mutant p53 binding was observed in Figure 3.1A. Because depletion of mutant p53 leads to localized resistance to MNase digestion, these data signify that mutant p53 is associated with remodeled chromatin at the proximal *VEGFR2* promoter and loss of mutant p53 leads to promoter closure (Figure 3.1B) consistent with the dramatic decrease in *VEGFR2* expression (see Figure 2.1A).

To confirm and extend these observations with an independent enzymatic technique, *in vivo* DNase I footprinting by ligation-mediated PCR (LM-PCR) was employed. This technique provides a direct visualization of virtually each nucleotide

in the genomic DNA region being queried. Increased DNase I cleavage (termed hypersensitivity) is a hallmark of active genes, corresponding to an open promoter configuration (reviewed in (Krebs and Peterson, 2000)), and is a feature of wild-type p53 target genes including *p21* (*CDKN1A*)(Braastad et al., 2003), for which it is known that the area surrounding the p53 response element undergoes nucleosomal remodeling subsequent to p53 recruitment (Laptenko et al., 2011). In the presence and absence of mutant p53, crosslinked chromatin was subjected to DNase I digestion. We found increased DNase I hypersensitivity at the proximal promoter between nucleotides -160 bp to +5 bp, corresponding to MNase amplicon 6 as well as the area that would theoretically be amplified by MNase primer set 5, in the presence of mutant p53 (Figure 3.1C, red). There were no detectable changes in DNase I hypersensitivity downstream of the transcriptional start site using a separate set of nested primers corresponding to *VEGFR2* exon 1 (Figure S3.1D). These experiments reveal that mutant p53 mediates promoter remodeling at the *VEGFR2* promoter and is required to sustain an open chromatin conformation.

Mutant p53 Interacts with the SWI/SNF Chromatin Remodeling Complex

In order to screen for the protein partners that may cooperate with mutant p53 to mediate changes in chromatin architecture, SILAC-based mass spectrometry was performed using H1299 cells expressing inducible the p53 R282W hotspot mutant. In this screen some subunits of the SWI/SNF chromatin remodeling complex were identified as mutant p53 interactors, namely BAF53A (ACTL6A) and a peptide mapping to the ATPase subunits BRG1 and BRM (Table 3.S2). No components of

other transcriptional machinery were identified in this screen. This is relevant to our study for a number of reasons: First, the SWI/SNF complex is a well-characterized ATP-dependent nucleosomal remodeler. Second, multiple components of the SWI/SNF complex have been identified as wild-type p53 binding partners including BRG1 (Lee et al., 2002; Naidu et al., 2009), INI1 (Lee et al., 2002), BAF60A and BAF155 (Oh et al., 2008), ARID1A (Guan et al., 2011), and BRD7 (Burrows et al., 2010). Third, *TP53* and SWI/SNF mutations have a tendency toward mutual exclusivity in cancers (Kadoch et al., 2013). Fourth, mutant p53 has many overlapping interacting partners with SWI/SNF components (Table 3.S3). Finally, mutant p53 and wild-type p53 often mediate opposing effects on their interacting partners, so in theory mutant p53 could dysregulate normal SWI/SNF complex function that wild-type 53 requires for transcriptional activities (Lee et al., 2002; Xu et al., 2007) by affecting its activity, interaction with other proteins, or chromosomal location.

Using immunoprecipitation experiments, we were able to co-immunoprecipitate SWI/SNF components with mutant p53 in several cell lines (Figure 3.2 and Figure S3.2). SWI/SNF core subunit BAF53A was co-immunoprecipitated by p53 R273H in MDA-468 cells and p53 R175H in SK-BR-3 cells (Figure 3.2A and 3.2B, respectively). SWI/SNF core subunit BAF155 was co-immunoprecipitated by p53 R273H in MDA-468 cells and p53 R280K in MDA-231 cells (Figure S3.2A and S3.2B, respectively). Reciprocal immunoprecipitation was performed for BAF155, which co-immunoprecipitated mutant p53 in MDA-468 and MDA-231 cells (Figure 3.2C and 3.2D, respectively). Additionally, BRG1 and BAF170 were able to be co-

immunoprecipitated with p53 R273H in HT29 cells (Figure S3.2C). Hence, mutant p53 associates with multiple SWI/SNF subunits, including core components BAF155, BAF170, and BAF53A that are present in both BAF and PBAF SWI/SNF complexes as well as core ATPases BRG1 (present in PBAF and BAF complex subclasses) and BRM (present in BAF complex subclasses)(Euskirchen et al., 2012). While at this point we cannot conclude that their association is direct, the fact that multiple SWI/SNF subunits were co-immunoprecipitated with mutant p53 suggests that their interaction is functional. This point is supported by the results described below.

SWI/SNF Chromatin Remodeling Complex Mediates Nucleosome Occupancy of *VEGFR2* Promoter and is Required for Optimal Mutant p53-Associated *VEGFR2* Expression

As mutant p53 association with the *VEGFR2* promoter mediated promoter remodeling and mutant p53 interacted with the SWI/SNF chromatin remodeling complex, it is plausible that the SWI/SNF complex facilitates remodeling of the *VEGFR2* promoter via nucleosomal repositioning. We first determined that mutant p53 and the SWI/SNF complex co-exist at the *VEGFR2* promoter. Two techniques were used to ascertain their co-occupation of this region: sequential chromatin immunoprecipitation (re-ChIP) and immunodepletion chromatin immunoprecipitation (ID-ChIP) using MDA-468.shp53 cells. Schematics for these procedures are depicted in Figure S3.2D and S3.2E. For the re-ChIP, the first immunoprecipitation was performed with IgG control or anti-p53 antibodies. The pellet was washed, eluted, and diluted for a second immunoprecipitation with anti-BAF170 or IgG control

antibodies. We found that ChIP-p53-ChIP-BAF170 was significantly elevated over ChIP-p53-ChIP-IgG and ChIP-IgG-ChIP-BAF170 signals, formally demonstrating that core SWI/SNF subunit BAF170 and mutant p53 co-localize at the *VEGFR2* promoter (Figure 3.2E). For the immunodepletion-ChIP, lysates were immunodepleted with IgG control antibodies or with p53 monoclonal antibodies to remove chromatin-bound p53 (see Figure S3.2E). Mutant p53 immunodepletion was confirmed in total cell extract (Figure 3.2H) and at the *VEGFR2* locus (Figure S3.2F). Each lysate was then immunoprecipitated with anti-BAF155, -BAF170, or IgG control antibodies. BAF155 and BAF170 were chosen because they are core SWI/SNF subunits for which ChIP-grade antibodies are available (Euskirchen et al., 2011). Both anti-BAF170 (Figure 3.2F) and anti-BAF155 (Figure 3.2G) signals were significantly reduced in the mutant p53 chromatin depleted samples, confirming that mutant p53 and BAF155, and mutant p53 and BAF170, are simultaneously present at the *VEGFR2* promoter.

We next queried whether mutant p53 recruits the SWI/SNF complex to the *VEGFR2* promoter. Using quantitative chromatin immunoprecipitation to determine the abundance of SWI/SNF core components BAF170 and BAF155 at the proximal (-150 bp site) and distal (-2350 bp site) *VEGFR2* promoter, corresponding to high and low mutant p53 sites, we found that BAF155 and BAF170 were enriched at the proximal promoter relative to the distal promoter (Figure 3.3A-B). Figure 3.S3A shows that mutant p53 signal was depleted at the -150 bp site in the experimental conditions. Since BAF155 and BAF170 signals did not change when mutant p53 was depleted, this indicated that mutant p53 does not affect the recruitment of the SWI/SNF complex to the *VEGFR2* promoter (Figure 3.3A-C, 3.S3A). Three additional

intragenic sites as well as one site 30 kb downstream of the 3'UTR were also analyzed, as the SWI/SNF complex has been shown to affect transcriptional efficiency by assisting with the RNA polymerase complex, but again no change was observed in the presence or absence of mutant p53 (Figure 3.3A-B, 3.S3A).

Since SWI/SNF localization to the *VEGFR2* promoter was unaffected by mutant p53, we considered the possibility that SWI/SNF recruits mutant p53 to the *VEGFR2* promoter to cooperate in the initiation of promoter remodeling. As we found that a greater impact of depletion of BRG1 and BRM together on VEGFR2 expression than siRNA-mediated reduction of either alone (see below) the two SWI/SNF ATPase components were co-depleted for this experiment. In fact, co-reduction of BRG1 and BRM significantly reduced occupancy of mutant p53 at its peak binding region in the VEGFR2 promoter (at -150 bp) (Figure 3.3D, $p < .01$) while p53 binding to a control site 30 kb downstream of the 3'-UTR was not significantly affected (Figure 3.3D). The decrease in mutant p53 binding with SWI/SNF knockdown should be contextualized by comparison to the maximal reduction of mutant p53 observed at the same -150 bp site when mutant p53 is depleted with shRNA (~30% to ~50%; Figures 3.3D to 3.1A, 3.1A-C, 3.S3A). Since mutant p53 levels were unchanged upon BRG1 and BRM co-depletion (Figure 3.3J), the reduction of mutant p53 presence at the VEGFR2 promoter was due to reduced presence of SWI/SNF complex.

To formally demonstrate that the SWI/SNF complex mediates nucleosomal displacement at the *VEGFR2* promoter, we performed MNase digestion followed by chromatin immunoprecipitation for histone H3, a core nucleosomal protein. BAF170

was chosen for depletion because it is a core SWI/SNF subunit, present in both BRG1- and BRM-containing SWI/SNF complexes (Euskirchen et al., 2012). Upon BAF170 depletion, there was a significant increase in histone H3 occupancy at the *VEGFR2* proximal promoter (MNase Amplicon 6; -78 to -10 bp) relative to the distal control site (MNase Amplicon 1; -390 to -330 bp) while no significant change between these sites in the control sample was detected (Figure 3.3E). Because depletion of a core SWI/SNF component resulted in increased nucleosome density at the *VEGFR2* promoter and decreased *VEGFR2* expression, we conclude that the SWI/SNF complex is required to sustain an open promoter conformation at a mutant p53 target gene.

The SWI/SNF Chromatin Remodeling Complex is Required to Activate Multiple Mutant p53-Dependent Genes

We hypothesized that mutant p53 enhances expression of additional genes that are also regulated by SWI/SNF complexes. We first determined whether *VEGFR2* expression requires SWI/SNF activity, employing an RNAi approach to deplete multiple SWI/SNF components including the BRM and BRG1 ATPases, of which only one is present per SWI/SNF complex (BRG1-containing or BRM-containing) and BAF155 and BAF170, which are components of all SWI/SNF complexes (Euskirchen et al., 2012). Upon depletion of all four of these SWI/SNF components, we observed significant reduction in *VEGFR2* RNA expression (Figure 3.3F-I). Interestingly, both BRM (Figure 3.3F) and BRG1 (Figure 3.3G) independently reduced *VEGFR2* levels, implying that both BRG1- or BRM-containing SWI/SNF

complexes mediate *VEGFR2* expression (suggesting that mutant p53 may cooperate with both PBAF and BAF complexes). We next determined whether co-depletion of BRG1 and BRM resulted in greater depletion of *VEGFR2* levels than depletion of either component individually. Compared to individual depletion of BRG1 or BRM, co-depletion resulted in even more dramatic reduction in *VEGFR2* levels of up to 60% (Figure 3.3J-K). Note that in these 2D culture conditions, for which we observe approximately 75% depletion of *VEGFR2* transcript on mutant p53 knockdown (Figure S1A), there may exist residual mutant p53-dependent transcriptional activation. This difference may be due to residual SWI/SNF complex, retained open promoter conformation, or unidentified factors. Because SWI/SNF recruits mutant p53 and because mutant p53 and SWI/SNF are both required to sustain *VEGFR2* promoter conformation and gene expression, our data indicate that mutant p53 enhances SWI/SNF-dependent *VEGFR2* expression.

We next sought to generalize the extent that mutant p53 relies on SWI/SNF complex function to mediate its transcriptional activities. Utilizing individual depletion of BRG1 or BRM, three of the top mutant p53 target genes from the global gene expression analysis were tested for impact of SWI/SNF on their gene expression (see Table 2.S1). Depletion of BRM (Figure 3.S3B) and BRG1 (Figure 3.S3C) caused a reduction in the expression of *IGFBP5*, *ceruloplasmin* (*CP*), and *mammaglobin-A* (*SCGB2A2*), which we verified as mutant p53 target genes (Figure 3.S3E). Co-depletion of both BRG1 and BRM led to greater reduction in the expression these genes (Figure 3.3L-N). Interestingly, when we examined expression of mutant p53 target genes *HMGCR* and *HMGCS1,* whose products play roles in the

mevalonate pathway (Freed-Pastor et al., 2012), depletion of the SWI/SNF complex had no significant effect on their expression (Figure 3.S3F).

To extend our findings more globally, we performed RNA-Sequencing on MDA-468 cells grown with siRNA to deplete mutant p53 (Mut p53 knockdown, KD) or siRNAs to co-deplete BRG1 and BRM (SWI/SNF KD). From a combined analysis of two biological replicates where the top 3000 affected genes were analyzed, 1785 genes were significantly upregulated and 1215 downregulated upon mutant p53 depletion, while 1902 genes were significantly upregulated and 1098 downregulated upon SWI/SNF depletion (Figure 3.4A-B). 1105 genes were co-upregulated by depletion of SWI/SNF or mutant p53, representing 61.9% of genes impacted by mutant p53 (Figure 3.4A-B). 531 genes were co-downregulated by depletion of SWI/SNF or mutant p53, representing 43.7% of genes impacted by mutant p53 (Figure 3.4A-B). Of 3000 genes affected by mutant p53, SWI/SNF depletion impacted 1636 (54.5%) of these genes in the same direction (Figure 3.4A-B). Each replicate is presented individually at a 1.67-fold expression cutoff in Supplemental Figure 3.4. Notably, fewer than about 1.5% of genes that were upregulated by SWI/SNF were downregulated by mutant p53, and fewer than about 1.5% of genes that were downregulated by SWI/SNF were upregulated by mutant p53, indicating that the SWI/SNF complex and mutant p53 are finely tuned to each other (Figure 3.S4B and S4D). We conclude that a common feature of numerous mutant p53-dependent genes is their requirement for SWI/SNF complex activity for maximal mutant p53-mediated expression. Mutant p53 likely mediates a good fraction of the genes it

activates by harnessing SWI/SNF chromatin remodeling complex function to remodel promoters into transcriptionally active conformations.

DISCUSSION

We have reported two aspects of mutant p53 function that could lead to clinical interventions: (1) classifying breast tumors by *TP53* mutational status could improve response to anti-VEGF therapy due to the combined effect of inhibiting mutant p53-induced pro-proliferative *VEGFR2* signaling compounded with antagonistic effects on tumor vasculature (see Chapter 2) and (2) targeting the SWI/SNF complex in mutant p53 tumors could impede mutant p53 transcriptional gain of function effects.

In this study, we investigated how mutant p53 impacts transcription of *VEGFR2* (which we identified as one of its strongest target genes; see Chapter 2). Mutant p53 was found to bind near the *VEGFR2* transcriptional start site, causing the promoter to adopt a transcriptionally active conformation. We identified subunits of the SWI/SNF chromatin remodeling complex as mutant p53 interactors that co-occupy the *VEGFR2* promoter along with mutant p53. SWI/SNF is required for maximal mutant p53 promoter occupancy, as depletion of SWI/SNF both reduces mutant p53 association with the *VEGFR2* promoter and results in significantly reduced *VEGFR2* expression. Using RNA sequencing, we report that approximately half of all mutant p53-dependent gene alteration requires the SWI/SNF complex. We surmise that mutant p53 impacts transcription of *VEGFR2* as well as myriad other

target genes by promoter remodeling through interaction with the SWI/SNF chromatin remodeling complex.

Mutant p53 mediates pro-oncogenic transcriptional profiles (reviewed in (Brosh and Rotter, 2009)). Interestingly, mutant p53 has been reported to stimulate additional receptor tyrosine kinases, including EGFR (Ludes-Meyers et al., 1996), IGF1R (Werner et al., 1996), MET (Muller et al., 2013), and PDGFRB (Weissmueller et al., 2014), all of which, along with VEGFR2, promote pro-proliferative signaling. As a tumor forms, acquisition of a hotspot mutation in *TP53* may facilitate transcriptional plasticity, whereby tumor cells increase capacity for gene expression changes and therefore undergo selection for the greatest pro-proliferative transcriptional program for the particular tumor context. This hypothesis explains, for instance, why such a wide array of genes and pathways has been reported to mediate mutant p53 gain of function.

Mutant p53 is associated with decreased overall survival in breast cancer (Langerod et al., 2007), which is most likely due to increased rate of metastases, a known phenotype in mutant p53 mouse models (Adorno et al., 2009; Lang et al., 2004; Olive et al., 2004; Weissmueller et al., 2014). *TP53* mutation facilitates the angiogenic switch by de-repressing HIF1A and VEGFA expression (Mukhopadhyay et al., 1995; Ravi et al., 2000), promoting expression of pro-angiogenic factors that enhance tumor angiogenesis, growth, and metastatic potential (Folkman, 2002). Our data suggest that p53 hotspot mutants may be selected over loss of function p53 mutants during the progression of breast cancer in part due to the advantages conferred by cell-autonomous VEGFR2 signaling. Critically, our data suggest that

mutant p53 cooperation with the SWI/SNF complex is critical to mediating *VEGFR2* expression.

The SWI/SNF complex associates genome-wide with transcription regulatory elements (Euskirchen et al., 2011) to regulate nucleosome occupancy (Tolstorukov et al., 2013). We observed SWI/SNF-dependent recruitment of mutant p53 to the *VEGFR2* promoter (Figure 3.3D). Since mutant p53 and SWI/SNF complexes are required to mediate chromatin remodeling at the *VEGFR2* proximal promoter, we propose that mutant p53 stimulates SWI/SNF-mediated nucleosomal displacement, possibly by facilitating recruitment of one or more transcriptional activators or histone modifiers that interact with mutant p53 (reviewed in (Freed-Pastor and Prives, 2012); Table 3.S3). SWI/SNF function is required at multiple mutant p53 target genes (Figure 3.3K-N). BRG1- and BRM-containing SWI/SNF complexes each are required for maximal expression of these mutant p53 target genes, as depletion of either ATPase decreases expression while co-depletion results in the greatest repression (Figure 3.3F-N, Figure 3.S3B-C). Our data define a model whereby mutant p53 facilitates gene activation via SWI/SNF-mediated promoter remodeling (Figure 3.4C).

Mutant p53 has been proposed to facilitate transcriptional plasticity (Quante et al., 2012), and our data supply a mechanism whereby mutant p53 may mediate genome-wide transcriptional changes by SWI/SNF-mediated nucleosomal remodeling. Because nucleosomal positioning is a critical factor in gene regulation, promoting or inhibiting transcription by regulating access to DNA, mutant p53 may co-opt SWI/SNF activity to mediate both gene activation and repression. As the SWI/SNF complex is reported to interact with many of the same transcriptional

regulators as mutant p53 (Table 3.S3), regulation of mutant p53 target genes could be extraordinarily complex, and protein recruitment patterns among loci or even at an individual locus may vary.

SWI/SNF proteins are tumor suppressive in some contexts. For instance, PBAF subunit BAF180 mediates p21 expression in breast tumor cells to suppress tumorigenesis (Xia et al., 2008), BRG1 is necessary for efficient RB-mediated cell cycle arrest (Strobeck et al., 2000), and BRG1 cooperates with ATM to promote the DNA damage response (Kwon et al., 2014). Moreover, mutations in SWI/SNF subunits and *TP53* have a tendency toward mutual exclusivity in multiple cancer types including breast cancer, suggesting that loss of SWI/SNF function may phenocopy p53 loss to mediate oncogenesis (Kadoch et al., 2013). It is possible that mutant p53 impedes tumor suppressive activities of the SWI/SNF complex, such as in the DNA damage response, which mutant p53 deregulates leading to genetic instability (Song et al., 2007). Therefore, in conjunction with chemotherapy or radiotherapy, restoration of SWI/SNF tumor suppressor function by targeting mutant p53 may be a therapeutic option in cancers expressing hotspot mutants of p53.

Oncology is headed toward highly adaptable treatment regimens based on the particular genetic alterations of a tumor. Defining the contributions of mutant p53 and VEGFR2 to breast cancer tumorigenicity are likely to be critical steps toward identifying specific tumor alterations that can be therapeutically harnessed. Outstanding questions include how SWI/SNF and mutant p53 positively regulate each other via recruitment of p53 to promoters and how the ensuing functional activation of promoter remodeling occurs. Future directions also include testing the

feasibility of development of small molecules to interrupt the mutant p53-SWI/SNF interaction to impede mutant p53 gain of function activities. Finally, whether patients with mutant p53-expressing breast tumors demonstrate improved survival with anti-VEGF treatment or such newly developed small molecules that impede mutant p53-dependent transcription will be of paramount importance.

EXPERIMENTAL PROCEDURES

Reagents

Plasmids

Doxycycline-inducible shp53 plasmids were generated as previously described (Freed-Pastor et al., 2012).

siRNAs

For siRNA knockdown experiments, Silencer® Select siRNAs were purchased from Life Technologies and are the following: siRNA to *TP53* (s605 and s606), *SMARCA4* (Brg1, s13139 and s13140), *SMARCA2* (BRM, s13133 and s13134), *SMARCC1* (BAF155, s13145 and s13146), and *SMARCC2* (BAF170, s13148 and s13149). Silencer® Select Negative Control #1 siRNA (Life Technologies) was used as control siRNA. DharmaFECT 1 (Thermo Scientific) was used as the transfection reagent for all siRNA knockdown experiments. siRNA sequences are listed in Table 3.S1.

Antibodies

p53 was detected using a combination of mAb 1801/mAb DO-1 (both in-house purified from hybridoma supernatants) or with polyclonal FL393 (sc-6243, Santa Cruz

Biotechnology). Anti-p53 PAb421 (in-house purified) was used along with mAb 1801/mAb DO-1 to deplete p53 in the immunodepletion ChIP. Anti-Actin (A2066), mouse IgG (I5381) and rabbit IgG (I5006) antibodies were purchased from Sigma. Anti-VEGFR2 (55B11) rabbit mAb was purchased from Cell Signaling Technology. Anti-BAF53A (ab131272), anti-Histone H3 (ab1791) and anti-Histone H2 antibodies (ab18255) were purchased from Abcam. Anti-BRG1 (G-7, sc-17796), BRM (N-19, sc-6450), BAF170 (H-116, sc-10757), and BAF155 (H-76, sc-10756) antibodies were purchased from Santa Cruz Biotechnology.

Drugs

Doxycycline (D9891) was purchased from Sigma Aldrich. For drug treatment experiments doxycyline was dissolved in H_2O and utilized at a final concentration of 10 µg/mL, which was determined to generate maximal depletion of endogenous mutant p53.

Cell Cultures

Cell Lines and Generation of Stable Cell Lines

MDA-468, MDA-231, SK-BR-3, HT29, and H1299 cells were maintained in DMEM + 10% Fetal Bovine Serum (FBS, Gemini Bio-Products). All cells were maintained at 37^oC in 5% CO_2. Unless otherwise stated we refer to these growth conditions as two-dimensional (2D) cultures to distinguish them from three-dimensional (3D) culture conditions described below.

Clonal MDA-468.shp53 cells are previously described (Freed-Pastor et al., 2012). To induce shRNA expression, cells were treated with 10 µg/ml doxycycline

from day 0 for time periods indicated in the figure legends. For siRNA knockdown experiments, cells were seeded 24 hours prior to transfection.

3D Cultures

The 3D cell culture protocol was performed as previously described (Debnath et al., 2003). For routine imaging, 8-well chamber slides were lined with 45 μL of growth factor reduced Matrigel (356231, BD Biosciences). Cells were seeded at 5,000 cells/well in assay medium (DMEM/F12 + 2% Horse Serum + 10 μg/mL Insulin + 0.5 μg/mL Hydrocortisone + 2% Matrigel), with 5 ng/ml EGF supplemented to MCF10A cultures. For RNA, protein, or chromatin analyses from 3D cultures, 35 mm plates were lined with 475 μl Matrigel and cells were seeded at a density of 175,000 to 225,000 cells/plate in assay medium + 2% Matrigel. Cells were re-fed with assay medium on day 4 and imaged or collected for analysis on day 8. When siRNA was utilized, cells grown in 2D conditions were transfected with 50 nM of siRNA and 24 hours later cells were plated in 3D culture conditions. Cells were harvested using Cell Recovery Solution (BD Biosciences). Where indicated drug concentrations in 3D cultures were maintained when refreshing media.

RNA Expression

Quantitative RT-PCR

For most experiments, RNA was isolated from cells using the Qiagen RNeasy Mini Kit. For RNA-Seq, RNA was isolated using the MagJET RNA Kit (Thermo Scientific). Complementary DNA was generated using the Qiagen Quantitect reverse transcription kit using 1 μg of input RNA as measured by NanoDrop

Spectrophotometer (Thermo Scientific). Real-time PCR was carried out on an ABI StepOne Plus machine using SYBR green dye. Transcript levels were assayed in triplicate and normalized to *RPL32* mRNA expression. Relative changes in cDNA levels were calculated using the Comparative-Ct Method ($\Delta\Delta C_T$ method). All qRT-PCR primers were designed with Primer Express (Applied Biosystems) from genomic DNA sequence from the UCSC Human Genome Browser hg19 assembly. Primer targeting was confirmed with the UCSC Human Genome Browser *in silico* PCR tool. All primer sequences were validated for amplification efficiency by comparison to a genomic DNA standard curve and amplify single targets as determined by melting curve analysis. Primer sequences are listed in Table 3.S1. All primers were purchased from Life Technologies.

Preparation and Sequencing of RNA-Seq Libraries

Total RNA extracted using MagJET RNA Kit (Thermo Scientific) was first checked for integrity on an Agilent Bioanalyzer 2100; samples with RNA integrity number (RIN) > 9.0 were used for subsequence processing. Total RNA was subjected to two rounds of poly(A) selection using oligo-d$(T)_{25}$ magnetic beads (New England Biolabs, NEB). A single read cDNA library was prepared following the Illumina TrueSeq small RNA protocol for strand-specific RNA sequencing with minor modifications (Hoque et al., 2013). Briefly, poly(A)+ RNA was fragmented in an alkaline buffer ($NaHCO_3$, pH 9.3) at 94°C for 2 min, followed by dephosphorylation with recombinant shrimp alkaline phosphatase (NEB) and then phosphorylation with T4 polynucleotide kinase (NEB). After addition of 3’ adapter (5’ adenylated) and 5’ adapter using truncated T4 RNA ligase II (NEB) and T4 RNA ligase I (NEB),

respectively, RNA was reverse transcribed (RT) using 3' adapter-specific primer. cDNA was then amplified by PCR for 15 cycles with a universal forward primer and a reverse primer with bar code. The cDNA libraries were purified from an 8% polyacrylamide gel and quantified on an Agilent Bioanalyzer.

Analysis of RNA-Seq Libraries

RNA-sequencing was performed using an Illumina HiSeq 2000 v3 instrument. At least 68 million reads per sample were acquired using 100 bp single end reads. HTSeq (Anders et al., 2014) was used to convert reads mapped with MapSplice in SAM format to gene read counts. Reads were converted to gene counts using hg19 genome as a reference. Differential gene expression was analyzed using EdgeR (Robinson et al., 2010). Gene expression counts were corrected for batch effect exactly as described in the EdgeR manual. The 3000 most affected genes (false discovery rate corrected values) in knockdown conditions as compared to control were selected. A Venn diagram program (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify genes that change between knockdown conditions.

For RNA-Seq expression change analysis of individual biological replicates, reads (in FASTQ formatted files) were received from the JP Sulzberg Columbia Genome Center and were processed by trimming barcodes and removing primers using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit). Reads were than mapped by MapSplice (Wang et al., 2010) to the hg19 reference genome with default settings. Mapped reads were then filtered by quality scores (higher than 10) using SAMTools (Li et al., 2009). GFOLD (Feng et al., 2012) was used to count mapped

reads (using default settings) with quality scores of 10 and higher. GFOLD was also used to find differentially expressed genes compared to control condition using the count files (using default settings). We defined significant genes as having a GFOLD number of 1.667 fold or more in each direction. A Venn diagram program (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify and represent genes that change between knockdown conditions.

Protein Analysis

Co-immunoprecipitations were performed based on a previously described method (Noll et al., 2012). Briefly, sub-confluent cultures of MDA-468 or SK-BR-3 ($1×10^6$ cells) were harvested, lysed in 500 μL of lysis buffer (20 mM Tris–HCl pH 8, 1 mM EDTA, 0.5% NP-40, 150 mM NaCl, 10% glycerol and protease inhibitors (Roche)), sonicated and centrifuged at 16,100 x g for 15 minutes at 4°C. Clarified lysates were incubated with 200 ng of either anti-p53 antibody (DO-1, Santa Cruz Biotechnology) or mouse IgG for 2 hours at 4°C with agitation followed by the addition of 10 μL of Protein-G–Sepharose beads (GE Healthcare) and incubation at 4°C for an additional 2 hours. Beads were washed three times with 400 μL of lysis buffer and protein complexes were eluted with SDS loading buffer at 95°C for 5 minutes. Western blot analysis of inputs and co-immunoprecipitated protein complexes was performed as described previously (Pishas et al., 2011). Where indicated, co-immunoprecipitations were performed as described in the Quantitative Chromatin Immunoprecipitation section below.

SILAC Mass Spectrometry

Cell Culture

In stable isotope labeling by amino acids in cell culture (SILAC) experiments, inducible p53 R282W mutant and wild-type p53 expressing H1299 cells were differentially labeled to incorporate isotopic forms of lysine and arginine present in the DMEM media. For triple labeling experiments, the mutant cells were grown in media containing normal (or 'light' (L)) isotopes of L-lysine-($^{12}C6^{14}N2$) (143 μg/ml, Sigma) and L-arginine- ($^{12}C6^{14}N4$) (83 μg/ml, Sigma) and media containing 'heavy' (H) isotopes of L-lysine-($^{13}C6^{15}N2$) and L-arginine-($^{13}C6^{15}N4$) (Cambridge Isotope Laboratory), respectively. The inducible wild-type p53-expressing cells were grown in media containing an intermediate isotopes (or 'medium' (M)) of L-lysine-(4,4,5,5-^{2}H) and L-arginine-($^{13}C6$) (Cambridge Isotope Laboratory). Cells were grown in SILAC media for at least 5-6 cell doublings to ensure complete incorporation of labeled amino acids. Cells grown in M and H media were then induced with 2.5 μg/ml of Ponasterone A (Invitrogen) for 24 hours before harvesting to induce the expression of p53 R282W and wild-type p53 respectively.

Immunoaffinity Purification of Protein Complexes

Cell pellets were lysed in ice-cold modified RIPA buffer (50mM Tris-HCl, pH 7.5-8, 150mM NaCl, 1% NP-40, Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablet (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail Tablet (Roche) and centrifuged at 20,000 x g for 20 min at 4ºC. Total protein concentrations were measured using a bicinchoninic acid (BCA) protein assay (Thermo Scientific). For the immunoaffinity experiments, equal quantities of extracts from each

differentially labeled cell line were affinity purified separately by overnight incubation at 4ºC with equal amount of anti-p53 (DO-1) conjugated to agarose beads (Santa Cruz Biotechnology). The beads were combined carefully after one wash step in RIPA buffer and were washed for additional three times with RIPA buffer thereafter. To elute the bound proteins from the anti-p53 (DO-1) agarose beads, a 1.5x bead-volume of 2x lithium dodecyl sulfate sample buffer with reducing agent was added and the matrix was boiled for 5 min. The proteins were separated on NuPAGE 4-12% Bis-Tris gels (Invitrogen) that were then stained with Colloidal Blue (Invitrogen) and destained overnight before being processed for mass spectrometry (see below).

Mass Spectrometry and Data Analysis

Eluted protein complexes were separated by 1D SDS-PAGE and digested with trypsin using published procedures (Shevchenko et al., 2006). Samples were analysed on an Orbitrap or Orbitrap XL (Thermo Fisher) coupled to a Proxeon Easy-nLC. Survey full scan MS spectra (m/z 300 – 1400) were acquired with a resolution of R=60,000 at m/z 400, an AGC target of 1e6 ions, and a maximum injection time of 500 ms. The ten most intense peptide ions in each survey scan with an ion intensity above 2000 counts and a charge state ≥ 2 were sequentially isolated to a target value of 1e4 and fragmented in the linear ion trap by collisionally induced dissociation (CID/CAD) using a normalized collision energy of 35%. A dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count, repeat and exclusion duration of 30 seconds.

Identification and Quantification of Peptides and Proteins

Proteins were searched using Mascot version 2.2 (Matrix Science, London, UK) against a concatenated target/decoy database prepared by sequence reversing the human International Protein Index (IPI) (version 3.68) with addition of common contaminants such as human keratins, porcine trypsin and proteases. Cysteine carbamidomethylation was searched as a fixed modification, N-acetylation and oxidized methionine were searched as variable modifications. Labeled arginine and lysine were specified as fixed or variable modifications, depending on the prior knowledge about the parent ion. SILAC peptide and protein quantification was performed automatically with MaxQuant version 1.0.13.13 (Cox and Mann, 2008) using default parameter settings. Maximum false discovery rates (FDR) were set to 0.01 for both protein and peptide.

Chromatin Analysis

Quantitative Chromatin Immunoprecipitation (ChIP)

Quantitative chromatin ChIP experiments were carried out as previously described (Gomes et al., 2006). Briefly, MDA-468 cells were lysed in RIPA Buffer (150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 0.5 mM phenylmethyl sulfonyl fluoride, 1 µM benzamidine, 3 µg/mL leupeptin, 100 ng/mL bacitracin, and 200 ng/mL a_2-macroglobulin) and sonicated to yield ~500 bp fragments. Protein A/G Sepharose beads were conjugated to anti-p53 antibodies (1801/DO-1) which were used subsequently to immunoprecipitate p53 from approximately 1 mg whole cell lysate. Quantitative ChIP was carried out on an

ABI StepOne Plus using SYBR green dye versus genomic standard DNA and input DNA. ChIP primers designed with Primer Express (Applied Biosystems) were derived from the USCS Human Genome Browser hg19 assembly. Primer sequence specificity was confirmed with the UCSC Human Genome Browser *in silico* PCR tool. All primer sequences were validated for amplification efficiency and amplify single targets as determined by melt curve analysis. ChIP primer sequences are provided in Table 3.S1. Samples were normalized to each other and to other amplicons using percent input DNA.

Micrococcal Nuclease-PCR

Approximately 1.5 million MDA-468.shp53 cells grown in 3D culture conditions were cross-linked for 10 minutes with 1% formaldehyde/PBS at room temperature followed by addition of 2.5 M glycine/PBS to 125 mM final concentration for 5 minutes. Cells were washed in PBS and harvested by scraping and nuclei were collected via extraction in 10 mL of hypotonic nuclei preparation buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.1% Nonidet-P40, 0.5 mM phenylmethyl sulfonyl fluoride) supplemented with 3mM $CaCl_2$ and were pelleted by centrifugation at 500 x g for 5 minutes. Nuclei were resuspended in 350 µL nuclei digestion buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM phenylmethyl sulfonyl fluoride) supplemented with 3mM $CaCl_2$. 0.5 units of micrococcal nuclease (Sigma N3755) diluted in 10 µL of nuclei digestion buffer were added to the sample. Incubation was performed for 10 minutes at 37°C to generate

primarily mononucleosomal length DNA fragments as determined by agarose gel electrophoresis. MNase activity was stopped by the addition of EGTA to a final concentration of 20 mM to chelate calcium ions. Chromatin was incubated at 65°C for 5 hours with proteinase K (40 µg proteinase K in 40 µL of Tris-EDTA buffer with 0.5% SDS) to reverse crosslinking and remove protein followed by 1 hour incubation with RNase A (100 units) at 37°C to remove RNA. DNA was extracted with phenol-chloroform-isoamyl alcohol extraction followed by isopropanol precipitation. DNA was resuspended in 40 µL 1X DNA loading dye, and 10 µL of resuspended material was separated via 2% agarose gel electrophoresis. DNA bands were visualized by ethidium bromide staining, and DNA bands corresponding to mononucleosomal-length (~147bp) fragments were excised. DNA was purified with QIAquick Gel Extraction Kit (Qiagen). qRT-PCR was utilized to determine ratio of MNase-resistant DNA between sample conditions. qPCR signal at the *VEGFR2* TSS -390 to -330 bp site (amplicon 1) was used to normalize -DOX (+Mut p53) and +DOX (-Mut p53) sample qPCR signal. Primers sequences were individually designed and tested for amplification efficiency (Table 3.S1).

Micrococcal Nuclease-ChIP

Approximately 10 million sub-confluent MDA-468.shp53 cells were cross-linked for 10 minutes with 1% formaldehyde/PBS at room temperature followed by addition of 2.5 M glycine/PBS to 125 mM final concentration for 5 minutes. Cells were washed in PBS and harvested by cell scraper. Nuclei were collected via extraction in 10 mL of hypotonic nuclei preparation buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 3mM $CaCl_2$, 0.1 mM

ethylenediaminetetraacetic acid, 0.15 mM spermine, 0.5 mM spermidine, 0.1% Nonidet-P40, 0.5 mM phenylmethyl sulfonyl fluoride) and were pelleted by centrifugation at 500 x gravity for 5 minutes. Nuclei were resuspended in 350 μL nuclei digestion buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 3mM $CaCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM phenylmethyl sulfonyl fluoride). 0.5 units of micrococcal nuclease (Sigma N3755) diluted in 10 μL of nuclei digestion buffer were added to the sample. Incubation was performed for 10 minutes at 37°C to generate primarily mononucleosomal length DNA fragments. MNase activity was stopped by the addition of EGTA to a final concentration of 20 mM to chelate calcium ions. Nuclei were disrupted via sonication, cell debris was cleared by centrifugation, and supernatant was collected. Samples were diluted in RIPA buffer and normalized by DNA content using a NanoDrop Spectrophotometer (Thermo Scientific). Chromatin immunoprecipitation was performed as previously explained using ChIP-grade antibody to Histone H3 (Abcam) or rabbit IgG (Sigma). Following final wash steps, immunoprecipitated chromatin was incubated at 65°C for 5 hours with proteinase K (40 μg proteinase K in 40 μL of Tris-EDTA buffer with 0.5% SDS) to reverse crosslinking and remove protein followed by 1 hour incubation with RNase A (100 units) at 37°C to remove RNA. DNA was extracted with phenol-chloroform-isoamyl alcohol extraction followed by isopropanol precipitation. DNA was resuspended in 30 μL of 1X DNA loading dye. 25 μL of resuspended material was separated via 2% agarose gel electrophoresis. DNA bands were visualized by ethidium bromide staining, and DNA bands corresponding to mononucleosomal-

length (~147bp) fragments were excised. DNA was purified with QIAquick Gel Extraction Kit (Qiagen). qPCR was utilized to determine ratio of MNase-resistant DNA between sample conditions. A standard curve of genomic DNA was utilized to determine nanograms (ng) of DNA immunoprecipitated. IP for Histone H3 in the siControl condition at the *VEGFR2* TSS -390 to -330 bp site (amplicon 1) was utilized to normalize samples for the *VEGFR2* TSS -78 to -10 bp site (amplicon 6).

In vivo DNase I Footprinting by Ligation-Mediated PCR (LM-PCR)

Approximately 1.5 million MDA-468.shp53 cells grown in 3D culture conditions were cross-linked for 10 minutes with 1% formaldehyde/PBS at room temperature followed by addition of 2.5 M glycine/PBS to 125 mM final concentration for 5 minutes. Cells were washed in PBS and harvested by scraping and nuclei were collected via extraction in 10 mL of hypotonic nuclei preparation buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.1% Nonidet-P40, 0.5 mM phenylmethyl sulfonyl fluoride) and were pelleted by centrifugation at 500 x g for 5 minutes. Nuclei were resuspended in 225 µL nuclei digestion buffer (300 mM sucrose, 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA acid, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM phenylmethyl sulfonyl fluoride). 2.5, 5, and 10 units of DNase I (Worthington Biochemical Corporation) diluted in 25 µL of nuclei digestion buffer was prepared separately and supplemented with 5 µL of 100mM $CaCl_2$. 220 µL of the resuspended nuclei were added to DNase I-containing mixtures and gently pipetted. Samples were then moved from ice to room temperature for 4 minutes followed by the addition of 250 µL of lysis buffer (50

mM Tris-HCl, pH 8.0, 20 mM EDTA, 1% SDS, 500 μg/mL proteinase K) to quench the reaction. Chromatin was incubated at 65°C for 5 hours to reverse crosslinking and eliminate protein followed by 1 hour incubation with RNase A (100 units, Qiagen) at 37°C to remove RNA. DNA was extracted with phenol-chloroform-isoamyl alcohol extraction followed by isopropanol precipitation. DNA was resuspended in Tris-EDTA-buffered water, and DNA concentration was determined by NanoDrop Spectrophotometer (Thermo Scientific).

1 μg of DNA was prepared for single-step primer extension with Footprinting Primer 1 using an annealing temperature of 59 ^{o}C to generate blunt-ended double stranded DNA using VentR (exo-) DNA polymerase (M0257, New England Biolabs) with primers listed in Table 3.S1. Deoxynucleotide triphosphates used in PCR steps were purchased from Roche Applied Science (#11969064001). A linker was ligated to these variable length DNAs using T4 DNA Ligase (Promega M1794) supplemented with ATP (P0759, New England Biolabs) for 12 hours at 16°C to generate DNA fragments of lengths that correspond to the DNase I cleavage site. DNA was purified by ethanol precipitation. A second PCR step using nested Promoter Footprinting Primer 2 (Forward primer) and Footprint Linker Primer (Reverse primer) was utilized to amplify the genomic DNA using PfuTurbo Hotstart DNA Polymerase (#600320, Agilent Technologies) for 30 cycles using a 64.5^{o}C annealing temperature. The Footprint Linker Primer anneals to the variable site in the genomic DNA where DNase I cut and the linker was ligated, allowing the amplification of variably sized products from the genomic DNA. A third nested primer, Footprinting Primer 3, was radiolabeled with [γ^{-32}P]-ATP (PerkinElmer) using T4

polynucleotide kinase (New England Biolabs) and purified from excess [γ^{-32}P]-ATP using microspin G-25 beads (GE Healthcare). PCR was performed at 72°C annealing temperature for 6 cycles with radiolabeled primer 3, which generates linear amplification (because there is no reverse primer) of the *in vivo* footprint sample. Note that Footprinting Primer 3 is nested within Footprinting Primer 2 and has a higher melting temperature and that Footprinting Primer 2 is nested within Primer 1 and has a higher melting temperature; these considerations offer additional specificity to the genomic amplicon.

Single stranded radiolabeled DNA was resolved by denaturing 8M urea polyacrylamide gel electrophoresis (6% polyacrylamide) and quantitated via phosphorimager exposure. Images were obtained with a Typhoon FLA7000 scanner (GE Healthcare Life Sciences). DNase I hypersensitivity signal represents γ^{-32}P decay detection by phosphorimager-based quantitation that was plotted using densitometry analysis in ImageQuant version 5.2 software (Molecular Dynamics). Primers were individually designed and PAGE-purified (listed in Table 3.S1). Optimal PCR conditions were determined empirically. A GC acyclonucleotide ladder, shown in Figure S3.1E, was used to confirm that the LM-PCR specifically amplifies the *VEGFR2* proximal promoter region depicted in Figure 3.1C. Acyclonucleotides were purchased from New England Biolabs (N0460). Procedure was designed with input from other sources (Carey et al., 2009; Patterson et al., 1997; Tagoh et al., 2006).

ACKNOWLEDGEMENTS

We thank Ella Freulich for excellent technical assistance and Lynn Biderman, Yan Zhu, Masha Poyurovsky as well as other members of the Prives laboratory for helpful discussions. This work was supported by NIH grant CA87497 and the MSTP program at Columbia University Medical School.

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FIGURE LEGENDS

Figure 3.1 Mutant p53 Associates with the *VEGFR2* Promoter and Leads to Promoter Remodeling

MDA-468.shp53 cells were cultured for 8 days in 3D culture in the presence (-Mut p53, black) and absence (+Mut p53, red) of doxycycline. Cells were treated with formaldehyde to crosslink chromatin and subjected to the indicated procedures.

(A) Scanning chromatin immunoprecipitation (ChIP) for mutant p53 was performed along 4 kilobases surrounding the *VEGFR2* transcriptional start site (TSS). ChIP was performed in the presence and absence of doxycycline for mutant p53 and also in the absence of antibodies to p53 using primers corresponding to the indicated data points. Immunoprecipitated chromatin was subjected to qPCR and percent input-normalized signal between -DOX and +DOX samples were plotted relative to the peak binding signal at the -150 bp *VEGFR2* site. Error bars represent standard error of the three independent experiments shown in Figure S3.1A-C.

(B) For micrococcal nuclease (MNase) PCR chromatin was digested with MNase and mononucleosome-sized DNA fragments were isolated. qPCR was performed for six amplicons averaging 66 bp along 446 bp of the *VEGFR2* promoter from -390 bp to +56 bp relative to the TSS, with signal normalized to Amplicon 1. Error bars represent standard error of three independent experiments. *$p < 0.05$ by one tailed t-test.

(C) *In vivo* DNase I footprinting by ligation-mediated PCR was performed at the *VEGFR2* promoter between approximately -160bp to +5 bp of the TSS. Densitometry analysis of the relative DNase I hypersensitivity signal is represented by a histogram (+Mut p53, red, -Mut p53, black).

Figure 3.2 Mutant p53 is Found in Protein Complexes with Members of the SWI/SNF Chromatin Remodeling Complex at the VEGFR2 Promoter

Extracts of MDA-468 (**A** and **C**), SK-BR-3 (**B**), or MDA-231 (**D**) cells were subjected to immunoprecipitation (IP) with anti-p53 antibodies (mAb DO-1; **A** and **B**) or anti-BAF155 antibody (**C** and **D**) followed by immunoblotting (IB) with anti-p53 (mAbs DO-1; **A** and **B** or DO-1 and 1801; **C** and **D**), anti-BAF53A (**A** and **B**) or anti-BAF155 (**C** and **D**) antibodies. Inputs represent 5% (**A**, **B** and **D**) or 3.3% (**C**) of total extract.

(E) ChIP-re-ChIP was performed in MDA-468.shp53 cells by performing initial ChIP for IgG or mutant p53 followed by re-ChIP with BAF170 or IgG antibodies. qPCR was performed at the *VEGFR2* promoter at site -150 bp from the TSS. Signal is shown as percent input of input material. Error bars represent standard error of two independent experiments.

(F-H) Immunodepletion ChIP was performed in MDA-468.shp53 by immunoprecipitating cross-linked cell extract with IgG or anti-p53 mAbs (DO-1/1801/PAb421). ChIP was then performed on the immunodepleted extracts with antibodies to BAF170 **(F)** or BAF155 **(G)**. ChIP-qPCR was performed at the *VEGFR2*

promoter as in **E**. Signal is shown as fold signal over ChIP for IgG. Error bars represent standard error of two independent experiments.

(H) Immunoblot for mutant p53 with histone 2A as loading control corresponds to panels **F** and **G.**

Figure 3.3 SWI/SNF is Required for Maximal *VEGFR2* Expression, Nucleosomal Remodeling and Expression of Other Mutant p53-Dependent Genes

A and B. MDA-468.shp53 cells were grown for 5 days in 2D cell culture in the presence (-Mut p53, black) and absence (+Mut p53, red) of doxycycline and then treated with formaldehyde and prepared for scanning ChIP to detect occupancy of BAF155 **(A)** or BAF170 **(B).** IgG was used as a control in either case. Immunoprecipitated chromatin was subjected to qRT-PCR using primers that spanned the length of the *VEGFR2* gene (from -2.35 kb to +30 kb downstream of the coding and the 3'-untranslated region (UTR)). Percent input-normalized signal between -DOX and +DOX samples were plotted relative to the peak binding signal at the -150 bp *VEGFR2* site. Error bars represent standard error of three independent experiments.

(C) Immunoblot of indicated proteins in **A** and **B.**

(D) ChIP for mutant p53 in MDA-468.shp53 cells grown in 2D culture was performed in the presence and absence of BRG1 and BRM. Negative site corresponds to +30 kb downstream of the *VEGFR2* 3'-UTR. **p < 0.01 by one-tailed t-test.

(E) MNase-assisted ChIP was performed on MDA-468.shp53 cells grown in 2D culture with control siRNA or siRNA to BAF170. MNase-digested chromatin was incubated with antibodies to histone H3 and IgG, and immunoprecipitated mononucleosomal-size DNA was purified by agarose gel electrophoresis. qRT-PCR was performed using MNase-PCR primers at the proximal promoter (-78 to -10 bp from TSS; Amplicon 6, red) and normalized to the distal promoter (-390 bp to -330bp from TSS; Amplicon 1, blue). Error bars represent standard error of three independent experiments.

(F-I) MDA-468.shp53 cells grown in 2D culture were transfected with 20 nM of two independent siRNAs to deplete BRM **(F)**, BRG1 **(G)**, BAF155 **(H)** , or BAF170 **(I)**. Total *VEGFR2* transcript was assayed by qRT-PCR and normalized to control siRNA (Ctrl). Error bars represent standard error of three independent experiments. Corresponding immunoblots for (**E-I**) are shown in Figure 3.S3D.

(J-N) MDA-468.shp53 cells grown in 2D culture were transfected with mixture of 50 nM of siRNA to co-deplete *BRM* and *BRG1* or with control (Ctrl) siRNA. *VEGFR2* protein **(J)** and RNA **(K)** are shown. Three other mutant p53 transcriptional targets *IGFBP5* **(L)**, *Ceruloplasmin* **(M)**, and *Mammaglobin-A* **(N)** were also assayed by qRT-

PCR and normalized to *RPL32* internal control. Error bars represent standard error of three independent experiments. **p < 0.01 by two-tailed t-test.

Figure 3.4 SWI/SNF Complex Mediates Mutant p53-Dependent Transcription at Many Mutant p53 Responsive Genes

(A) RNA-Sequencing was performed on two independent replicates of MDA-468.shp53 cells grown for 4 days with either control siRNA, siRNA to deplete mutant p53 (Mut p53 knockdown, KD), or siRNAs to co-deplete BRG1 and BRM (SWI/SNF KD). The 3000 most affected genes in both knockdown conditions, as compared to siControl, were analyzed. The total number of upregulated and downregulated genes for each knockdown condition are depicted. The number of co-upregulated or co-downregulated genes in both Mut p53 KD and SWI/SNF KD conditions are demonstrated by Venn diagram.

(B) The table lists the number of co-regulated genes (common genes) and antagonistically regulated genes (defined as genes up- or down-regulated by mutant p53 depletion that were respectively down- or up-regulated by SWI/SNF depletion) from the RNA-Seq data. Percent of co-regulated genes was calculated by dividing the number of co-regulated genes by the number of genes affected in the KD condition.

(C) Proposed model depicting how mutant p53 interacts with SWI/SNF at mutant p53-responsive genes to promote transcription. Mutant p53 is recruited by SWI/SNF

to SWI/SNF regulated genes. Mutant p53 recruits other transcription factors (TF), histone acetyltransferases (HATs), or other chromatin modifiers which promote SWI/SNF-dependent promoter remodeling.

Figure 3.S1 Mutant p53 Associates with the *VEGFR2* Promoter and Leads to Promoter Remodeling, (Related to Figure 3.1)

(A-C) MDA-468.shp53 cells were cultured for 8 days in 3D culture in the presence (-Mut p53, black) and absence (+Mut p53, red) of doxycycline. Chromatin was crosslinked with formaldehyde and subjected to scanning chromatin immunoprecipiation (ChIP) analysis. Three biological replicates of the ChIP experiment from Figure 3.1A are shown to demonstrate binding patterns of mutant p53 to the *VEGFR2* promoter along 4 kilobases surrounding the *VEGFR2* transcriptional start site (TSS). ChIP was performed in the presence and absence of doxycycline for mutant p53 and also in the absence of antibodies to p53. Immunoprecipitated chromatin was subjected to qPCR and percent input-normalized signal between -DOX and +DOX samples were plotted relative to the peak binding signal at the -150 bp *VEGFR2* site.

(D) *In vivo* DNase I footprinting of *VEGFR2* exon 1 in MDA-468.shp53 cells grown in the presence (-Mut p53) or absence (+Mut p53) of doxycycline to deplete mutant p53. Approximate genomic position is indicated in relation to the transcriptional start site. Densitometry analysis of the relative DNase I hypersensitivity signal is represented by a histogram (+Mut p53, red, -Mut p53, black). Samples were run on

the same gel in non-adjacent lanes as indicated by dashed line.

(E) *In vivo* DNase I footprinting acycloCTP and acycloGTP ladder of the *VEGFR2* genomic region represented in Figure 3.1C to demonstrate the specificity of the footprinting. Acyclonucleotide ladder primers (Table 3.S1) were used to amplify the genomic region representing the *VEGFR2* promoter region in Figure 3.1C. Radiolabeled *VEGFR2* promoter footprinting primer 3 was then used along with acycloCTP or acycloGTP-supplemented PCR reaction to perform linear amplification. Footprinting products were resolved on a 6% polyacrylamide/8M urea sequencing gel. The position relative to the *VEGFR2* TSS (+1 site) is indicated. Genome sequence is from the UCSC Genome Browser hg19 assembly.

Figure 3.S2 Mutant p53 Forms a Protein Complex with Members of the SWI/SNF Chromatin Remodeling Complex, (Related to Figure 3.2)

(A) Mutant p53 was immunoprecipitated from MDA-468.shp53 cells following chromatin IP procedure. Input represents 3.3% of input material.

(B) Mutant p53 was immunoprecipitated from MDA-231.shp53 cells following chromatin IP procedure. Input represents 5% of input material.

(C) Mutant p53 was immunoprecipitated from HT29 cells following chromatin IP procedure. Input represents 25% of input material. Black lines adjoin lanes from the same immunoblot.

(D) ChIP-re-ChIP workflow.

(E) Immunodepletion ChIP workflow.

(F) Immunodepletion ChIP for mutant p53 was performed in MDA-468.shp53 cells by immunodepleting cross-linked cell extract with p53 or IgG antibodies. ChIP was then performed on the immunodepleted extracts with antibodies to mutant p53 (FL-393 polyclonal p53 antibody) or rabbit IgG control. qPCR was performed at the *VEGFR2* promoter at the site -150 bp from the transcriptional start site. ChIP signal is shown as fold increase over IgG ChIP signal. Error bars represent standard error of two independent experiments.

Figure 3.S3 SWI/SNF is Required for *VEGFR2* Expression and Nucleosomal Remodeling and for the Expression of Select Mutant p53-Dependent Genes, (Related to Figure 3.3)

MDA-468.shp53 cells were grown for 5 days in cell culture under the listed experimental conditions.

(A) Cells grown in the presence (-Mut p53, black) and absence (+Mut p53, red) of doxycycline were fixed with formaldehyde and prepared for scanning chromatin immunoprecipitation. Cell extracts were incubated with anti-p53 antibody FL-393 or a control rabbit IgG. Immunoprecipitated chromatin was subjected to qPCR using primers that spanned the length of the *VEGFR2* gene. Relative position from

VEGFR2 transcriptional start site along with exon position are indicated. Percent input-normalized signal between -DOX and +DOX samples were plotted relative to the peak binding signal at the -150 bp *VEGFR2* site. Error bars represent standard error of three independent experiments. The same samples were used for experiments in Figure 3.3A-B with immunoblot shown in Figure 3.3C.

(B-C) Cells were transfected with 20 nM of two independent siRNAs to deplete **(B)** BRM (red) or **(C)** BRG1 (grey). Expression of three novel mutant p53 transcriptional targets are shown: *IGFBP5*, *ceruloplasmin*, and *mammaglobin-A*. RNA expression was assayed by qRT-PCR and normalized to control siRNA condition. Error bars represent standard error of three independent experiments.

(D) Immunoblots for the experiments in (**B**), (**C**), and Figure 3.3E-I.

(E) MDA-468.shp53 cells were grown with and without doxycycline to deplete endogenous mutant p53. RNA expression was assayed by qRT-PCR and normalized to control siRNA condition for *IGFBP5*, *ceruloplasmin*, and *mammaglobin-A* genes.

(F) MDA-468.shp53 cells were transfected with mixture of 50 nM of siRNA to *BRM* and *BRG1* as well as with control siRNA. HMGCR and HMGCS1 RNA expression was assayed by qRT-PCR and normalized to control. Error bars represent standard error of three independent experiments.

Figure 3.S4 SWI/SNF Complex Mediates Mutant p53-Dependent Transcription at Many Mutant p53 Responsive Genes, (Related to Figure 3.4)

In two independent experiments (**A-B** and **C-D**), RNA-Sequencing was performed on MDA-468.shp53 cells grown for 4 days with control siRNA, siRNA to deplete mutant p53 (Mut p53 knockdown, KD), and siRNA to co-deplete BRG1 and BRM (SWI/SNF KD). At least 68 million reads per sample were acquired using 100 bp single end reads. Genes with a GFOLD change of at least 40% (1.667-fold) compared to control were categorized as either upregulated or downregulated.

(A) Genes that were co-upregulated or co-downregulated by mutant p53 or SWI/SNF were examined and overlapping expression patterns were determined for the first replicate of this experiment.

(B) Table lists number of co-regulated genes (defined as genes that were upregulated in both conditions or downregulated in both conditions) and antagonistically regulated genes (defined as genes up- or down-regulated by mutant p53 depletion that were respectively down- or up-regulated by SWI/SNF depletion) from the RNA-Seq data for the first replicate.

(C) Genes that were co-upregulated or co-downregulated by mutant p53 or SWI/SNF were examined and overlapping expression patterns were determined for the second replicate of this experiment.

(D) Table lists number of co-regulated genes and antagonistically regulated genes from the RNA-Seq data for the second replicate. The combined data for these two replicates using FDR criteria are presented in Figure 3.4A-B.

Table 3.S1 Primer, Oligonucleotide, and siRNA List

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) primers, scanning chromatin immunoprecipitation (ChIP) primers, micrococcal nuclease (MNase) PCR and MNase-ChIP primers, *in vivo* DNase I footprinting by ligation-mediated PCR primers and ligation linker sequence, RNA sequencing library primers, and siRNA sequences are shown. For the ChIP primers, base pair position is approximate and based on UCSC hg19 genome assembly. For microccocal nuclease primers, total amplicon length was calculated. For the RNA sequencing index primer, the barcode location, which was variable, is indicated.

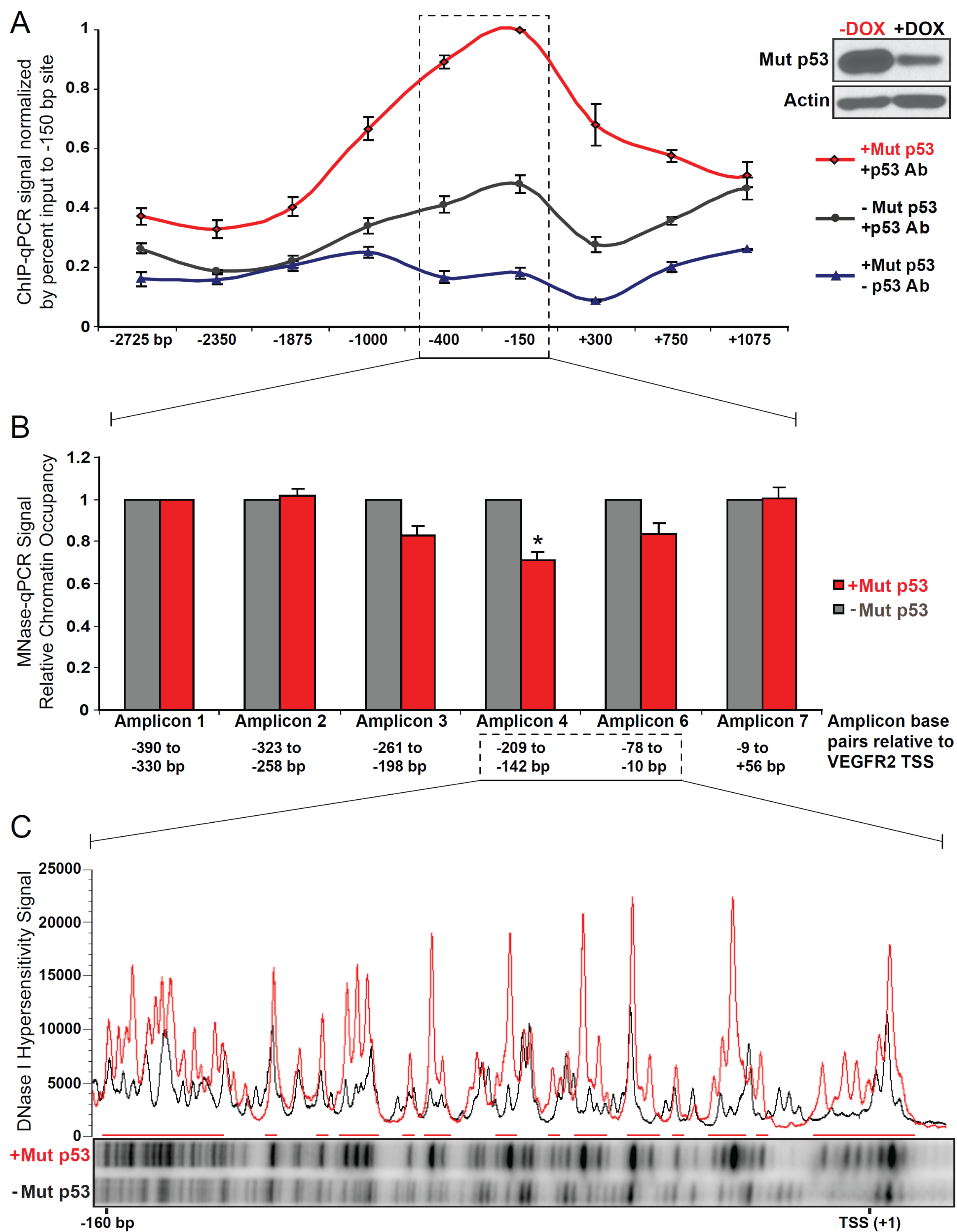
Table 3.S2 SILAC Mass Spectrometry List of Mutant p53 Interactors

H1299-p53-R282W cells with inducible mutant p53 R282W were grown with and without induction of p53 R282W using stable isotope labeling by amino acids in cell culture (SILAC) and immunoprecipitation was performed as described in the Methods section. Immunoprecipitated material was processed and analyzed by mass spectrometric analysis as described in the Methods section. Genes corresponding to mass spectra peptides with H/L normalized ratio > 2.0 are listed along with the official full name and NCBI gene alias. SWI/SNF components are listed in bold. BRM and BRG1 are both listed because an enriched peptide maps to both proteins.

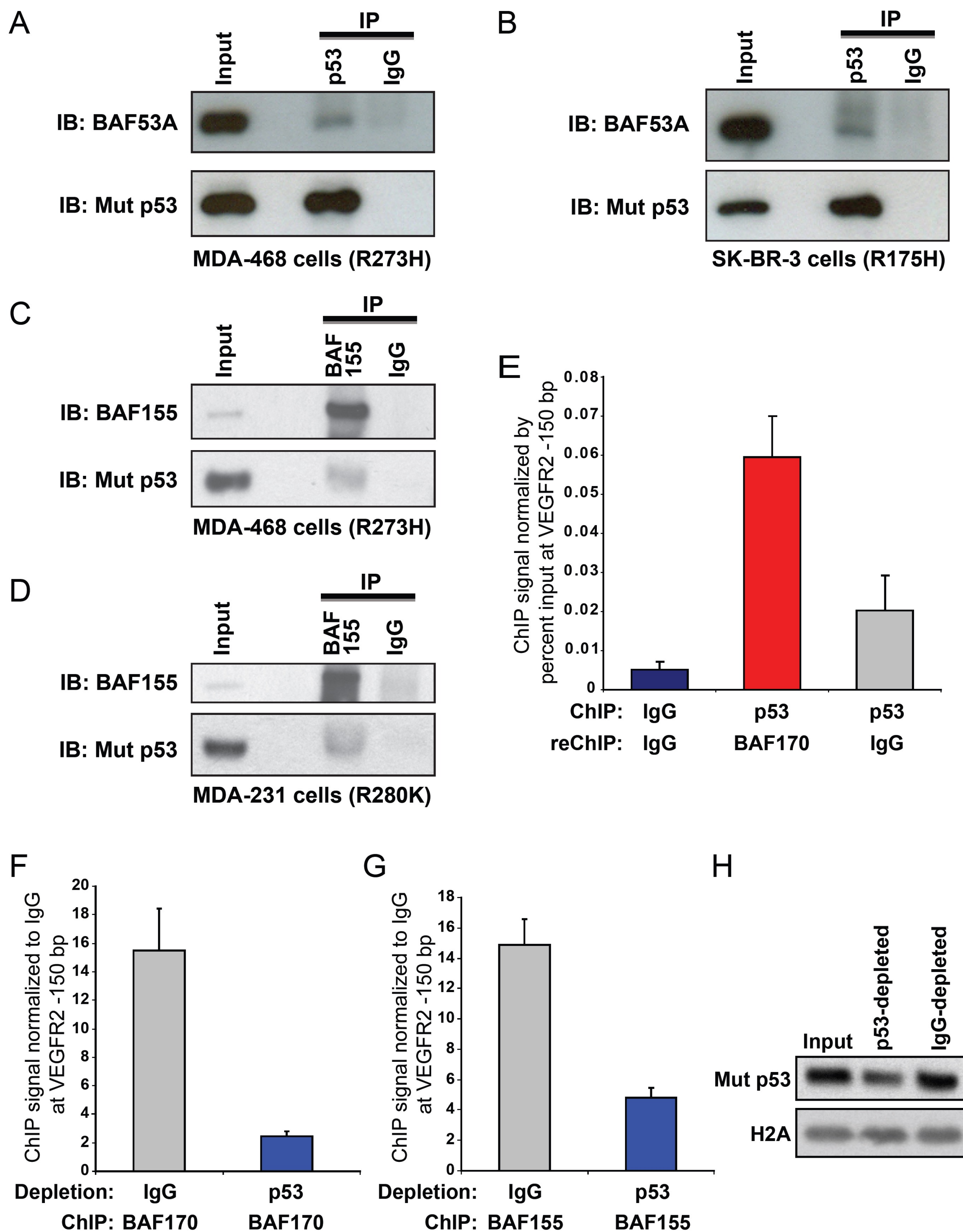
Table 3.S3 BioGRID Analysis of p53 and SWI/SNF Interaction Networks

BioGRID release 3.2.118 (Stark et al., 2006) was utilized to compile lists of *TP53* and SWI/SNF interactors based on published protein-protein or genetic interactions from human samples. Gene List 1 included *TP53*, for which there were 798 published p53 interactors (not shown). Gene List 2 included the listed SWI/SNF components (SWI/SNF gene aliases are listed) for which there were a total of 417 published SWI/SNF interactors (not shown). From the 798 *TP53* and 417 SWI/SNF interactors, there were 115 genes that overlapped between *TP53* and SWI/SNF groups (Common Interacting Partners). The 115 genes are separated into three columns and listed in alphabetical order. Note that SWI/SNF components and *TP53* are on the list (bolded and underlined), as different SWI/SNF components have been shown to interact with wild-type p53 (see main text). Nine proteins (bolded in red) that have been reported to interact with mutant p53 that are on the list are shown separately with the indicated references.

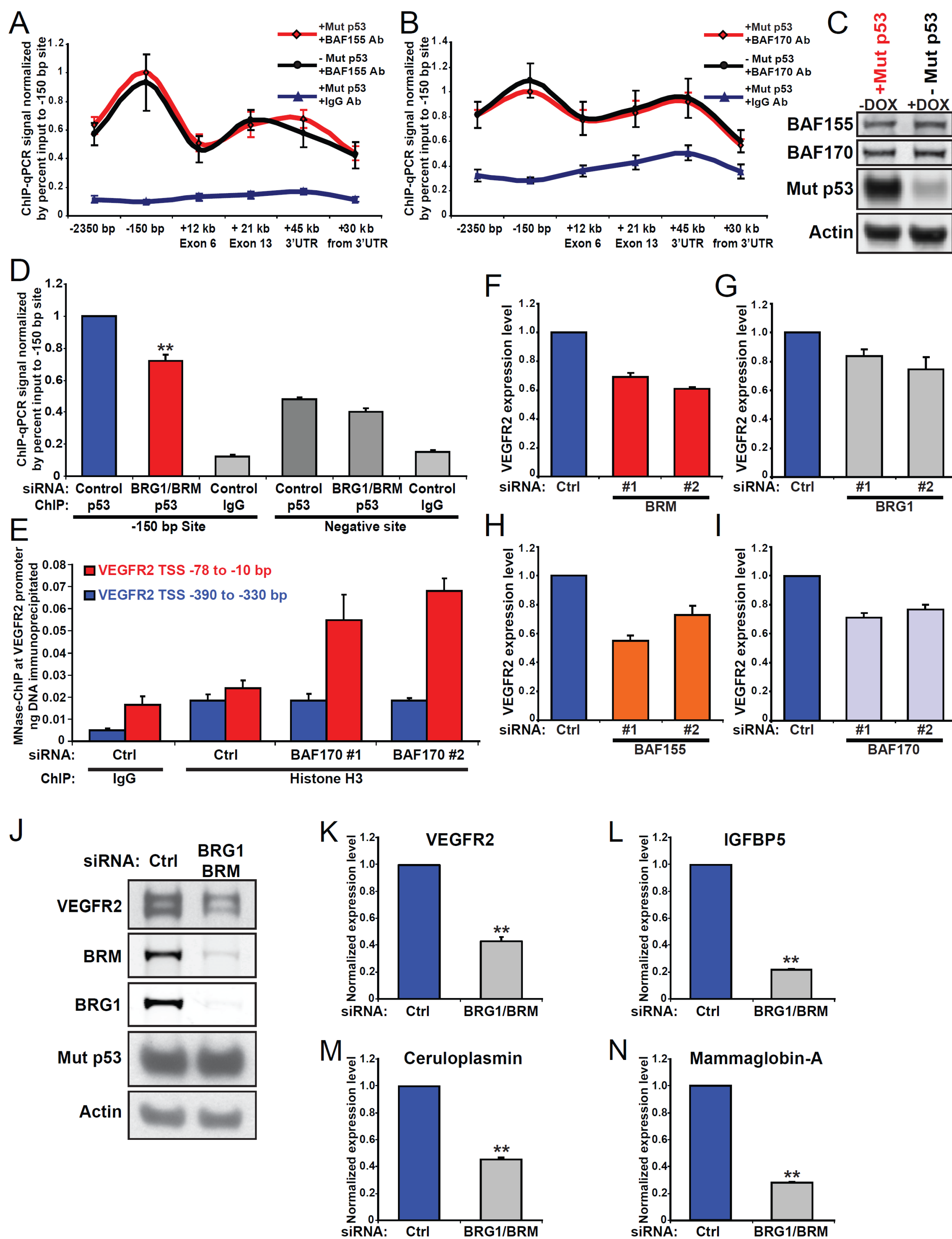
Pfister et al., Figure 3.1



Pfister et al., Figure 3.2

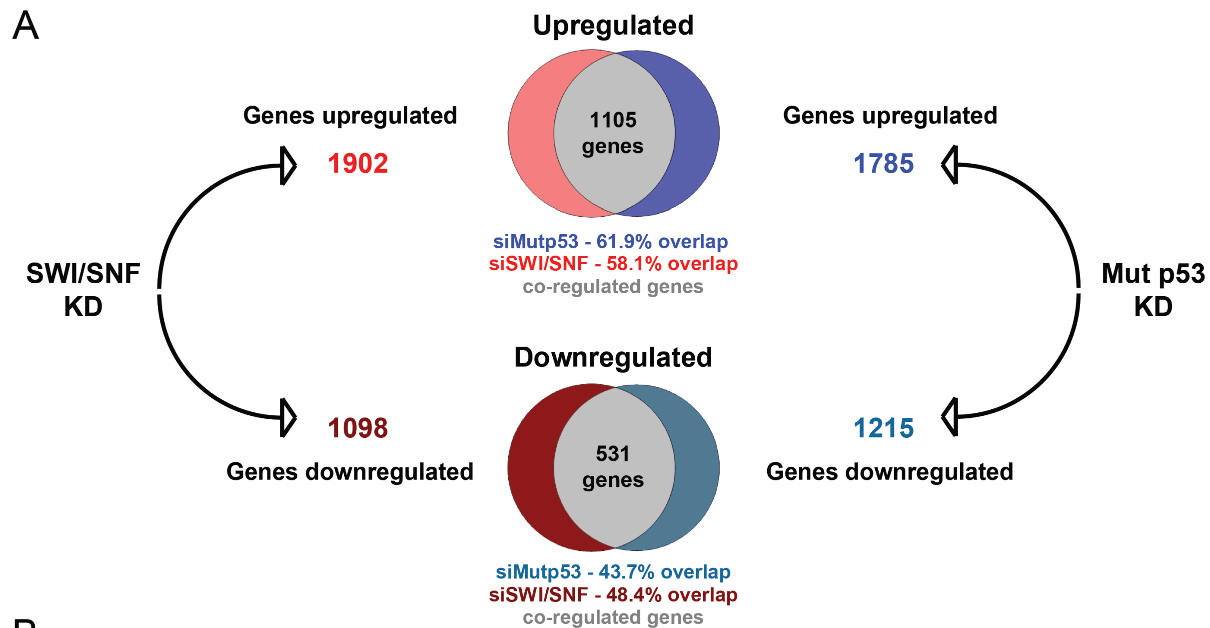


Pfister et al., Figure 3.3



Pfister et al., Figure 3.4

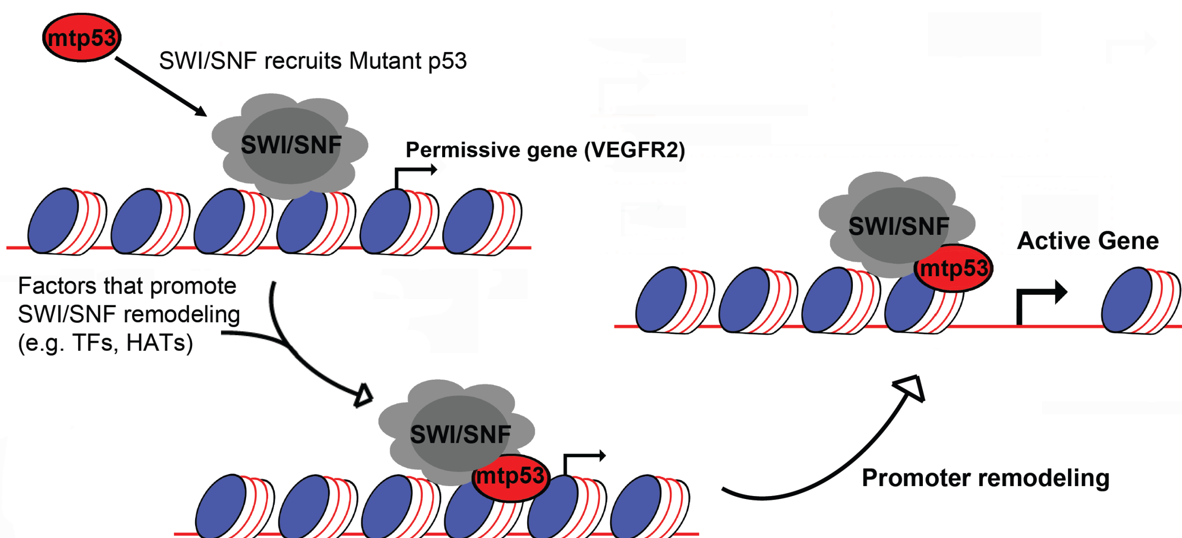
A



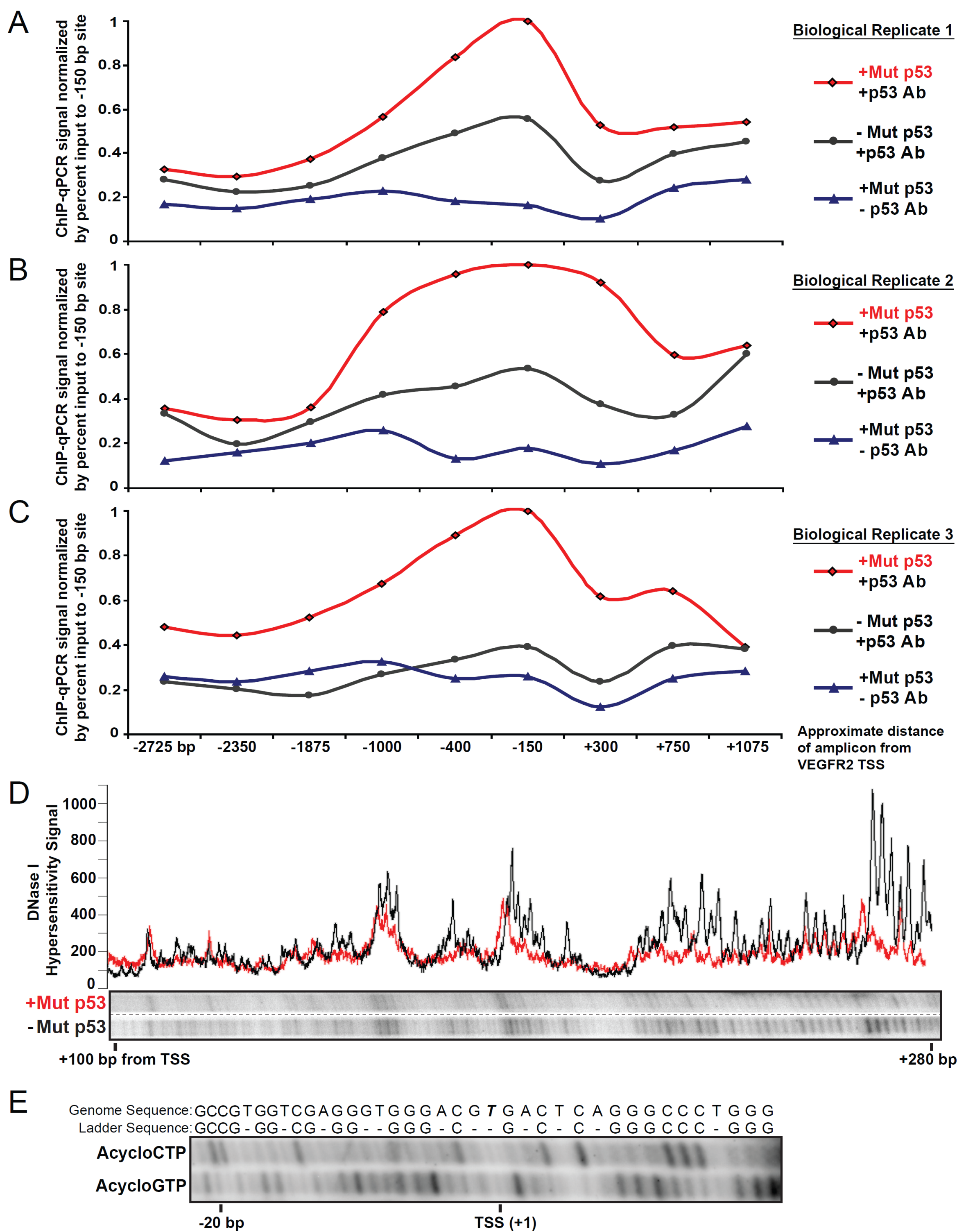
B

	Sample vs. si Control	Top 3000 genes per KD condition	Common genes	% genes co-regulated	Total Genes antagonistically regulated
Upregulated	siMutp53	1785	1105	**61.90%**	0
Genes	siSWI/SNF	1902	1105	**58.10%**	1
Downregulated	siMutp53	1215	531	**43.70%**	1
Genes	siSWI/SNF	1098	531	**48.36%**	0

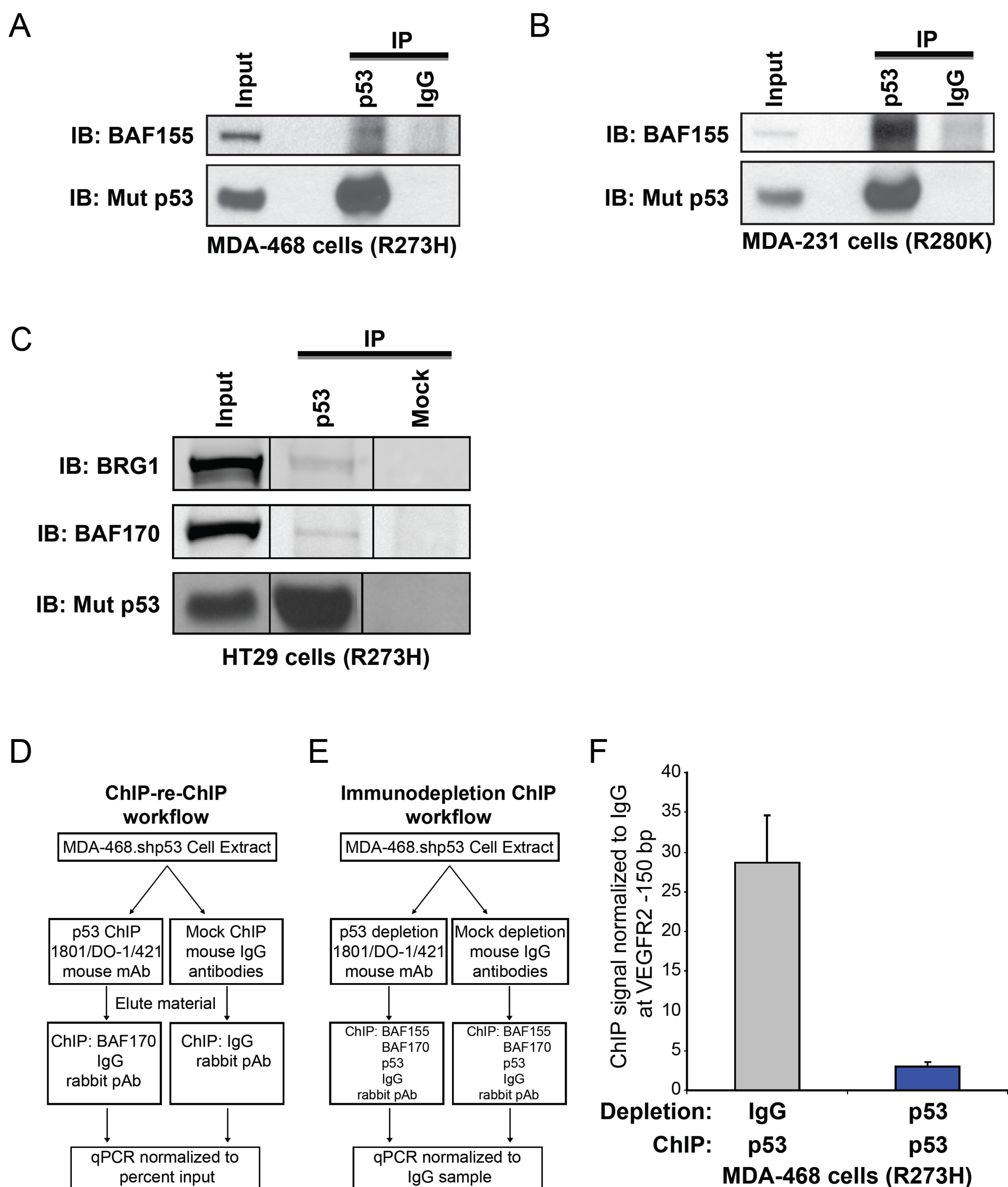
C



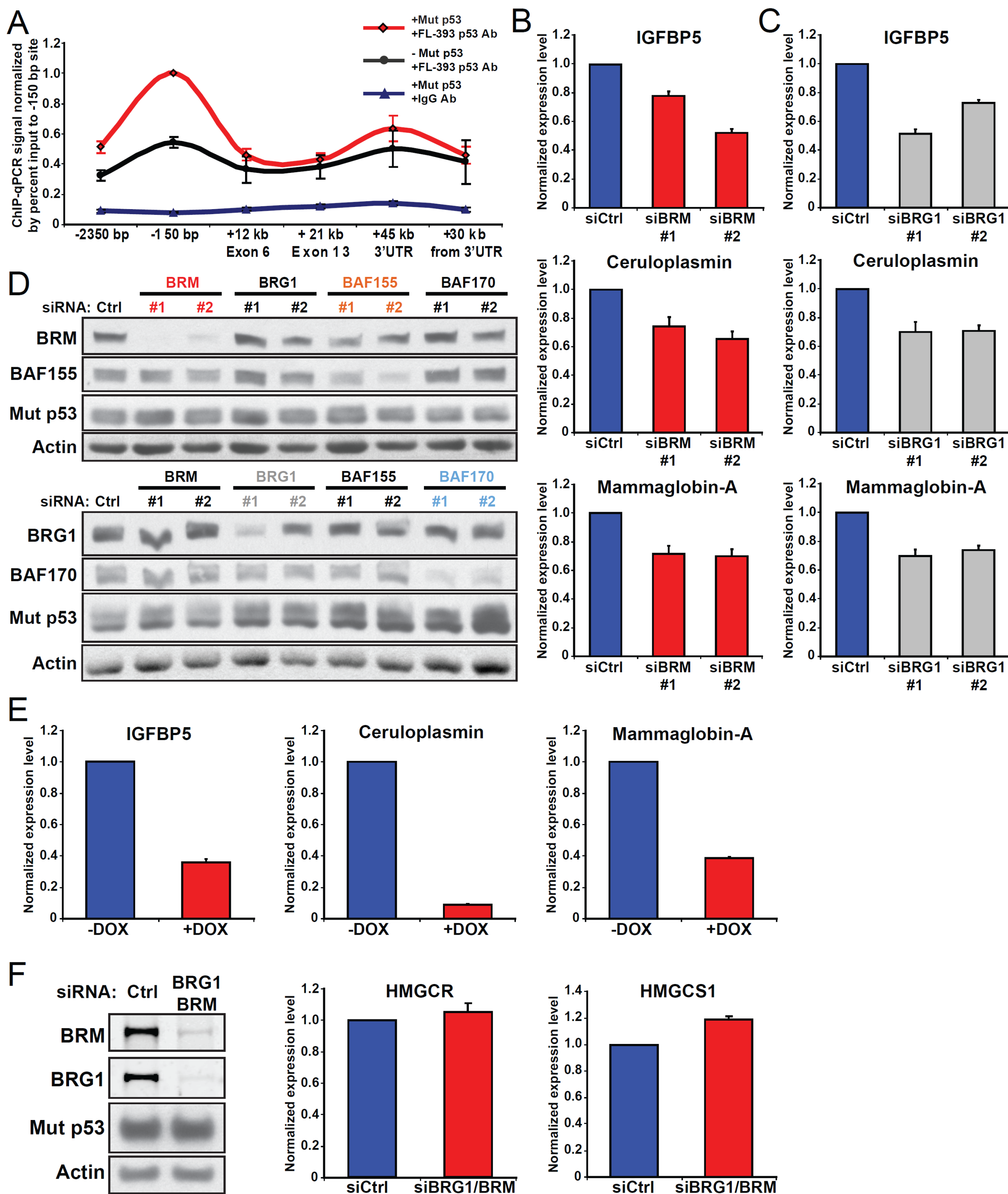
Pfister et al., Figure 3.S1



Pfister et al., Figure 3.S2

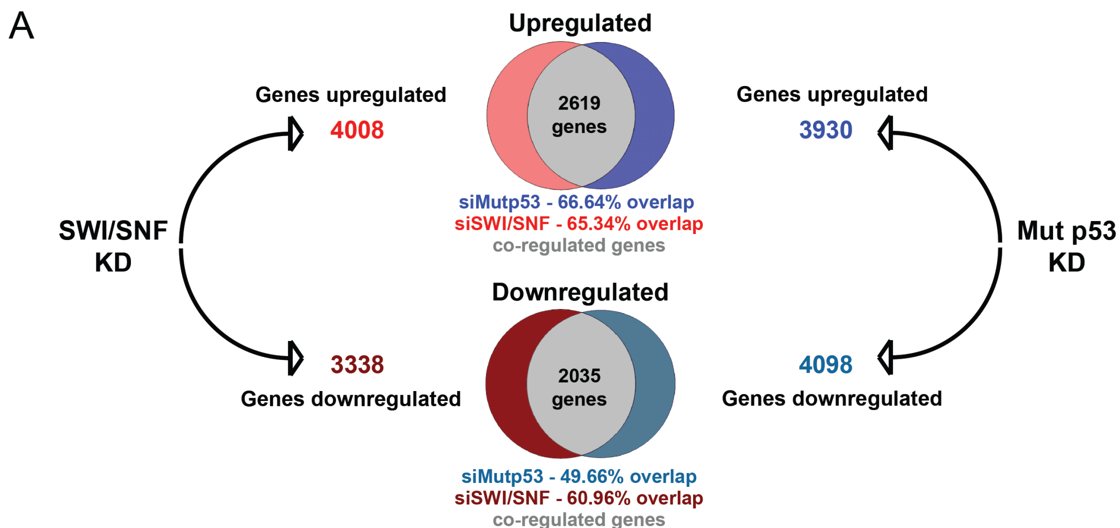


Pfister et al., Figure 3.S3



Pfister et al., Figure 3.S4

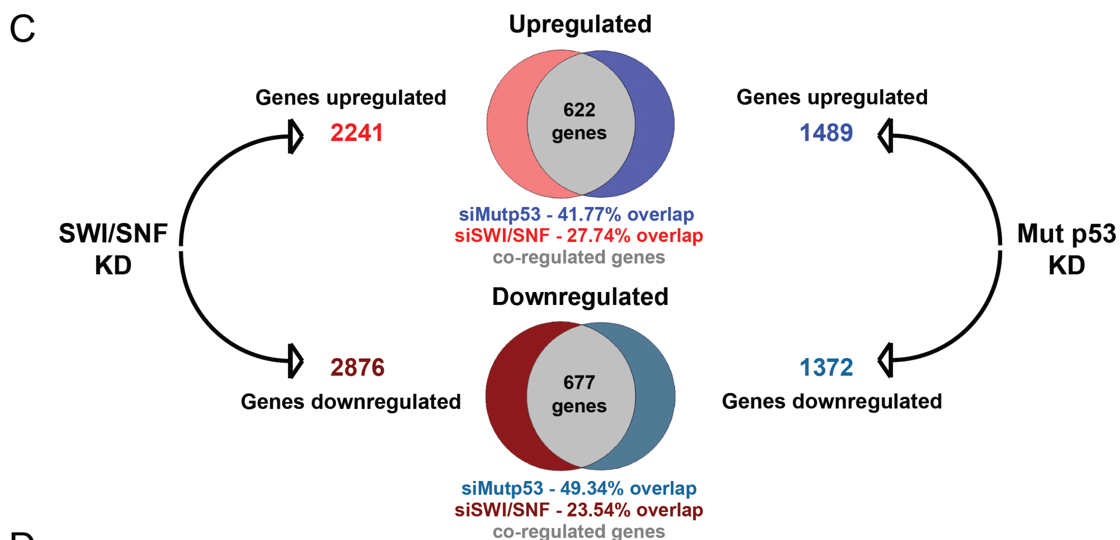
A



B

	Sample vs. siControl	Total genes affected at >40% expression change	Common genes	% genes co - regulated	Total genes antagonistically regulated	% genes antagonistically regulated
Upregulated	siMutp53	3930	2619	**66.64%**	51	**1.30**
Genes	siSWI/SNF	4008	2619	**65.34%**	57	**1.42**
Downregulated	siMutp53	4098	2035	**49.66%**	57	**1.39**
Genes	siSWI/SNF	3338	2035	**60.96%**	51	**1.53**

C



D

	Sample vs. siControl	Total genes affected at >40% expression change	Common genes	% genes co - regulated	Total genes antagonistically regulated	% genes antagonistically regulated
Upregulated	siMutp53	1489	622	**41.77%**	36	**2.42**
Genes	siSWI/SNF	2241	622	**27.74%**	13	**0.58**
Downregulated	siMutp53	1372	677	**49.34%**	13	**0.95**
Genes	siSWI/SNF	2876	677	**23.54%**	36	**1.25**

Pfister et al., Table 3.S1

	qRT-PCR Primers:	
Forward	*RPL32*	TTCCTGGTCCACAACGTCAAG
Reverse	*RPL32*	TGTGAGCGATCTCGGCAC
Forward	*VEGFR2* exonic primer	CCTCCCCCGCATCACAT
Reverse	*VEGFR2* exonic primer	GCTCGTTGGCGCACTCTT
Forward	*VEGFR2* intronic primer	TCCTTTTCTAGGACTCTGGTTTGC
Reverse	*VEGFR2* intronic primer	CGGCATCTCAGGACATGCT
Forward	*IGFBP5*	GATCTTCCGGCCCAAACA
Reverse	*IGFBP5*	TCTTCACTGCTTCAGCCTTCAG
Forward	*Ceruloplasmin (CP)*	CACCATCAGAGTAACCTTCCATAACA
Reverse	*Ceruloplasmin (CP)*	CCCCAATCGGCTCAATACTG
Forward	*Mammaglobin-A (SCGB2A2)*	TGGCTGCCCCTTATTGGA
Reverse	*Mammaglobin-A (SCGB2A2)*	TTGTATTCAGTCTTAGACACTTGTGGATT
Forward	*HMGCR*	GGCCCAGTTGTGCGTCTT
Reverse	*HMGCR*	CGAGCCAGGCTTTCACTTCT
Forward	*HMGCS1*	GGGCAGGGCATTATTAGGCTAT
Reverse	*HMGCS1*	TTAGGTTGTCAGCCTCTATGTTGAA

	Scanning ChIP Primers	Sequence:
Forward	*VEGFR2* TSS -2725 bp	CCCAGTTCCTGGTTCAATGC
Reverse	*VEGFR2* TSS -2725 bp	AGCAGGCTCATTCATTCAACAG
Forward	*VEGFR2* TSS -2350 bp	CTACTACTCTGCTGTGGCATCTGAA
Reverse	*VEGFR2* TSS -2350 bp	GCAAAGTGCCCCAAATGTGT
Forward	*VEGFR2* TSS -1875 bp	CTCTCCAAACCAGGTTCCATCT
Reverse	*VEGFR2* TSS -1875 bp	AAATAGGATGGACTCTGGCAAAGT
Forward	*VEGFR2* TSS -1000 bp	TGGTGAAGAATGGTCCTTTAGGTT
Reverse	*VEGFR2* TSS -1000 bp	AATCTTCCAGATGCCTATGTCTTTTAC
Forward	*VEGFR2* TSS -400 bp	TCTCCCTTGTGGCTCCAAAC
Reverse	*VEGFR2* TSS -400 bp	CGCGCGCGTCTGAAG
Forward	*VEGFR2* TSS -150 bp	GTTCTCTCCTGGGCGACTTG
Reverse	*VEGFR2* TSS -150 bp	CCATTTACATCTCCCCATTTCC
Forward	*VEGFR2* TSS +300 bp	TAGACAGGCGCTGGGAGAAA
Reverse	*VEGFR2* TSS +300 bp	AGCAGCACCTTGCTCTGCAT
Forward	*VEGFR2* TSS +750 bp	GCGAGAACAGGCGGTGAA
Reverse	*VEGFR2* TSS +750 bp	GGCCCGGACTAGGATGTTG
Forward	*VEGFR2* TSS +1075 bp	GGTCTCCAAGTAACAGCCAACTG
Reverse	*VEGFR2* TSS +1075 bp	CCACAGCGCTTTGAAAGATG
Forward	*VEGFR2* TSS +12 kb Exon 6	GGAAGTTCAGTCAACTCTTTTTTTCA
Reverse	*VEGFR2* TSS +12 kb Exon 6	TGGGTTTTTAGGTCTCGGTTTACA
Forward	*VEGFR2* TSS +21 kb Exon 13	TTGCAGGACCAAGGAGACTATGT
Reverse	*VEGFR2* TSS +21 kb Exon 13	CGCAATGTCTTTTCTTGGTCTTC
Forward	*VEGFR2* TSS +45 kb 3'UTR	TCTTCTCTCTGCCAACTCCTTTG
Reverse	*VEGFR2* TSS +45 kb 3'UTR	GCTTTTGCTGGGCACCAT
Forward	+30 kb from *VEGFR2* 3'-UTR	GGGCAAAAGGCCTGAACAA
Reverse	+30 kb from *VEGFR2* 3'-UTR	ATTTGCCTTCTTGCCATCTGTATAT

	MNase-PCR and MNase-ChIP Primers		Amplicon
Forward	*VEGFR2* MNase Amplicon 1	TGCAGATTCTCGGCCACTTCAGAC	61 bp
Reverse	*VEGFR2* MNase Amplicon 1	CTCACCAGGCGCGTCAAAG	
Forward	*VEGFR2* MNase Amplicon 2	TCTTCGCAGCGCTCCTGGTGATG	66 bp
Reverse	*VEGFR2* MNase Amplicon 2	GGCGCTGAGCAACTCCAAGATTTAATC	
Forward	*VEGFR2* MNase Amplicon 3	CAGCGCCCGTTACCGAGTAC	64 bp
Reverse	*VEGFR2* MNase Amplicon 3	CAGGAGAGAACATCCCAGAGCAACA	
Forward	*VEGFR2* MNase Amplicon 4	GTTCTCTCCTGGGCGACTTG	68 bp
Reverse	*VEGFR2* MNase Amplicon 4	CCATTTACATCTCCCCATTTCC	
Forward	*VEGFR2* MNase Amplicon 6	CTCCGGCCCCGCCCCGCAT	69 bp
Reverse	*VEGFR2* MNase Amplicon 6	TGGGAGCTGGTGCCGAAACTCTA	
Forward	*VEGFR2* MNase Amplicon 7	GCTCCCACCCTGCACTGAGT	66 bp
Reverse	*VEGFR2* MNase Amplicon 7	AACGCAGCGACCACACATTGA	

	***In vivo* DNase I Footprinting by LM-PCR Primers**	
	Blunt end ligation linker sequence #1 (annealed to #2)	AGCTTCGTGAGCATGGTGATCTGAATTC
	Blunt end ligation linker sequence #2 (annealed to #1)	GAATTCAGATC
Reverse	Footprink linker Primer	AGCTTCGTGAGCATGGTGATCTGAATTC
Forward	*VEGFR2* Promoter Footprinting Primer 1	AGGCAGAGGAAACGCAGCGA
Forward	*VEGFR2* Promoter Footprinting Primer 2	AGGAAACGCAGCGACCACACATTG
Forward	*VEGFR2* Promoter Footprinting Primer 3	ACGCAGCGACCACACATTGACCGCTCTC
Forward	*VEGFR2* Exon 1 Footprinting Primer 1	GTCTCCACGCAGAGCCACAG
Forward	*VEGFR2* Exon 1 Footprinting Primer 2	CTCTGCATCCTGCACCTCGAGC
Forward	*VEGFR2* Exon 1 Footprinting Primer 3	TCCTGCACCTCGAGCCGGGCGAAATG
Forward	*VEGFR2* Acyclonucleotide Ladder	ACGCAGCGACCACACATTGACCGCTCTC
Reverse	*VEGFR2* Acyclonucleotide Ladder	GTTGTTGCTCTGGGATGTTCTCTCCTG

	RNA Sequencing Library Primers	Barcode location underlined
	5'-Adapter	GUUCAGAGUUCUACAGUCCGACGAUCNNNN
	3'-Adapter	5rApp/NNNNTGGAATTCTCGGGTGCCAAGG/3ddC/
	Reverse Transcription Primer	GCCTTGGCACCCGAGAATTCCA
Forward	Index Forward Primer	AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA
Reverse	Index Reverse Primer, contains barcode	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA

	siRNA sequences:	Life Technologies Silencer Select® siRNA Reference	Gene
Sense	GUAAUCUACUGGGACGGAATT	s605	*TP53*
Antisense	UUCCGUCCCAGUAGAUUACCA	s605	*TP53*
Sense	GAAAUUUGCGUGUGGAGUATT	s606	*TP53*
Antisense	UACUCCACACGCAAAUUUCCT	s606	*TP53*
Sense	CCGCAUAGCUCAUAGGAUATT	s13133	*BRM*
Antisense	UAUCCUAUGAGCUAUGCGGGC	s13133	*BRM*
Sense	GCCCAUCGAUGGUAUACAUTT	s13134	*BRM*
Antisense	AUGUAUACCAUCGAUGGGCTT	s13134	*BRM*
Sense	GGAAUACCUCAAUAGCAUUTT	s13139	*BRG1*
Antisense	AAUGCUAUUGAGGUAUUCCTG	s13139	*BRG1*
Sense	GGCUUGAUGGAACCACGAATT	s13140	*BRG1*
Antisense	UUCGUGGUUCCAUCAAGCCTG	s13140	*BRG1*
Sense	CCAACACCUGUACCCAAUATT	s13145	*BAF155*
Antisense	UAUUGGGUACAGGUGUUGGGT	s13145	*BAF155*
Sense	CAAGAGUAUUUAACUAGCATT	s13146	*BAF155*
Antisense	UGCUAGUUAAAUACUCUUGGG	s13146	*BAF155*
Sense	GCUACUAUCCUGACAGUUATT	s13148	*BAF170*
Antisense	UAACUGUCAGGAUAGUAGCCC	s13148	*BAF170*
Sense	GCAAUGCACCGCUCACUAATT	s13149	*BAF170*
Antisense	UUAGUGAGCGGUGCAUUGCTG	s13149	*BAF170*

Pfister et al., Table 3.S2

Genes corresponding to Mass Spectra Peptides	Official Full Name	NCBI Gene Alias
ATOH1	Atonal homolog 1	ATH1; HATH1; MATH-1; bHLHa14
ANXA6	Annexin A6	ANX6; CBP68
XPOT	Exportin, tRNA	XPO3
VAPA	Vesicle-associated membrane protein-associated protein A, 33kDa	VAP-A; VAP33; VAP-33; hVAP-33
VAPB	Vesicle-associated membrane protein-associated protein B and C	ALS8; VAP-B; VAMP-B
PEF1	Penta-EF-hand domain containing 1	ABP32; PEF1A
HSPB1	Heat shock 27kDa protein 1	HS.76067; HEL-S-102
NGDN	Neuroguidin, EIF4E binding protein	NGD; LCP5; CANu1; lpd-2; C14orf120
BRM	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	SMARCA2; SNF2; SWI2; hBRM; NCBRS; Sth1p; BAF190; SNF2L2; SNF2LA; hSNF2a
BRG1 *peptide maps to BRM and BRG1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	SMARCA4; SNF2; SWI2; MRD16; RTPS2; BAF190; SNF2L4; SNF2LB; hSNF2b; BAF190A
BAF53A	Actin-like 6A	ACTL6A; Arp4; ACTL6; INO80K; ARPN-BETA
BCL7A	B-cell CLL/lymphoma 7A	BCL7
BCL7B *peptide maps to BCL7A and BCL7B	B-cell CLL/lymphoma 7B	
BCL7C	B-cell CLL/lymphoma 7C	

Pfister et al., Table 3.S3

Gene List 1:	Gene List 2:	Gene Alias:
TP53	SMARCA2	BRG1
	SMARCA4	BRM
	ACTL6A	BAF53A
	SMARCC1	BAF155
	SMARCC2	BAF170
	SMARCB1	INI1/hSNF5
	PBRM1	BAF180
	ARID1A	BAF250A
	ARID1B	BAF250B
	ARID2	BAF200
	SMARCE1	BAF57
	SMARCD1	BAF60A
	SMARCD2	BAF60B
	SMARCD3	BAF60C

Common Interacting Partners A-I	Common Interacting Partners I-S	Common Interacting Partners S-Z
ACTB	ITCH	SIRT7
ACTL6A	KAT2A	SMAD1
AR	KAT2B	**SMAD2**
ARID1A	KAT5	**SMAD3**
ATF3	KDM1A	**SMARCA4**
ATM	MAP1LC3B	**SMARCB1**
AURKB	MAPK14	**SMARCC1**
BMI1	MDM2	**SMARCD1**
BRCA1	MED17	**SMARCD2**
CAD	MED21	**SP1**
CARM1	MLL	STK11
CDK2	**MYC**	SUMO1
CDK8	NCOA1	SUMO2
CDK9	NCOR1	TAF1
CDKN2A	NPM1	TAF10
CHD3	NR0B2	TAF6
COPS5	NR3C1	TAF9
CREB1	NR4A1	**TBP**
CREBBP	PCNA	TFAP4
CSNK2A1	PHB	TOP2B
DDX5	**PML**	TOPORS
ECT2	PPP1CA	**TP53**
ELAVL1	PPP1CC	TP53BP1
EP300	PRMT5	**TP63**
EP400	RB1	TRIM28
ESR1	RB1CC1	TRRAP
EWSR1	RBBP4	UBB
H2AFX	RBBP5	UBC
HDAC1	RBBP7	UBD
HDAC2	RBX1	VCP
HDAC9	RFC1	**VDR**
HECW2	RNF2	WDR77
HHV8GK18_gp81	RPA1	WWOX
HIF1A	SART1	XPC
HMGB1	SETD7	YY1
HNRNPA1	SIN3A	ZMIZ2
HSPB1	SIN3B	ZMYND11
ING1	SIRT1	
ING2	SIRT2	

Characterized Mutant p53 Interactor	References reporting mutant p53 interaction partners (see supplemental references):
EP300	(Di Agostino et al., 2006)
MYC	(Frazier et al., 1998)
PML	(Haupt et al., 2013)
SMAD2	(Adorno, et al. 2009)
SMAD3	(Adorno, et al. 2009
SP1	(Chicas et al., 2000)
TBP	(Ragimov et al., 1993; Truant et al., 1993; Lee et al., 2000)
TP63	(Gaiddon et al., 2001; Strano et al., 2002; Adorno et al., 2009)
VDR	(Stambolsky et al., 2010)

Chapter 4

PERSPECTIVES AND FUTURE DIRECTIONS

We have reported two aspects of mutant p53 function that could lead to clinical interventions: (1) classifying breast tumors by *TP53* mutational status could improve response to anti-VEGF therapy due to the combined effect of inhibiting mutant p53-induced pro-proliferative *VEGFR2* signaling compounded with antagonistic effects on tumor vasculature (Chapter 2) and (2) targeting the SWI/SNF complex in mutant p53 tumors could impede mutant p53 transcriptional gain of function effects (Chapter 3).

We investigated how mutant p53 impacts transcription of *VEGFR2* (which we identified as one of its strongest target genes; see Chapter 2). Mutant p53 was found to bind near the *VEGFR2* transcriptional start site, causing the promoter to adopt a transcriptionally active conformation. We identified subunits of the SWI/SNF chromatin remodeling complex as mutant p53 interactors that co-occupy the *VEGFR2* promoter along with mutant p53. SWI/SNF is required for maximal mutant p53 promoter occupancy, as depletion of SWI/SNF both reduces mutant p53 association with the *VEGFR2* promoter and results in significantly reduced *VEGFR2* expression. Using RNA sequencing, we report that approximately half of all mutant p53-dependent gene alteration requires the SWI/SNF complex. We surmise that mutant p53 impacts transcription of *VEGFR2* as well as myriad other target genes by promoter remodeling through interaction with the SWI/SNF chromatin remodeling complex.

Mutant p53 Transcriptional Plasticity

Mutant p53 mediates pro-oncogenic transcriptional profiles (Brosh and Rotter, 2009). Our data supply a mechanism whereby mutant p53 may mediate genome-wide

transcriptional changes by SWI/SNF-mediated nucleosomal remodeling. This is important as mutant p53 has been proposed to facilitate transcriptional plasticity (Quante et al., 2012). Because nucleosomal positioning is a critical factor in gene regulation, promoting or inhibiting transcription by regulating access to DNA, mutant p53 may co-opt SWI/SNF activity to mediate both gene activation and repression. It is possible that mutant p53 may stimulate the activity of the SWI/SNF complex by recruiting additional factors such as p300. We are currently investigating this possibility to further define how mutant p53 cooperates with the SWI/SNF complex.

What we know is that mutant p53 and SWI/SNF both regulate largely overlapping sets of genes in cells expressing endogenous mutant p53. We also know that mutant p53 depletion leads to promoter closure at the *VEGFR2* locus (Figure 3.1). SWI/SNF and mutant p53 co-occupy the VEGFR2 promoter (Figure 3.2). SWI/SNF depletion (in the presence of mutant p53) also leads to promoter closure and correlates with decreased *VEGFR2* expression (Figure 3.3). Knockdown of mutant p53 does not affect SWI/SNF promoter recruitment, but SWI/SNF knockdown leads to decreased mutant p53 recruitment to the *VEGFR2* promoter (Figure 3.3). These data suggest a model whereby mutant p53 may amplify expression of genes which are primed by the presence of SWI/SNF. SWI/SNF enhances mutant p53 promoter occupancy, and then mutant p53 (likely through its transactivation subdomains or C-terminal domain) recruits additional factors that promote SWI/SNF-dependent promoter recruitment. Remember that in the absence of mutant p53, the VEGFR2 promoter is closed and the gene is not abundantly expressed.

The SWI/SNF complex associates genome-wide with transcription regulatory elements (Euskirchen et al., 2011) to regulate nucleosome occupancy (Tolstorukov et al., 2013). Interestingly, BRG1 and BRM both impact *VEGFR2* expression and independently affect the expression of three other mutant p53 target genes. This suggests the at least in this cell line, BRG1 and BRM can co-substitute for each other in promoting mutant p53 gain of function.

SWI/SNF proteins are tumor suppressive in some contexts. For instance, PBAF subunit BAF180 mediates p21 expression in breast tumor cells to suppress tumorigenesis (Xia et al., 2008), BRG1 is necessary for efficient RB-mediated cell cycle arrest (Strobeck et al., 2000), and BRG1 cooperates with ATM to promote the DNA damage response (Kwon et al., 2014). Moreover, mutations in SWI/SNF subunits and *TP53* have a tendency toward mutual exclusivity in multiple cancer types including breast cancer, suggesting that loss of SWI/SNF function may phenocopy p53 loss to mediate oncogenesis (Kadoch et al., 2013).

It is interesting to consider that the tumor suppressive functions of SWI/SNF may be counterbalanced by the dependence of a cancer cell to utilize SWI/SNF function for its own pro-survival purposes. It is conceivable that alteration of SWI/SNF function through interaction with mutant p53 may obviate SWI/SNF tumor suppressor function and allow the complex to function as an oncogene. This may explain, for instance, why SWI/SNF components are frequently amplified in multiple tumor types (see Figure 1.4). Interestingly, in tumors with mutated SWI/SNF residues, other functional SWI/SNF components can retain oncogenic potential for the cell. This has been described for BRG1 and BRM (the ATPases of the SWI/SNF complex). When BRG1 is mutated,

intact SWI/SNF complex is still present in tumor cells (Wilson et al., 2014). This is thought to be because BRM may substitute for BRG1 (Wilson et al., 2014). As mentioned in Chapter 1, this hypothesis led to the discovery using an shRNA screen that in BRG1 mutant tumors, BRM is the most important genetic vulnerability that can be targeted (Hoffman et al., 2014; Wilson et al., 2014). The authors describe the concept of cancer-selective paralog dependency, whereby loss of one genetic paralog (BRG1) reveals a dependence to the paralogous gene (BRM)(Hoffman et al., 2014). It is interesting to consider that such genetic vulnerabilities could be harnessed to antagonize the growth of mutant p53-expressing tumors.

Wild-type p53 interacts with SWI/SNF, which is important in mediating expression of p53 target gene p21 (Lee et al., 2002). Multiple components of the SWI/SNF complex have been identified as wild-type p53 binding partners including BRG1 (Lee et al., 2002; Naidu et al., 2009), SNF5 (Lee et al., 2002), BAF60A and BAF155 (Oh et al., 2008), ARID1A (Guan et al., 2011), and BRD7 (Burrows et al., 2010). At the *p21* promoter, which both distal and proximal p53 response elements contain high levels of nucleosomal occupancy (Laptenko et al., 2011). Upon p53 activation, nucleosomal occupancy is rapidly lost (Laptenko et al., 2011). Nucleosomal displacement is most likely to occur subsequent to p53 DNA binding as it is unlikely that the alternative - that nucleosomes and p53 compete for the same site - occurs, especially considering that p53 can bind to its response element while the response element is engaged by a nucleosome (Laptenko et al., 2011). Furthermore, p53 recruits p300 in order to acetylate nucleosomal histones to mediate transcriptional activation (Espinosa and Emerson, 2001). It can be surmised that p53 binds to its response

element, recruits p300 which acetylates key histone residues, which stimulates SWI/SNF recruitment and subsequent nucleosomal repositioning to facilitate the recruitment of other transcriptional components that culminate in the formation of the RNA pol II pre-initiation complex. SWI/SNF may be stably present throughout these processes or be recruited following p53 response element binding, and SWI/SNF could dissociate from the promoter once remodeling occurs. SWI/SNF dissociation from the p21 promoter following promoter remodeling (subsequent to p53 response element binding) could explain the observation that BRG1 promoter occupancy decreases following p53 recruitment (Naidu et al., 2009).

In this work, we describe mutant p53 interaction with BRG1, BRM, BAF155, BAF170, and BAF53A (Chapter 3). As mutant p53 and wild-type p53 often mediate opposing effects on their interacting partners, in theory mutant p53 could dysregulate normal SWI/SNF complex function that wild-type 53 requires for transcriptional activities (Lee et al., 2002; Xu et al., 2007) by affecting its activity, interaction with other proteins, or chromosomal positioning. Mutant p53 has been proposed to facilitate transcriptional plasticity (Quante et al., 2012), and functional interaction of mutant p53 with a chromatin remodeling complex like SWI/SNF that has broad genomic distribution (Euskirchen et al., 2011) may explain the ability of mutant p53 to mediate gene expression at multiple loci. Because nucleosomal positioning is a critical factor in gene regulation, promoting or inhibiting transcription by regulating access to DNA-binding proteins (Wilson and Roberts, 2011), mutant p53 could theoretically co-opt SWI/SNF activity to mediate both gene activation and repression.

Mutant p53 Gain of Function

It is well established that mutant p53 promotes metastasis in mouse models, which is consistent with the observations that mutant p53 expression correlates with worse survival in human cancers (Alsner et al., 2008; Elledge et al., 1993; Langerod et al., 2007; Olivier et al., 2006; Petitjean et al., 2007). Hotspot mutations of p53 lead to increased cell proliferation, (Bossi et al., 2006; Bossi et al., 2008; Freed-Pastor et al., 2012; Haupt et al., 2009; Preuss et al., 2000; Scian et al., 2004; Strano et al., 2002; Yan and Chen, 2009; Yan et al., 2008), resistance to apoptosis (Bossi et al., 2008; Lim et al., 2009) which can be mediated through mutant p53 interaction with Ets-2 (Do et al., 2012), increased migration (Adorno et al., 2009; Weissmueller et al., 2014), and increased cellular invasion through Matrigel (Muller et al., 2009; Muller et al., 2013). The weight of these studies cannot be ignored. The contributions of specific missense mutations to cancer formation - and how different missense mutations correlate with other mutations or gene expression patterns - is essential to making significant strides in the understanding and treatment mutant p53-expressing cancers.

These studies identify a variety of mechanisms through which mutant p53 promotes oncogenesis. As each tumor is unique in its development, it should be considered at this point that mutant p53 may be a promiscuous transcription factor that is utilized by the tumor cell - based on its specific mutations, dominant signaling pathways, and interaction with the microenvironment - in a manner that is selectively advantageous. Mutant p53 has been reported to stimulate multiple receptor tyrosine kinases, including EGFR (Ludes-Meyers et al., 1996), IGF1R (Werner et al., 1996), MET (Muller et al., 2013), and PDGFRB (Weissmueller et al., 2014), all of which, along

with VEGFR2, promote pro-proliferative signaling. As a tumor forms, acquisition of a hotspot mutation in *TP53* may facilitate transcriptional plasticity, whereby tumor cells increase capacity for gene expression changes and therefore undergo selection for the greatest pro-proliferative transcriptional program for the particular tumor context. This hypothesis explains, for instance, why such a wide array of genes and pathways has been reported to mediate mutant p53 gain of function.

One example from this work involves VEGF pathway signaling. *TP53* mutation facilitates the angiogenic switch by de-repressing HIF1A and VEGFA expression (Mukhopadhyay et al., 1995; Ravi et al., 2000), promoting expression of pro-angiogenic factors that enhance tumor angiogenesis, growth, and metastatic potential (Folkman, 2002). Our data suggest that p53 hotspot mutants may be selected over loss of function p53 mutants during the progression of breast cancer in part due to the advantages conferred by cell-autonomous VEGFR2 signaling. SWI/SNF activity is required for mutant p53-mediated VEGFR2 expression, and our data also suggest that *TP53* mutated tumors may respond to anti-VEGF pathway drugs.

Specific mutant p53 domains are either required or dispensible to mutant p53 target gene activation, and the mechanisms of this regulation are incompletely defined (Table 1.1). It is imperative to investigate domain-specific p53 interactions in order to understand mechanistically how p53 regulates various genes as well as to integrate new findings in the context of previous literature. Mutant p53 likely mediates transcription by co-opting sets of transcription factors to initiate gene activation at the transcription factor's location. Co-activators recruited by the transcription factor or mutant p53 then stimulate gene expression. The extent that mutant p53 co-opts

individual transcription factors for target gene activation is unclear and likely dependent on the specific mutation in p53 and the active cell signaling pathways leading to subsets of active transcription factors in the cell. It is also possible that mutant p53, following recruitment by a transcription factor or chromatin modulator, recruits additional factors that can stimulate the function of the initial recruiting factor.

Wild-type p53 is known to direct transcription through interaction with the Mediator complex (Meyer et al., 2010; Zhang et al., 2005). The pre-initiation complex is composed of the Mediator complex, the general transcription factors TFII-A, -B, -D, -E, -F, and -H, and RNA polymerase II (Esnault et al., 2008; Roeder, 1996). The human Mediator complex interacts directly with TFIID in the process of forming the pre-initiation complex (Johnson et al., 2002). p53 has been reported to interact with various Mediator components (Gu et al., 1999), including Med17 (TRAP80)(Ito et al., 1999) and Med1 (RB18A)(Drane et al., 1997; Meyer et al., 2010). Med17 interacts with p53 TAD1 (Ito et al., 1999) and Med1 interacts with the p53 CTD (mapped to residues 363-393) (Meyer et al., 2010). Interestingly, increasing titrations of Med1 lead to decreased p53-dependent p21 expression and increased p53-dependent Bax expression (Frade et al., 2000) and increased MDM2 expression (Frade et al., 2002). Notably, the D5 domain of Med1 has been reported to interact with mutant p53 in Raji lymphoma cells (R213Q, Y234H), although this interaction has not been reviewed by the mutant p53 literature nor subsequently reported on through this point in time (Lottin-Divoux et al., 2005).

Further studies should investigate the role of the Mediator complex in mediating mutant p53 gain of function. It is likely that the Mediator complex is necessary to mediate mutant p53-dependent gene expression at SWI/SNF remodeled promoters.

The extent that mutant p53 may interact with and regulate the Mediator complex will be the basis for many further studies. The reason this is critically important is that protein-protein interactions with mutant p53 do not exist in cells that do not have mutant p53. If a protein is found that is required for mutant p53 gene transactivation, then an intervention could be developed for the mutant p53-protein complex that would only exist in cells expressing mutant p53 gain of function mutants.

Anti-VEGF Therapy in TP53 Mutant Breast Cancer

It is an exciting observation that *TP53* status may predict response to anti-VEGF therapies in breast cancer (Chapter 2). Needless to say, larger and more comprehensive clinical trials must be conducted in order to define an effect. Furthermore, these data must be stratified by *TP53* mutation types, which will require large patient cohorts. To improve the confidence in this hypothesis, mouse models should be considered. It would be optimal to generate breast tissue-specific conditional VEGFR2 knockout mice to study in different p53 genetic backgrounds. A more feasible alternative would be to study the extent that *in vivo* knockdown of VEGFR2 affects tumor growth, metastasis, and survival in xenotransplants of breast tumor cells expressing inducible short hairpin RNAs to deplete VEGFR2. Other models of VEGFR2 inhibition such as pharmacological inhibition or VEGF antagonism affect the tumor vasculature, so the impact of autocrine VEGFR2 signaling on tumorigenesis can be isolated. Tumor cell-specific depletion of VEGFR2 on the other hand would be restricted to the tumor cells. This could be compared to mutant p53 depleted cells. The proposed experiment has never been conducted and will provide valuable insight with the

implication that cancers with enhanced autocrine VEGFR2 signaling will respond more favorably to anti-VEGF treatments than cancers that do not have high epithelial VEGFR2 expression.

It is also possible to investigate the mechanisms in which VEGFR2 affects cell growth. We observed that VEGFR2 knockdown prevents optimal cell growth, and this could occur through decreased cellular proliferation, increased apoptosis, increased cellular senescence, or a combination of these mechanisms. Interestingly, withdrawal of VEGF has been shown to shift tumor cells into a senescent state (Hasan et al., 2011). It is also important to examine the cell signaling changes mediated by mutant p53 and VEGFR2. Such experiments could include extensive immunoblotting for the PI3K, MAPK, FAK, and PKC signal cascades in the presence and absence of mutant p53 and VEGFR2. It is always important to consider expressing a potential phenotype-mediating gene in a rescue model to have more confidence in an observation, so VEGFR2 could be re-introduced in the absence of mutant p53 (as in Figure 2.3A-C).

It would be informative to analyze the gene expression changes that occur upon VEGFR2 inhibition (or other mutant p53 implicated receptor tyrosine kinases) and compare these to mutant p53 depletion. It is likely that VEGFR2 signaling mediates some of the oncogenic gene expression changes mediated by mutant p53. Because VEGFR2 inhibition by itself is enough to restrict growth of mutant p53-expressing breast cancer cells, it is important to identify any coordinating factors that may mediate this effect. Such a factor may be common in multiple mutant p53-upregulated signaling pathways (eg: by EGFR or MET). This potential factor could then be specifically targeted in mutant p53 tumors.

Final Remarks

TP53 is among the most important genes in cancer. However, it cannot continue to be primarily studied without consideration to other mechanisms taking place in the cell. New hypotheses regarding mutant p53 function should be derived from large databases of mutations in human cancer. Synthetic lethal shRNA screens will be increasingly important in identifying proteins that cooperate with mutant p53 or other oncogenes. Cell biological studies should take account what exists in the clinical literature so that the time from bench to bedside is reduced. The goal is that selection criteria for therapeutics may be pre-selected for an individual with specific alterations in certain genes. Clinical trials must increasingly obtain genetic information from patients and consult the scientific literature to understand which subsets of patients could most benefit from a particular drug and then adjust the clinical trial criteria for those genetic subtypes. There is much to be said about increasing collaboration between these areas.

Oncology is headed toward highly adaptable treatment regimens based on the particular genetic alterations of a tumor. Defining the contributions of mutant p53 and VEGFR2 to breast cancer tumorigenicity are likely to be critical steps toward identifying specific tumor alterations that can be therapeutically harnessed. Outstanding questions include how SWI/SNF and mutant p53 positively regulate each other via recruitment of p53 to promoters and how the ensuing functional activation of promoter remodeling occurs. Future directions also include testing the feasibility of development of small molecules to interrupt the mutant p53-SWI/SNF interaction to impede mutant p53 gain of function activities. Finally, whether patients with mutant p53-expressing breast tumors demonstrate improved survival with anti-VEGF treatment or such newly developed

small molecules that impede mutant p53-dependent transcription will be of paramount importance.

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