Characterization of consecutive p53 responses separated by a period of recovery in human cells and samples from acute myeloid leukemia patients undergoing allogenic stem cell transplantation

by

John Chmara

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Abstract

The p53 tumor suppressor is a transcription factor that exerts its anti-neoplastic effects by regulating the expression of specific mRNAs and miRNAs. Previous work from our lab using a temperature sensitive variant of p53 that permitted rapid and reversible control of p53 activity suggested that p53-induced mRNAs were unstable while miRNAs were stable. In this way, p53-induced mRNAs returned to baseline rapidly after transient p53 activation while p53-responsive miRNAs remained elevated, modulating subsequent p53 responses. In the present work, this model was tested using a reversible small molecule activator of p53 (Nutlin-3a) in HCT116 colon cancer cells. In addition, the p53 response was monitored in buccal epithelium and blood samples obtained throughout the course of daily radiation treatments in patients with acute myeloid leukemia in preparation for bone marrow transplantation. These models didn't fully recapitulate earlier findings but provide important insight into the p53 response *in vitro* and *in vivo.*

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Statement of Contributions

Teeghan Rambo, a second-year undergraduate researcher, performed the immunoblot assays of the dual p53 response to Nutlin-3a treatment in HCT116 cells.

Ficoll-Pacque® separations of patient blood samples were conducted by staff at the Ottawa Hospital under direction of Dr. Mitchell Sabloff.

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AML	Acute myeloid leukemia
ATM	Mutated in ataxia telangiectasia
BAX	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bp	Base pair
BSA	Bovine serum albumin
BTG2	B-cell translocation gene 2
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CD	Cluster of differentiation/cell differentiation
cDNA	Complementary DNA
CDKN1A	Cyclin-dependent kinase inhibitor 1A
ChIP	Chromatin immunoprecipitation
Cip1	Cyclin-dependent kinase inhibitory protein 1
CK1	Casein kinase 1
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CR	Complete remission
CREB	cAMP response element-binding protein
CTD	C-terminal domain
CUREB	Carleton University Research Ethics Board
DBD	DNA binding domain
DDB2	DNA damage-binding protein 2
DNA	Deoxyribonucleic acid
DNA-PK	DNA-activated protein kinase
dNTP	Deoxynucleotide
dTTP	Deoxythymidine triphosphate
ENCODE	Encyclopedia of DNA elements
GAPDH	Gylceraldehyde 3-phosphate dehydrogenase
GDF15	Growth differentiation factor 15
GMP	Granulocyte/macrophage progenitor
Gy	Gray
H2B	Histone 2B
HAT	Histone acetyltransferase
HDAC1	Histone deacetylase 1
HiDAC	High-dose cytarabine
HSC	Hematopoietic stem cell
Hsp27	Heat shock protein 27
Ile	Isoleucine
IR	Ionizing radiaton
K	Lysine
KRT	Cytokeratin
kD	Kilodalton

Leu	Leucine
LT-HSC	Long-term renewing hematopoietic stem cell
Lys	Lysine
MDM2	Murine double minute 2
MEF	Mouse embryonic fibroblast
MEP	Megakaryocyte/erythrocyte progenitor
miR	MicroRNA
miRNA	MicroRNA
MPP	Multipotent progenitor
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NIH	National Institute of Health
NK	Natural killer
NLS	Nuclear localization signal
NOXA(PMAIP1)	Phorbol-12-myristate-13-acetate-induced protein 1
OD	Oligomerization domain
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCAF	P300/CBP-associated factor
PCR	Polymerase chain reaction
Phe	Phenylalanine
PRD	Proline-rich domain
PUMA	p53 upregulated modulator of apoptosis
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RE	Response element
RNA	Ribonucleic acid
RT	Reverse transcriptase/reverse transcription
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH3	Src homology 3
Ser	Serine
SEER	Surveillance, epidemiology, and end results program
SEM	Standard error of the mean
SV40	Simian virus 40
SUMO	Small ubiquitin-like modifier
TAD	Transactivation domain
TAF	TBP-associated factor
TBI	Total body irradiation
TBP	TATA-binding protein
TBST	Tween-Tris-buffered saline
TFIID	Transcription factor II D
Thr	Threonine
Trp	Tryptophan
UNG	Uracil N-glycosylase
UTR	Untranslated region
UV	Ultra-violet

Waf1	Wild-type activating fragment 1
WBC	White blood cell
WHO	World Health Organization

1. Introduction

1.1 p53

1.1.1 History of p53

The p53 tumor suppressor protein was initially discovered in 1979 by a variety of groups (Lane and Crawford, 1979; Kress *et al.,* 1979; Linzer and Levine, 1979; Melero *et al.,*1979; Smith *et al.,* 1979) as a protein that complexed with the large tumor antigen of SV40 (T antigen). At the time cell lines were being transformed with the SV40 virus, and it appeared that the T antigen carried by the SV40 virus was important in maintenance of the transformed state of the cells. This therefore necessitated research into the interactions of the T antigen in transformed cells, which these groups carried out by a variety of immunoprecipitation experiments. These experiments yielded 2 bands by SDS-PAGE: a 94kD protein, being the SV40 T antigen, and another 53kD protein which was, as of yet, undefined (Lane and Crawford, 1979).

The cDNA of the murine p53 gene was cloned in 1983 (Oren and Levine, 1983; Zakut-Houri *et al.*, 1983), and at the time p53 was actually considered an oncogene. This came about for a variety of reasons, among them studies such as one conducted in 1983 by Varda Rotter showing that p53 protein accumulated in high quantities in tumors harvested from mice no matter how the tumor was generated, while normal thymocytes displayed only low p53 protein levels (Rotter, 1983). Rotter also showed that the phosphorylation status of the p53 protein in tumors differed from that in normal cells. At the time, these results put p53 squarely in the oncogene/cellular tumor antigen category (Rotter, 1983). Adding to this, *Nature* published a series of articles in December of 1984

which all demonstrated how cells that were transfected with both an activated *ras* gene along with p53 lead to transformation of primary cell lines (Eliyahu *et al.,* 1984; Jenkins *et al.,* 1984; Parada *et al.,* 1984). Further complicating the matter, attempts to clone the transcript were substantially less advanced than they are today, and so cloning was done in cellular environments which were conducive to large quantities of p53 (Levine and Oren, 2009). This meant conducting cloning experiments to generate cDNA for the p53 transcript in cancer cell lines, leading researchers to use mutated cDNA for further experimentation, continuing the misconception that p53 functioned as an oncogene.

It was not until 1985 that the existence of p53 as an oncogene was called into question. While investigating the expression of p53 in Friend virus-induced erythroleukaemic cells, Mowat *et al.* found that a surprisingly large proportion of cell lines expressed no p53, or a truncated variant. They also found that cells which did not express p53 appeared to have experienced gene rearrangements which inactivated the p53 gene, which was curious as p53 was thought at the time to contribute to tumorigenicity (Mowat *et al.,* 1985). Later in 1988 Finlay *et al.* showed that one particular p53 cDNA clone could not transform cells, but became 'activated' upon obtaining mutations (Finlay *et al.,* 1988). Later in 1988, it was shown that wild-type p53 had no transformative capabilities, and only cDNA clones carrying point mutations were able to transform primary cell lines (Eliyahu *et al.,* 1988). In 1989 results surfaced that firmly categorized p53 as a tumor suppressor. One discovery which contributed to this change was that human colorectal cancers, which frequently harbor deletions in the 17p chromosomal arm, also had high expression of the remaining p53 allele. This p53 allele, however, was mutated, and so no wild-type p53 remained in the tumor tissue (Baker *et al.,* 1989). The

loss of wild-type alleles in tumor cells is typically telling of tumor suppressors, but in addition to this finding, there was also evidence that wild-type p53 overexpression could inhibit tumor formation in normal tissues when co-expressed in cells under tumorigenic pressure such as overexpression of *ras* (Finlay *et al.,* 1989; Eliyahu *et al.,* 1989). Later, researchers showed that a lack of functional p53 can be seen in the cancer-prone disorder of Li-Fraumeni syndrome (Srivastava *et al.,* 1990), and also that p53-null mice are prone to spontaneous development of tumors (Donehower *et al.,* 1992). All of the research mentioned here, along with more that will be discussed in greater detail later, firmly positioned the p53 protein as a key piece of the puzzle regarding cancer biology requiring further investigation.

1.1.2 p53 structure

The *TP53* human gene is roughly 19kb, contains 12 exons, and is located on the short arm of chromosome 17 at 17p13.1 (NCBI gene ID 7157, http://www.ncbi.nlm.nih.gov/gene/7157). The full-length human p53 protein contains 393 amino acids split into six major domains: two transactivation domains (TAD), one proline-rich domain (PRD), one sequence-specific DNA-binding domain (DBD), one oligomerization domain (OD), and the C-terminal domain (CTD)(Figure 1)(Garcia and Attardi, 2014). There are also five highly conserved regions referred to as *BOX-I*, *BOX-II, BOX-III, BOX-III, BOX-IV, and BOX-V* (Soussi *et al.,*1987; Soussi *et al.,* 1990).

The first TAD was discovered in 1990 by three groups of researchers (Fields and Jang, 1990; O'Rourke *et al.,* 1990; Raycroft *et al.,* 1990). They did this by creating fusion proteins linking fragments of p53 cDNA to the *GAL4* DNA-binding domain, and

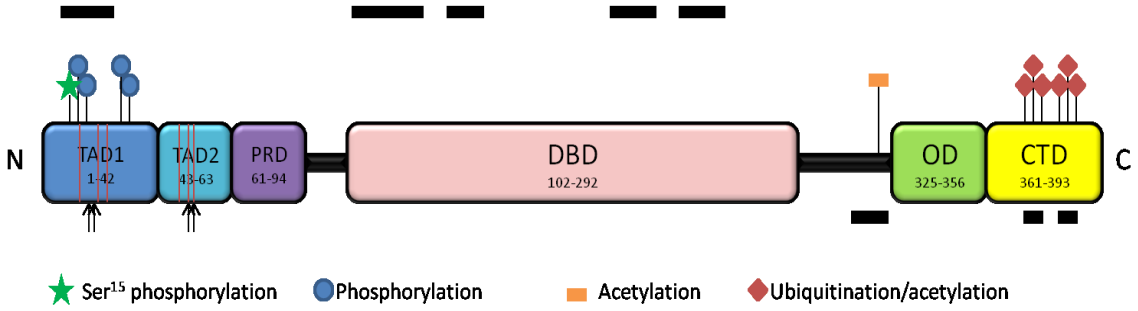


Figure 1: The human p53 protein.

General depiction of human p53 protein, along with pertinent post-translational modifications. Green star represents phosphorylation at Ser^{15}. Blue circles represent phosphorylation at Thr^{18} as well as serines 20, 33, and 37. Orange rectangle represents acetylation at Lys^{320}. Red diamonds represent acetylation/ubiquitination at lysines 370, 372, 373, 381, 382, and 386. Black rectangles below the figure represent NLS1 (316-322), NLS2 (370-377), and NLS3 (380-386). Black rectangles above the figure represent evolutionarily conserved *BOX* regions *I-V*. Black arrows below the figure represent residues 22/23 of TAD1 and 53/54 of TAD2. Red lines in TAD1 represent Phe^{19}, Trp^{23}, and Leu^{26}, while red lines in TAD2 represent Ile^{50}, Trp^{53}, and Phe^{54}. TAD1: transactivation domain 1 (1-42). TAD2: transactivation domain 2 (43-63). PRD: proline-rich domain (61-94). DBD: DNA-binding domain (102-292). OD: oligomerization domain (325-356). CTD: C-terminal domain (361-393).

Fields and Jang observed that amino acids 1-73 of p53 could effectively activate transcription (Fields and Jang, 1990). Unger *et al.* further narrowed down the transactivation domain to amino acids 1-42 two years later (Figure 1)(Unger *et al.,* 1992) using the same approach. TAD2 was discovered later by Zhu *et al.* in 1998 to be comprised of amino acids 43-63 (Figure 1)(Zhu *et al.,* 1998) by employing p53 constructs missing portions of their N-terminal region. This was particularly surprising as, previously, it had been shown that hydrophobic amino acid residues at position 22 and 23 were required for effective transcriptional activation by p53 (Figure 1)(Lin *et al.,* 1994). In 1996 Chen *et al.* showed that a cell line lacking the first 22 amino acids of p53 could still induce apoptosis to similar levels as wild-type (Chen *et al.,* 1996), and after investigating further Zhu *et al.* found that induction of the apoptotic response was closely linked to the availability of wild-type amino acids 43-63 (Zhu *et al.,* 1998). Interestingly, similar to TAD1, TAD2 contained two hydrophobic residues at position 53 and 54 (Figure 1), both appearing to be integral to maintaining some transcriptional activation in the absence of TAD1 function (Zhu *et al.,* 1998). It should also be mentioned that the transactivation domains are one of the domains subject to post-translational modifications which allow for regulation of their activity, but that will be discussed in detail later in this introduction.

Brady *et al.* continued the line of inquiry of the importance of key hydrophobic residues to the TADs by creating mouse MEFs mutant for p53 at position 22/23, 53/54, or the quadruple mutant (Brady *et al.,* 2011). They found that the quadruple mutants had

essentially no transactivation potential, seeming nearly identical to the p53 null case. Those cells bearing the 22/23 mutation had substantially reduced transactivation capability, and cells with the 53/54 mutation appeared similar to wild-type MEFs (Brady *et al.,* 2011). It should be mentioned, however, that cases between murine and human p53 may be different when discussing p53 transactivation domains. Although Brady *et al.* identified deficits in the expression of distinct mRNAs in cells lacking the murine equivalent of either 22/23 or 53/54, Smith *et al.* found that the same mutations in the human protein led to decreased expression of similar proteins in colorectal carcinoma cells, suggesting that there were no distinct targets that were differentially activated by the individual TADs (Smith *et al.,* 2007). While the work of both groups support a role for both transactivation domains, it remains unclear how these interact in the control of specific genes.

The PRD has had a somewhat more varied history. Originally investigated by Walker and Levine in 1996 (Walker and Levine, 1996), they claimed the PRD included amino acids 61-94 of human p53 which contains 5 repeats of PXXP (Figure 1), where P designates a proline residue and X represents any other amino acid. Notably, proline-rich regions such as the PRD of p53 are commonly involved in protein-protein interactions, and this particular motif was suggested to be similar to SH3-binding motifs of other proteins (Sakuramo *et al.,* 1997). Walker and Levine found that the PRD was dispensable for transactivation, but was necessary for effective suppression of growth (Walker and Levine, 1996). Later it was contested whether or not the PRD was actually important for growth suppression, but that the PRD was involved in apoptosis, and not in cell cycle arrest (Sakuramo *et al.,* 1997; Venot *et al.,* 1998; Baptiste *et al.,* 2002). Venot *et al.* also

provided evidence that the PRD was important for maintenance of transcription repression for some genes, as well as proper DNA binding and transcription activation of some pro-apoptotic genes (Venot *et al.,* 1998). Berger *et al.* later demonstrated how the PRD was integral to p53 regulation via its key inhibitor, MDM2 (Berger *et al.,* 2001). They showed that p53 lacking the PRD had increased susceptibility to inhibition by MDM2, leading to greater MDM2-mediated p53 degradation, and that this was caused by an increase in the affinity of the p53 lacking the PRD for MDM2 (Berger *et al.,*2001). Interestingly, Toledo *et al.* generated mice lacking a section of the p53 PRD and found that they were deficient in their ability to undergo cell cycle arrest, which was opposing what had been found previously (Toledo *et al.,*2006). Much more recently, work has been done by Campbell *et al.* in 2013 suggesting that the PRD is involved in mediating growth suppression and DNA repair in the event of DNA damage, but not other cellular stressors such as hypoxia (Campbell *et al.,* 2013). It should, however, be noted that the deletion of the PRD in question was from residues 58-88, which does contain the end of TAD2, and that this may potentially play a part in the phenotype seen.

The p53 DNA-binding domain includes amino acids 102-292 of the p53 protein (Figure 1)(Pavletich *et al.,* 1993). It also contains the vast majority of mutations found in tumours, as changes to the ability of p53 to bind DNA sequences all but abolishes its function (Bargonetti *et al.,* 1991; Kern *et al.,* 1991; Hollstein *et al.,* 1991; El-Deiry *et al.,* 1992; May and May, 1999). In fact, of the 280 p53 mutations that were analyzed by Hollstein *et al.* in 1991, 98% of them were found within the region between residues 110-307, and nearly one third of them came from CpG hotspots at residues 175, 196, 213, 248, 273, and 282 (Hollstein *et al.,* 1991). This has held true for nearly 30000 somatic

mutations (IARC, http://p53.iarc.fr/TP53SomaticMutations.aspx). The sequence-specific DNA-binding capabilities of p53 were first demonstrated by two groups in 1991 (Bargonetti *et al.,* 1991; Kern *et al.,* 1991). El-Deiry *et al.* reported the consensus sequence bound by p53: a 10bp motif of 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', where Pu represents a purine nucleotide and Py represents a pyrimidine nucleotide. These would appear twice in each bound segment, separated by 0-13bp (El-Deiry *et al.,* 1992). Depending on the source and method, the number of targets of p53 (deemed p53 response elements, or REs) differs drastically. For instance, in 2006 Wei *et al.* used a ChIP paired-end tag method to identify 542 loci in the human genome which had high confidence of direct p53 binding (Wei *et al.,* 2006). However, many interactions of p53 with DNA can occur transiently with low fidelity when mismatches are taken into consideration, and this allows for potentially further regulation of p53 (Tebaldi *et al.,* 2015). Upon analyzing the human genome for p53 REs of varying levels of fidelity to the consensus sequence, using previous studies, the method employed by Tebaldi *et al.* predicted a drastically larger number of p53 REs than Wei *et al.* did. For example, their method used ENCODE data to predict p53 REs in roughly 44000 distant enhancer elements (Tebaldi *et al.,* 2015), although these were variable in their predicted functionality.

The oligomerization potential of p53 was initially shown by Kraiss *et al.* in 1988 (Kraiss *et al.,* 1988), and was originally deemed to incorporate amino acids 311-363 of p53 (Pavletich *et al.,* 1993). Following this, the OD of p53 was isolated to only amino acids 325-356 of the p53 protein (Figure 1)(Clore *et al.,* 1994; Jeffrey *et al.,* 1995). p53 carries out its functions in a homotetramer, and when the ability of p53 to complex with

itself is removed through removal of the OD, it becomes deficient in transactivation potential (Pietenpol *et al.,* 1994).

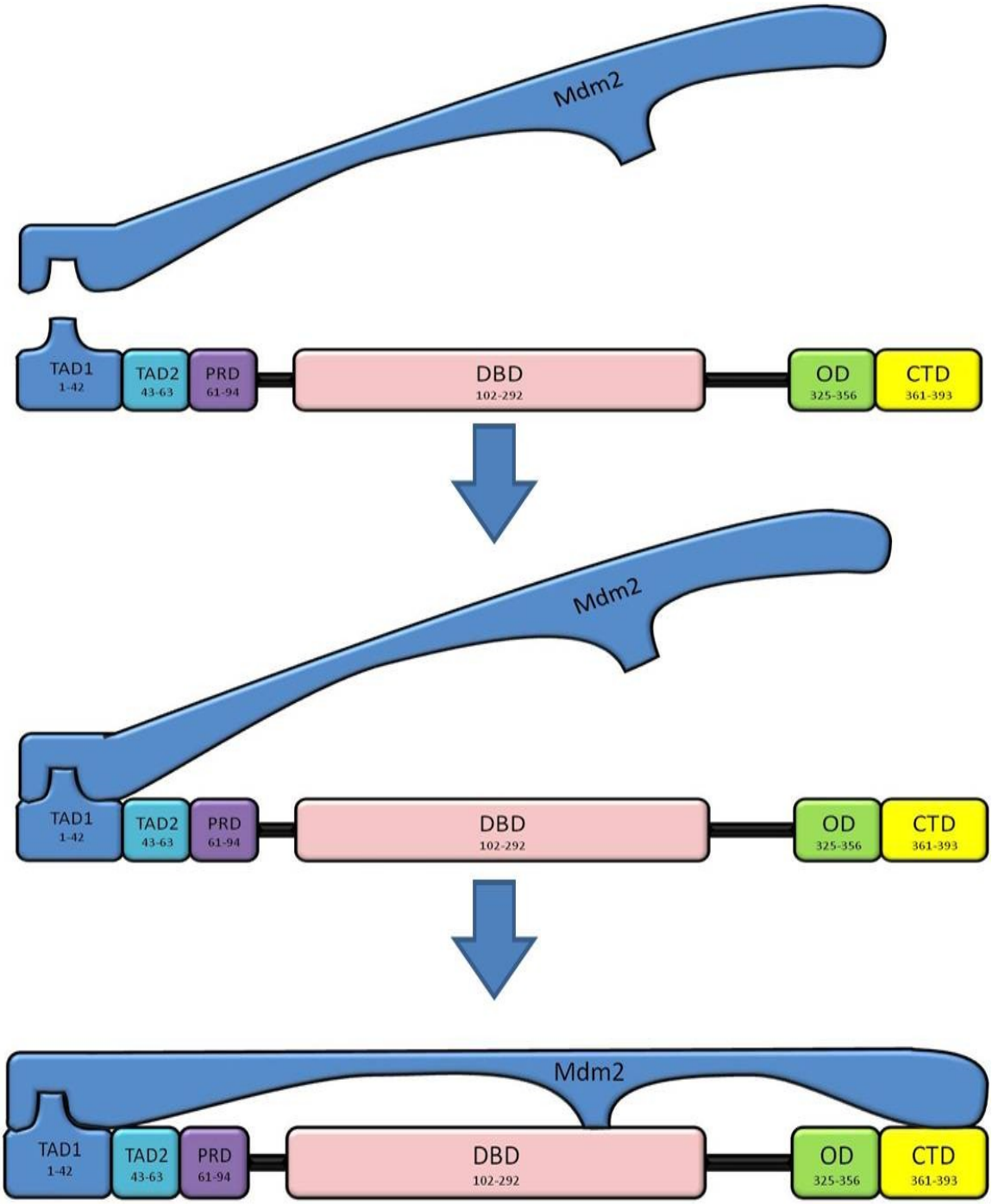
The C-terminal end of the p53 protein is the subject of some debate. Generally, however, it is accepted to undergo a variety of post-translational modifications which modulate p53 function and its regulation (Joerger and Fersht, 2008). Consisting of residues 361-393 (Figure 1)(Weinberg *et al.,* 2004), the CTD of p53 was shown to nonspecifically bind DNA (Foord *et al.,* 1991), and that removal or occupation of the CTD by binding other substrates or phosphorylation can lead to activation of sequence-specific DNA binding by p53 (Hupp *et al.,* 1992). In fact, use of an antibody titled Pab-421 came into use as an activator of sequence-specific DNA binding as it bound the C-terminus of p53 (Hupp *et al.,* 1992). This lead to the creation of a model of allosteric regulation of DNA binding by p53 proposed by Hupp and Lane in 1994, where they provided evidence suggesting that 'latent' DNA binding by p53 can be activated by changes in conformation by disrupting interactions between the core domains of p53 and the CTD (Hupp and Lane, 1994). However, multiple studies provided a variety of arguments against this hypothesis, such as that of Ayed *et al.* who demonstrated that there is no conformational difference between 'latent' and 'active' forms of p53 (Ayed *et al.,* 2001). Instead, Weinberg *et al.* found that the DBD of p53 could bind sequence-specific DNA inherently, but that the CTD competed with the DBD, and that in the presence of both specific sequences of DNA and non-specific DNA, the CTD binding to non-specific sequences out competed sequence-specific DBD binding (Weinberg *et al.,* 2004). As the CTD becomes post-translationally modified, it loses the ability to bind DNA non-specifically, and therefore allows the DBD to bind DNA in a sequence-specific manner,

thereby regulating the DNA binding potential of p53 (Frielder *et al.,* 2005). Interestingly, a model was proposed in 2004 by McKinney *et al.* in response to mounting evidence of a positive effect of the CTD on p53 regulation and their own results. In this model, the ability of the CTD to non-specifically bind DNA is required by p53 for it to effectively scan or 'linearly diffuse' along long stretches of DNA (McKinney *et al.,* 2004).

The CTD has been shown to be implicated in a variety of other regulatory roles than just DNA binding regulation. For example, one major role of the CTD of p53 is as the site of ubiquitin ligation by the E3 ubiquitin ligase MDM2 (Rodriguez *et al.,* 2000). MDM2 serves as the primary regulator of p53 levels and activity, and will be discussed at length later in this introduction (Figure 2). The CTD also serves as the target of MDM2-mediated neddylation: the modification of lysine residues with the ubiquitin-like NEDD8 protein, which serves to inhibit p53 transcriptional activity (Xirodimas *et al.,* 2004; Guihard *et al.,* 2012). Sumoylation of the CTD of p53 also provides a variety of regulatory opportunities to activate or inhibit p53 transcriptional activation in response to the addition of SUMO1, or SUMO2/3 proteins (Chen and Chen, 2003; Stindt *et al.,* 2011). Lysine methylation in the CTD also appears to be important in p53 function, as demonstrated by Chuikov *et al.* (Chuikov *et al.,* 2004). The results of their studies suggest that K372 methylation is important for p53 stabilization, nuclear localization and transcriptional activation, as well as that it may preclude acetylation in response to DNA damage. The CTD has also been shown to be involved in recruiting coactivators and histone acetyltransferases following acetylation (Barlev *et al.,* 2001; Mujtaba *et al.,* 2004). However, as stated earlier, there is some debate as to the importance of the effects of the CTD. For example, in 2005 Krummel *et al.* generated mice which had germline

Figure 2: Simplified interaction between human p53 protein and MDM2.

MDM2 inhibits p53 sequentially in two ways. First, the MDM2 N-terminus binds to the *BOX-I* region of the p53 TAD1 and inhibits the transactivation potential of p53 by disrupting the p53 TAD. Once the N-terminus of MDM2 binds the *BOX*-I domain of p53, a conformational change takes place in MDM2 which favors the binding of the MDM2 acidic domain to the *BOX-IV/BOX-V* region of the p53 DBD. This leads to polyubiquitination of the p53 CTD, and the eventual degradation of the p53 protein. TAD1: transactivation domain 1. TAD2: transactivation domain 2. PRD: proline-rich domain. DBD: DNA-binding domain. OD: oligomerization domain. CTD: C-terminal domain. (Modified from Meek, 2015)


Mdm2
TAD1
1-42
TAD2
43-63
PRD
61-94
DBD
102-292
OD
325-356
CTD
361-393
Mdm2
TAD1
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TAD2
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mutations of p53 such that all seven CTD lysines were mutated to arginines, and discovered the mice to be phenotypically normal, which was astonishing given beliefs about the CTD (Krummel *et al.,* 2005). The conflicting reports surrounding the effects and importance of the CTD highlight our lack of concrete understanding, as well as differences in experimental methodologies between researchers (Joerger and Fersht, 2008).

Three nuclear localization signals have also been identified in the C-terminus of p53 (Shaulsky *et al.,* 1990). These include residues 316-322 (NLS1), 370-377 (NLS2), and 380-386 (NLS3)(Figure 1). NLS1 appears to direct the transport itself, whereas NLS2 and NLS3 appear to increase the efficiency of the import process (Shaulsky *et al.,*1990).

1.1.3 p53 function and regulation

Despite some conflicting results and gaps in understanding, such as those mentioned previously, there is general consensus on p53 function and regulation. There is also drastically more information available than can be covered here: a simple search of PubMed using the search term p53 will return over 80000 articles which discuss p53 function and/or regulation in some manner, including over 9000 review articles.

What is important to know, and what is agreed upon by all, is that the primary function of p53 is in the role of a transcription factor. Despite some functions related to p53 directly contributing to apoptotic functions of the cell by interacting with other cytoplasmic proteins and contributing to membrane permeabilization (reviewed in Vaseva and Moll, 2009; Green and Kroemer, 2009), the p53 protein largely carries out its

immense cellular influence through its transactivation capabilities. However, the p53 protein is kept at relatively low levels in resting cells through interactions with other proteins, primarily with the E3 ubiquitin ligase MDM2, leading to ubiquitination, proteasome-mediated degradation, as well as inhibiting transactivation by forming an MDM2-p53 complex (Figure 2)(Meek, 2015). The importance of the *in vivo* MDM2-p53 interaction was made evident by the rescue of embryonic lethality in MDM2-null mice by deletion of p53 (Jones *et al.,* 1995; Montes de Oca Luna *et al.,* 1995).

The p53 response becomes activated in response to a wide variety of cellular stresses, which typically lead to disruption of the p53-MDM2 interaction, thereby allowing p53 protein levels to rise. The first cellular stress to be identified as activating the p53 response, and also the most extensively studied, is DNA damage (Maltzman and Czyzyk, 1984). Besides DNA damage, p53 also responds to other stressors such as activation of oncogenes, ribosomal stress, hypoxia, or heat shock (reviewed in Hu *et al.,* 2012; reviewed in Meek, 2015). Following activation, p53 has been shown to lead to cell cycle arrest (Baker *et al.,* 1990; Diller *et al.,* 1990; reviewed in Meek, 2015), apoptosis (Yonish-Rouach *et al.,* 1991; reviewed in Meek, 2015), or senescence (Figure 3)(Bond *et al.,* 1996; reviewed in Meek, 2015). However, although somewhat less intensely investigated, p53 has also been shown to be involved in a variety of other pathways such as regulation of proliferation and differentiation of stem cells (reviewed in Aloni-Grinstein *et al.,* 2014), ageing (Tyner *et al.,* 2002), and immune response via antagonism of NF-κB (Komarova *et al.,* 2005; Liu *et al.,* 2009).

Not only did Maltzman and Czyzyk identify the increase of p53 protein in response to DNA damage caused by either UV irradiation or chemical induction, they

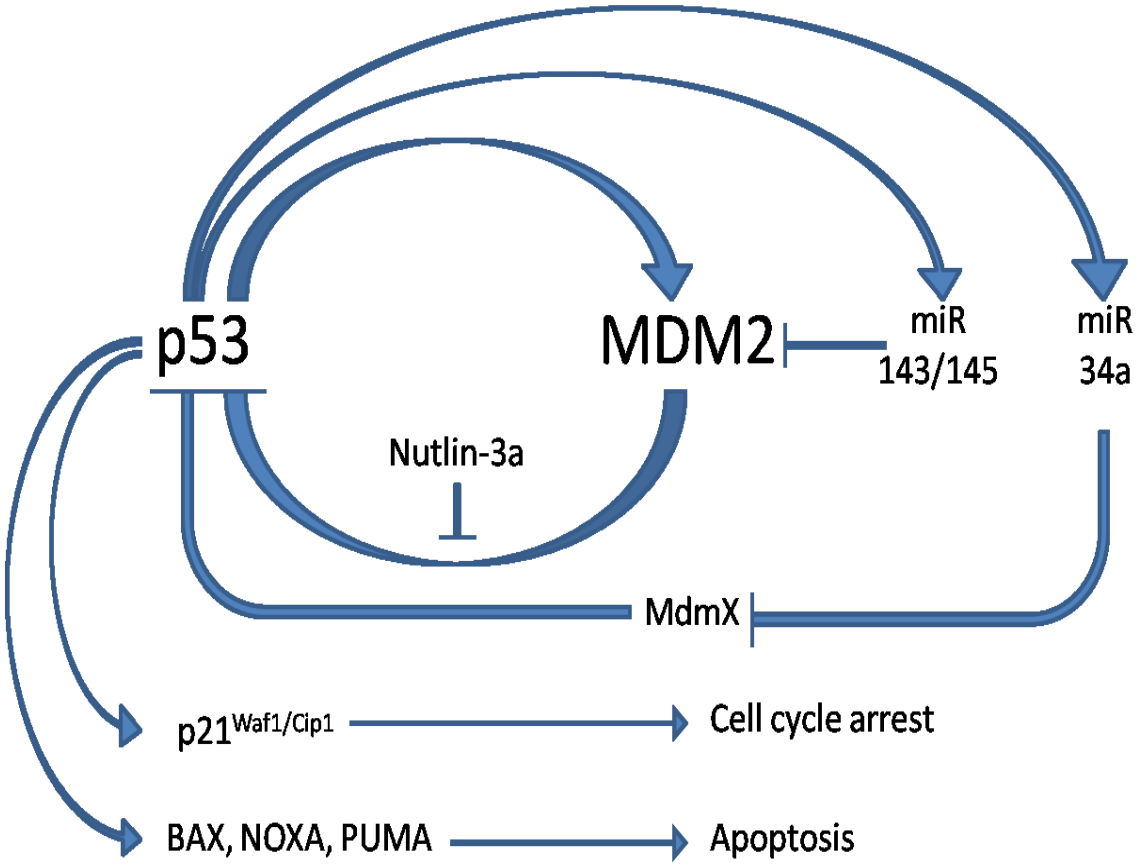


Figure 3: Model of the autoregulatory feedback mechanism of p53 and MDM2.
p53 activity is kept in check by transactivation inhibition by MDM2 and MdmX, along with nuclear export and polyubiquitination by MDM2 and associated MdmX. In the event of cellular stressors such as DNA damage, this interaction is disrupted, and p53 accumulates and becomes active. This leads to transactivation of numerous transcripts such as $p21^{Waf1/Cip1}$ and Bcl-2 family proteins such as BAX, NOXA, and PUMA. These can direct the cell towards cell cycle arrest, senescence, or apoptosis. p53 activation also leads to transactivation of MDM2 and, inhibiting p53-mediated transactivation. Nutlin-3a disrupts this feedback loops, and it is thus capable of indirectly activating p53. The p53 protein also transactivates miRNAs, which may feedback onto p53 regulators such as MDM2 and MdmX to modulate the p53-mediated transcriptional response.

also presented evidence that this was due to a dramatic increase in p53 protein stability (Maltzman and Czyzyk, 1984). This increase of protein stability is primarily a result of the disruption of the interaction between MDM2 and p53, although the destabilization of p53 is not the only result of p53-MDM2 interactions. Upon identifying that a 90kD protein which co-purified with p53 in immunoprecipitation experiments was actually homologous to *mdm-2* (murine double minute 2), Momand *et al.* demonstrated its ability to disrupt p53 transactivation (Momand *et al.,* 1992). Shortly after it was shown that MDM2-p53 complexes lose the ability to strongly bind DNA in a sequence-specific manner, and that this potentially contributes to disruption of p53 transactivation in the presence of MDM2 (Zauberman *et al.,* 1993). This was expanded upon by Oliner *et al.* by showing that the disruption of transactivation was due to MDM2 blocking the transactivation domains of p53 (Oliner *et al.,* 1993). That same year, Wu *et al.* demonstrated the fact that p53 transactivates MDM2 via a p53-responsive element in the 5' end of the MDM2 sequence, and that MDM2-p53 complexes lose this ability (Wu *et al.,* 1993). This lead to the formation of what is now a well-known auto-regulatory feedback loop, whereby p53 activation leads to transactivation of its own repressor, which in turn decreases the transactivation function of the available p53, as well as shutting off the upregulation of MDM2 (Figure 3). This feedback loop would help maintain p53 levels at low levels to permit cell proliferation and allow cells to survive (Wu *et al.,* 1993).

The interacting regions of the MDM2-p53 complex at the amino-terminus were outlined by Kussie *et al.*, showing that the amino-terminus of the MDM2 protein forms essentially a trough of hydrophobic residues which interacts with residues in TAD1 of

p53 (Figure 2)(Kussie *et al.,* 1996). Of particular importance are three p53 residues: Phe^{19}, Trp^{23} and Leu^{26}, which are an integral part of the p53-MDM2 complex, and are invariant across species (Figure 1)(Kussie *et al.,* 1996) and will be discussed in detail later when explaining *BOX-I* mimetics. These residues also play an important role in the specific mechanism by which p53 causes transactivation, and are a part of *BOX-I* (residues 13-26)(Liu *et al.,* 2001). The TAD1 region of p53 has been shown to recruit subgroups of the TFIID transcription machinery, specifically TBP-associated factors (TAFs) such as $TAF_{II}40$ and $TAF_{II}60$ and their human homologs, and that mutation of residues 22 and 23 in human p53 leads to disruption of the association between the p53 TAD1 and interacting TAFs (Lu and Levine, 1995; Thut *et al.,* 1995).

The repression of p53 by MDM2 does not end only in inhibition of interaction with TAFs and, by extension, of transactivation. In 1997 two separate groups demonstrated that MDM2 also targets p53 for degradation by functioning as an E3 ubiquitin ligase (Honda *et al.,* 1997; Haupt *et al.,* 1997), and it was later discovered that the lysine residues that are targets for ubiquitination by MDM2 reside in the C-terminus of p53 (Kubbutat *et al.,* 1998): namely the six lysine residues Lys^{370}, Lys^{372}, Lys^{373}, Lys^{381}, Lys^{382}, and Lys^{386} (Figure 1)(Rodriguez *et al.,*2000). It should be mentioned, however, that there is no indication that the C-terminus of p53 is involved in the binding of MDM2 to p53. There is also evidence that low levels of MDM2 lead to the monoubiquitination and nuclear export of the p53 protein (Li *et al.,* 2003).

In order for MDM2 to target p53 for degradation, more needs to happen than simply the binding of the N-terminus of MDM2 to the N-terminus of p53. Burch *et al.* presented evidence of a second MDM2 binding site in p53 (Burch *et al.,* 2000), and

Shimizu *et al.* identified this domain in a flexible, unfolded linker region of the p53 DBD between two conserved domains referred to as *BOX-IV* and *BOX-V* (Figure 2)(Shimizu *et al.,* 2002). This region was actually shown to have a much lower affinity for MDM2 binding, and only bound to MDM2 upon a conformational change in MDM2 caused by the binding of its N-terminus to a substrate. A trio of papers emerged in 2006 which further discussed the secondary MDM2 binding domain in p53 (Yu *et al.,* 2006; Kulikov *et al.,* 2006; Wallace *et al.,* 2006). All three papers concluded that the MDM2 binding domain of p53 interacted with the central acidic region of MDM2, and Kulikov *et al.* discovered three key findings. Firstly, MDM2 binding to the DBD of p53 is implicated in the necessary deacetylation of the p53 CTD prior to polyubiquitination, which will be discussed in more detail later. Secondly, MDM2 binding the DBD of p53 is necessary for effective polyubiquitination (and subsequent degradation) of p53 protein. Thirdly, although it was known that MDM2 was commonly phosphorylated, Kulikov *et al.* demonstrated how MDM2 phosphorylation at Ser^{253} and Ser^{256} were key for effective binding of MDM2 to the DBD of p53 (Kulikov *et al.,* 2006). Collectively, MDM2 and p53 interact in a variety of ways and there are several levels of control, but the initiating event is the binding of MDM2 to the *BOX-I* region within TAD1 of p53.

Wallace *et al.* conducted experiments using peptides which were found to block the binding of MDM2 to p53, leading to increased p53 levels and activity (Wallace *et al.,* 2006). Intriguingly, they found that using peptides which would mimic the TAD1 region bound by MDM2 (*BOX-I)* did not inhibit ubiquitination, despite disrupting the *BOX-I* binding of MDM2 (Wallace *et al.,* 2006) (This particular category of peptide will be discussed later, and was used as an activator of the p53 response in our studies). Instead,

Wallace *et al.* found that only peptides which mimic the *BOX-IV/BOX-V* region of p53 (thereby disrupting MDM2 binding there) disrupted p53 ubiquitination by MDM2, and that peptides that mimic *BOX-I* actually lead to an increase in ubiquitination, despite causing p53 to become transcriptionally active. Following this, a model emerged of allosteric regulation, whereby interaction between the N-terminus of MDM2 and the *BOX-I* region of p53 did not directly contribute to ubiquitination. Instead, it causes a conformational change in MDM2 which favors interactions between the MDM2 acidic domain and the p53 *BOX-IV/BOX-V* region, leading to polyubiquitination and subsequent degradation (Figure 2)(Wallace *et al.,* 2006). There is some evidence that binding of the N-terminus of MDM2 to p53 may potentially occur outside of the *BOX-I* region, as Chi *et al.* demonstrated that residues in TAD2 also have binding affinity for MDM2 (Chi *et al.,* 2005). These residues were Ile^{50}, Trp^{53}, and Phe^{54}, which is particularly interesting as the 53/54 residues were discussed previously as being integral to TAD2 function (Figure 1).

In order for the inhibition of p53 by MDM2 to be circumvented, a number of post-translational modifications need to occur. For the sake of simplicity, only those post-translational modifications which are pertinent to this project will be outlined here, full reviews on post-translational modifications can be found elsewhere (reviewed in Jenkins *et al.,* 2012; Reed and Quelle, 2014).

When p53 was first recognized as a protein of interest to the scientific community, it had been identified as a phosphoprotein, and consequently investigations began into the specific residues found to be phosphorylated. For example, Samad *et al.* identified four possible phosphorylation in murine p53 (Samad *et al.,* 1986), which Meek and Eckhart expanded to include serines 7, 9, 12, 18, 23, 37, 310, 312, and 389 (Meek

and Eckhart, 1988). It should be noted here that murine p53 incorporates an additional 3 residues in the N-terminal, and so homologous residues between murine and human p53 actually have different residue positions. For example, serine 18 and 23 in murine p53 correspond to serine 15 and 20 in human p53. Serines 6, 15 , 37, and 166 were shown to be phosphorylated by DNA-activated protein kinase (DNA-PK) in human p53, although only Ser^{15} and Ser^{37} were phosphorylated efficiently (Lees-Miller *et al.,* 1992). Other phosphorylation events pertinent to this discussion include Ser^{33} phosphorylated by multiple c-Jun N-terminal kinases (JNKs)(Hu *et al.,* 1995), Ser^{20} (Shieh *et al.,*1999; Craig *et al.,* 1999), and Thr^{18} (Craig *et al.,* 1999, Dumaz *et al.,* 1999), of which both Ser^{20} and Thr^{18} are conserved residues in the *BOX-I* region and are phosphorylated by casein kinase I (CK1)(Figure 1). Dumaz *et al.* also found that Thr^{18} and Ser^{20} are preferentially phosphorylated following initial phosphorylation of Ser^{15} (Dumaz *et al.,* 1999).

How phosphorylation of p53 relates to its function has been demonstrated by a number of research groups (Figure 1). Fiscella *et al.* initially demonstrated the importance of the Ser^{15} phosphorylation event by showing that p53 variants with an alanine substitution at Ser^{15} caused a decrease in cell cycle arrest capability, as well as a decrease in the ability of p53 protein to accumulate (Fiscella *et al.,* 1993). This was elaborated on by Shieh *et al.* who identified a number of key findings regarding p53 phosphorylation. First and foremost, they discovered that Ser^{15} phosphorylation is not a steady-state condition of resting cells, but that it can occur following DNA damage by UV light, ionizing radiation (IR) or chemical induction (Shieh *et al.,* 1997). Secondly, they demonstrated that Ser^{15} or Ser^{37} phosphorylation disrupt p53-MDM2 complex formation, and that this leads to an increase in the transactivation activity of p53 by

reducing the inhibitory effect the MDM2-p53 complex has on p53-dependent transactivation. At the same time, Mayo *et al.* showed that MDM2 is also phosphorylated by DNA-PK, and that phosphorylation at Ser^{17} in particular (which is near the p53-binding site) was potentially even more critical to the disruption of the MDM2-p53 complex than serine 15 or 37 phosphorylation of p53 (Mayo *et al.,*1997). Craig *et al.* also found that Ser^{15}, as well as Thr^{18} and Ser^{20} phosphorylation reduces the binding of MDM2 to p53. Interestingly, Pise-Masison *et al.* found that phosphorylation at only Ser^{15}, in the absence of Ser^{37} phosphorylation, lead to a decreased interaction with TFIID (Pise-Masison *et al.,* 1998). Conversely, phosphorylation of both residues disrupted MDM2 binding and increased the interaction between p53 and TFIID. Taken together, the results presented regarding the multiple phosphorylation sites in the p53 N-terminal and MDM2 clearly demonstrate the importance of phosphorylation in the disruption of the p53-MDM2 complex and subsequent activation of p53.

Evidence emerged suggesting that phosphorylation of p53 not only disrupted MDM2 binding, but also directed subsequent post-translational modifications: acetylation of C-terminal lysines. Gu and Roeder demonstrated that the histone acetyltransferases (HATs) CBP/p300 and p300/CBP-associated factor (PCAF) directly lead to the acetylation of p53 in the CTD (Gu and Roeder, 1997). Lys^{373} and Lys^{382} were identified as being preferentially acetylated, while lysines 370, 372, and 381 underwent lower amounts of acetylation, and the positions of these residues appear to be evolutionarily conserved (Figure 1). Acetylation of the CTD was also shown to dramatically increase sequence-specific DNA binding of p53. These results were supported by Sakaguchi *et al.,*

who then identified another acetylated residue at Lys^{320} (Figure 1), which was preferentially acetylated by PCAF, and demonstrated the activating effect that p53 acetylation events by p300 and PCAF in the CTD had on sequence-specific DNA binding (Sakaguchi *et al.,* 1998). Commensurate with acetylation taking place at many of the same lysines required for ubiquitination and degradation, Ito *et al.* found that acetylated lysines in the C-terminal of p53 must be deacetylated by a complex of MDM2 and HDAC1 prior to degradation (Ito *et al.,* 2002). This lead to a model of competition between acetylation by p300/CBP and ubiquitination by MDM2 and HDAC1.

CBP/p300 have also been shown to interact directly with the N-terminal of p53 (Avantaggiati *et al.,* 1997; Gu *et al.,* 1997; Lill *et al.,* 1997; Scolnick *et al.,* 1997). This interaction was abolished in the presence of mutated residues 22 and 23 of TAD1 (Gu *et al.,* 1997) or residues 53 and 54 (Scolnick *et al.,* 1997), but it was identified that the interaction with wild-type p53 led to an increase in transactivation activity, and that the interaction with p300/CBP takes place at its C-terminal transactivation domain (Avantaggiati *et al.,* 1997; Gu *et al.,* 1997; Scolnick *et al.,* 1997). Inhibiting the interaction of p300/CBP with p53 through dominant negative isoforms or competitive inhibition was also shown to suppress p53-dependent transactivation (Avantaggiati *et al.,* 1997; Lill *et al.,* 1997; Scolnick *et al.,* 1997). Lill *et al.* also presented evidence that p53-DNA complexes were dependent on p300/CBP binding, and that this interaction is imperative for p53-dependent cell cycle arrest and transcription-dependent, early apoptosis (Avantaggiati *et al.,* 1997; Lill *et al.,* 1997).

In order for any of this to happen, however, Ser^{15} first needs to become phosphorylated, and this can be caused by DNA-PK as mentioned before, or by the gene

product mutated in ataxia telangiectasia (ATM)(Siliciano *et al.,* 1997; Banin *et al.,* 1998; Canman *et al.,* 1998). DNA-PK can also phosphorylate Ser^{15} in response to DNA damage caused by UV light, IR or chemical induction, but Siliciano *et al.* demonstrated that patients with mutant ATM generated very low levels of Ser^{15} phosphorylation specifically in response to IR (Siliciano *et al.,* 1997). However, Canman *et al.* found that Ser^{15} phosphorylation only occurred in association with functional ATM (Canman *et al.,* 1998), potentially demonstrating some evidence of cell-specific or context-specific regulation. One potential reason for the differences in activation of DNA-PK and ATM is that ATM activity is regulated by the availability of manganese ions, and not the presence of sheared double-stranded DNA, as is the case with DNA-PK (Banin *et al.,* 1998). Another role for ATM in response to DNA damage is the phosphorylation of MDM2 on Ser^{395}, which has been shown to inhibit its ability to affect the degradation of p53 (Maya *et al.,* 2001).

The link between phosphorylation and acetylation lies in Ser^{15}. In response to p53 phosphorylation by DNA-PK, binding of p300/CBP increases, and this is largely dependent on Ser^{15} phosphorylation, although Ser^{37} phosphorylation plays a very minor role as well (Lambert *et al.,* 1998). Also, this increase in p300/CBP binding to p53 was correlated with increased acetylation, and as before the acetylation was dependent on Ser^{15} phosphorylation. These results were supported by Dumaz and Meek, who demonstrated that while Ser^{15} phosphorylation itself increases binding of p300/CBP, it does not directly affect MDM2 binding (Dumaz and Meek, 1999).

Once the p53-MDM2 interaction has been disrupted, p53 then has to carry out its functions, which previously had been broadly categorized as being involved in cell cycle

arrest, apoptosis, senescence, differentiation of stem cells, ageing, etc. In order for p53 to affect these changes, it needs to transactivate genes related to those functions (Figure 3). The most well-studied target of p53 transcriptional activation is the CDKN1A gene encoding the $p21^{Waf1/Cip1}$ protein, which is the major mediator of p53-dependent G_1 arrest (el-Deiry *et al.,* 1993; Deng *et al.,* 1995; Waldman *et al.,* 1995). The p53-mediated apoptotic response is associated predominantly with the induction of pro-apoptotic Bcl-2 family proteins such as Bax, Noxa, and PUMA (Figure 3)(McCurrah *et al.,* 1997; Yin *et al.,* 1997; Oda *et al.,* 2000; Nakano and Vousden, 2001; Chipuk *et al.,* 2005), but p53 can also interact directly with Bcl-2 family proteins in mitochondria to stimulate apoptosis (reviewed in Vaseva and Moll, 2009; Green and Kroemer, 2009). Of note, the response of these pro-apoptotic proteins to p53 activation may not be equal in their importance. For instance, disrupting Bax induction does not abolish p53-mediated apoptosis, but deletion of Noxa or PUMA substantially reduces apoptotic response to DNA damage (Oda *et al.,* 2000; Nakano and Vousden, 2001; Chipuk *et al.,* 2005).

An extra layer of regulation comes with the discussion of Mdm4, or MdmX. Originally identified in mice as a protein with similar structure to MDM2, both the mouse (Shvarts *et al.,* 1996) and human (Shvarts *et al.,* 1997) MdmX proteins were demonstrated to have p53-binding capabilities leading to inhibition of transactivation *in vivo* (Figure 3). However, levels of MdmX did not respond to UV irradiation, unlike MDM2 (in response to p53 transactivation). MdmX and MDM2 were shown to form hetero-oligomers through their respective C-terminals, which lead to increased stability of MDM2 (Tanimura *et al.,*1999). Critically, loss of MdmX results in early embryonic lethality, much like loss of MDM2, and was identified as being a result of unregulated

p53 activity (reviewed in Marine and Jochemsen, 2005). As mentioned previously, MdmX was found to inhibit p53 transactivation, and this appeared to be related to the binding of MdmX to the same TAD region as MDM2, inhibiting interaction with p300/CBP (Böttger *et al.,* 1999; Sabbatini and McCormick, 2002).

The interaction of MdmX with MDM2 also appears to be integral for effective polyubiquitination of p53, as although MdmX has no ubiquitin ligase function itself, the disruption of the complex results in increased p53 levels and reduced p53 polyubiquitination (Kawai *et al.,* 2007; Wang *et al.,* 2011). MdmX has also been shown to be phosphorylated by ATM on multiple serine residues following DNA damage, leading to an increase in MdmX ubiquitination by MDM2 and its subsequent degradation (Pereg *et al.,* 2005).

With the emergence of microRNAs (miRNAs) came investigation into the role of miRNAs in the regulation of the p53 response. Multiple articles in 2007 surfaced to report members of the miR-34 family (a, b, and c) as being the most prominently induced miRNAs in response to p53 activation (Figure 3)(reviewed in Hermeking, 2007). These miRNAs were found to affect cell cycle arrest, senescence, and apoptosis, and miR-34a was shown to be encoded by a separate transcript from miR-34b/c. Most of the groups also found that miR-34a-5p is most significantly induced following p53 induction, although ectopic induction of miR-34a or miR-34b/c can induce G_1 arrest, senescence, or apoptosis, and inhibition of production of miR-34a-5p resulted in reduced rates of DNA damage-mediated apoptosis. Members of the miR-34 family have been shown to target transcripts for proteins such as Bcl-2 and Mdm4, both of which have been discussed previously (reviewed in Rokavec *et al.,* 2014). Of particular interest, MDM2 is a target of

miR-143-3p/miR-145-5p, which are both generated from the same primary transcript, and activated by p53 (Figure 3)(Zhang *et al.,* 2013). Overexpression of miR-143-3p/miR-145-5p resulted in decreased MDM2 levels, and knockdown of miR-143-3p/miR-145-5p resulted in increased MDM2 levels, and consequently had the inverse result on p53 levels. Similarly, adding the miR-143-3p/miR-145-5p recognition sites to reporter constructs lead to inhibition of the reporter construct when in the presence of miR-143-3p/miR-145-5p, and this interaction was abolished upon mutation of the miR-143-3p/miR-145-5p recognition sites. Considering miR-143-3p/miR-145-5p result in inhibition of MDM2, it logically follows that they would have anti-proliferative and pro-apoptotic functions, and this is exactly what was found (Zhang *et al.,* 2013). The response of miR-143-3p/miR-145-5p to p53 activation is not at the transcriptional level, though; instead, the rise in miR-143-3p/miR-145-5p levels is a result of posttranscriptional regulation. The p53 protein can stimulate the processing of miRNAs in response to DNA damage by interacting with part of the miRNA processing machinery, the Drosha complex (Suzuki *et al.,* 2009). This interaction was shown to increase processing of primary miRNAs to precursor miRNAs, an integral step in the maturation process of miRNAs.

A simplified model for the overall response of p53 to DNA damage is that, at steady-state levels, p53 is bound tightly by MDM2, both at the N-terminal *BOX-I* region, as well as at the *BOX-IV/BOX-V* core MDM2 binding region. This causes the nuclear export of p53, inhibition of p53-dependent transactivation, and polyubiquitination of the p53 CTD. However, in response to DNA damage, ATM (or ATR) phosphorylates of MDM2 and MdmX, inhibiting degradation of p53 and causing the degradation of MdmX

by MDM2. ATM then causes the phosphorylation of Ser^{15}, which then begins a cascade of phosphorylation and acetylation events. Primarily, Ser^{15} phosphorylation increases the binding affinity for p300/CBP, which competes for binding with MDM2. Other residues in the N-terminal of p53 become phosphorylated as a result of Ser^{15} phosphorylation as well, p300/CBP outcompetes MDM2 for p53 binding, and acetylates lysine residues in the p53 CTD, which increases the stability of the p53 protein and increases sequence-specific DNA binding. Following this, p53 goes on to transactivate downstream effectors such as p21, Bax, Noxa, and PUMA, resulting in cell cycle arrest, senescence, or apoptosis. The activation of p53 also results in the transactivation of miRNAs, as well as the increase in miRNA processing through Drosha, following which the nascent miRNAs go on to inhibit other transcripts, including those transactivated by p53, providing another layer of feedback regulation.

1.1.4 Nutlin and MDM2

Considering p53 is such an important tumor suppressor with wide-ranging functions, there is little wonder why researchers have dedicated considerable effort to identifying drugs which will stimulate its activity. The major target for drug development is the disruption of the p53-MDM2 interaction. To this end, groups synthesized antisense oligonucleotides and peptides homologous to p53 to inhibit MDM2 either at the transcript or the protein level, respectively (Chen *et al.,* 1998; Wasylyk *et al.,* 1999; Chène *et al.,* 2000; Tortora *et al.,* 2000). These all effectively activated p53 functions such as transactivation of downstream effectors. Those peptides or molecules which disrupt the

MDM2-p53 interaction by occupying the same region on MDM2 as *BOX-I* of p53 are referred to as *BOX-I* mimetics.

Vassilev *et al.* screened chemicals for small-molecule inhibitors of the p53-MDM2 interaction (Vassilev *et al.,* 2004). Phe^{19}, Trp^{23} and Leu^{26} of the p53 N-terminal were all previously discussed as being integral to the p53-MDM2 complex, and project into the hydrophobic cleft of MDM2 (Kussie *et al.,* 1996). Vassilev *et al.* identified a group of compounds they termed Nutlins which disrupted the p53-MDM2 interaction at the hydrophobic cleft of MDM2 by mimicking the Phe^{19}, Trp^{23} and Leu^{26} residues (Figure 3)(Vassilev *et al.,* 2004). Treatment of cells with Nutlin-1 lead to increases in cellular p53, MDM2, and $p21^{Waf1/Cip1}$ in a p53- and MDM2-dependent manner, which was related to decreased degradation of p53, as well as G_1 and G_2 arrest. It is important to note that Nutlin treatment did not result in Ser^{15} or Thr^{18} phosphorylation (Vassilev *et al.,* 2004; Tovar *et al.,* 2006; Kumamoto *et al.,* 2008). Despite the fact that p53 is not phosphorylated on critical N-terminal serines, there is evidence that Nutlin-3a can lead to induction of p53 acetylation, presumably due to the lack of MDM2-directed ubiquitination of lysine residues on p53 that also serve as targets for acetylation (Haaland *et al.,* 2014). In keeping with this, Nutlin-3a treatment also leads to histone H2B and Hsp27 acetylation, both potential targets for MDM2-mediated deacetylation and ubiquitination. Also, p53 acetylation-defective mutant cells (lacking p53 C-terminal 6 lysines) are resistant to Nutlin-3a (Haaland *et al.,* 2014). It would appear that acetylation, but not phosphorylation, is necessary for effective activation of p53 in the context of Nutlin treatment.

An investigation into differentially expressed genes following Nutlin treatment revealed 2942 genes were affected: 1737 up-regulated, and 1205 down-regulated. Included in those up-regulated were *BTG2, GDF15, p53,* and *BAX* (Kumamoto *et al.,* 2008). Along with those genes found up-regulated following Nutlin-3a treatment, miR-34a and miR-34b/c were found to be induced, and miR-34a was found to be induced prior to miR-34b/c. Interestingly, although treatment with Nutlin is capable of inducing cell cycle arrest, its ability to induce apoptosis varies between cell lines, ranging from 80% after 48h with 10μM Nutlin-3 in SJSA-1 cells to less than 10% in HCT116 or A549 cells even after 72h with 10μM Nutlin-3a (Tovar *et al.,* 2006). Upon investigating potential causes for these differences, Tovar *et al.* discovered that 53 genes were differentially expressed between tested cells that respond to Nutlin with high levels of apoptosis (SJSA-1 and MHM) and those that respond with low levels of apoptosis (HCT116 and U2OS), 14 of which were noted as being related to apoptosis function, including *PUMA, NOXA,* and *BAX*. It was suggested that SJSA-1 and MHM cell lines overexpress MDM2 while maintaining wild type p53. In partial contrast, HCT116 cells have wild-type p53 with normal MDM2 expression, so they may have different downstream alterations in the p53 pathway that ultimately affect the outcome of Nutlin treatment (Tovar *et al.,* 2006). Curiously, although resistance to Nutlin-mediated apoptosis is seen in some cell lines, it was demonstrated that Nutlin-3a not only binds MDM2, but is also capable of binding anti-apoptotic Bcl-2 family proteins, thereby directly contributing to the induction of apoptosis (Ha *et al.,* 2011; Shin *et al.,* 2012).

Nutlin-3a was also shown to cause cellular senescence, with 50% senescence after treatment with 10μM for 3 days, and 100% senescence with 10μM within 7 days

(Kumamoto *et al.,* 2008). However, senescence in U2OS, which was previously shown to resist Nutlin-induced apoptosis, did not occur until 14 days after the beginning of treatment, which was attributed to the previously discussed possibility of defects downstream in p53 signalling.

Nutlin-3 is now being used in clinical trials, although in another form. Many human tumors present with amplification or overexpression of MDM2 (reviewed in Momand *et al.,* 1998), and therefore disrupting the MDM2-p53 complex in these cases with Nutlin-3a may prove to be therapeutic. Vu *et al.* generated a new small-molecule antagonist of MDM2 named RG7112 by using the Nutlin structure as a basis (Vu *et al.,* 2013). RG7112 was found to be as effective as Nutlin-3a with only a quarter of the same dose, and was found (like Nutlin) to inhibit tumor growth. Since its synthesis and characterization, RG7112 has been used for clinical trial testing, and in one Phase I study was administered to 116 patients (Andreeff *et al.,* 2016). All patients suffered some form of side-effect, while tumor suppression was seen in 30 patients with acute myeloid leukemia. Not surprisingly, p53 transactivation activity was only identified in those patients with wild type p53 in their leukemic cells.

1.1.5 Consecutive p53 responses separated by a period of recovery

Much of the discussion about p53 in this paper surrounding its extensive regulation has mentioned either DNA or protein, and very little has been said about mRNA. One reason for this is that, as discussed, p53 mRNA levels change very little, while protein levels range widely due to post-translational regulation. Shortly after p53 activation by reparable DNA damage, p53 itself is inhibited by increased MDM2 levels,

and p53 levels drop accordingly. Therefore, the synthesis of p53-regulated mRNAs may occur for a relatively short period of time followed by recovery, such that downstream effectors are only transiently expressed.

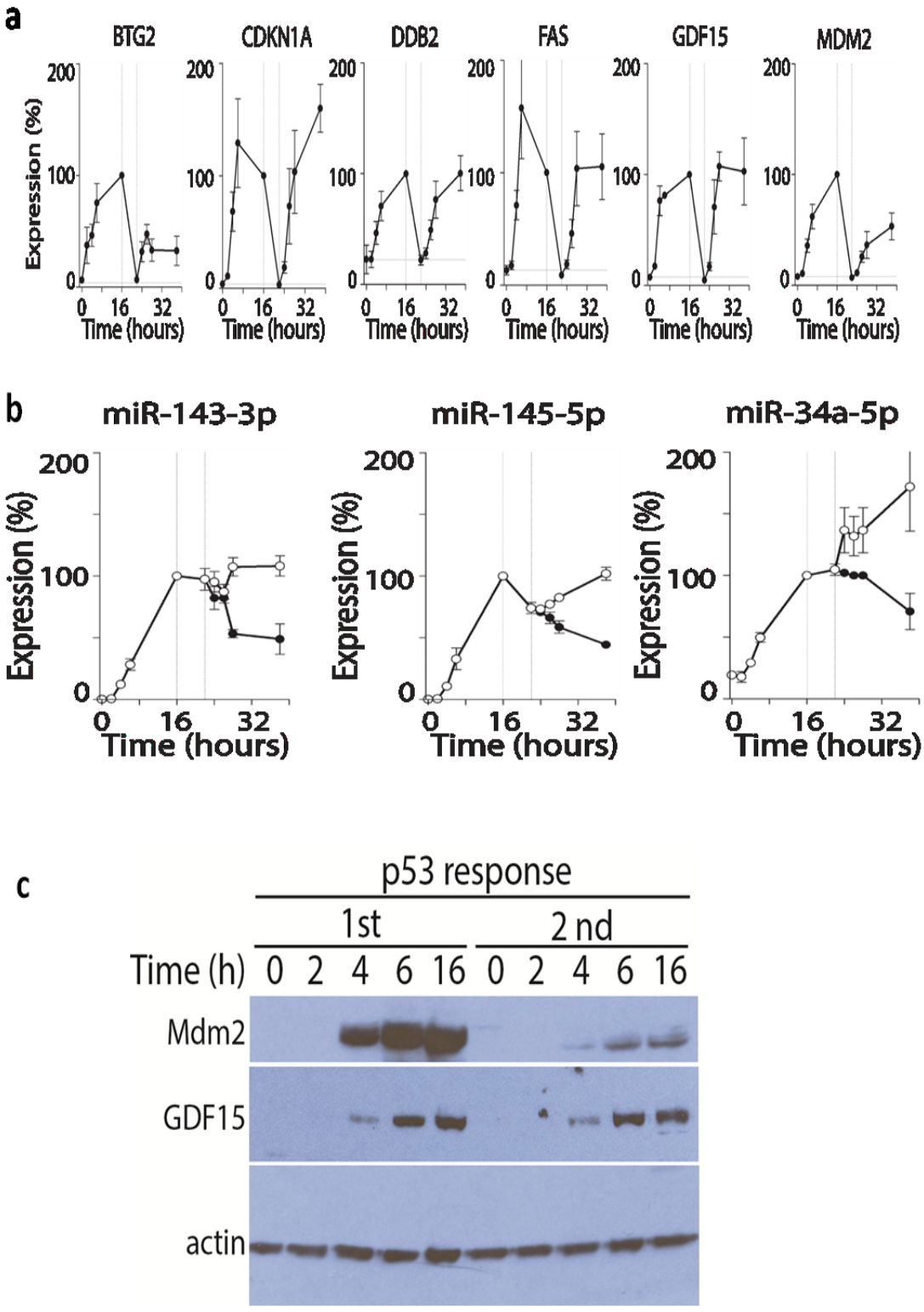
Melanson *et al.* investigated the induction and subsequent decay of the p53 response by employing HT29-tsp53 cells (Melanson *et al.,* 2011). These cells express a murine p53 variant which includes a valine-to-alanine substitution at residue 135, leading to a temperature-sensitivity for nuclear import (Michalovitz *et al.,* 1990; Gannon and Lane, 1991). In these cells, incubation at 32^{o}C (permissive temperature) leads to nuclear accumulation of p53 protein, target gene transactivation, and G_1 arrest, whereas incubation at 38^{o}C (restrictive temperature) prevents these effects. RNA was harvested from cells that were incubated for 16h at the permissive temperature with or without a 6h period of recovery (Melanson *et al,* 2011). These RNA samples were analyzed with oligonucleotide microarrays. Twenty nine known targets of p53 were induced and 21 of these transcripts had apparent half-lives of less than 2h (Melanson *et al.,* 2011). Heterologous reporter constructs containing the 3'UTRs from representative mRNAs recapitulated the rapid turnover of p53-regulated mRNAs. Analysis of the 3'UTRs of p53-regulated mRNAs identified the presence of motifs associated with mRNA decay pathways in short-lived mRNAs that were absent in the more stable transcripts (Melanson *et al.,* 2011). Therefore, most p53-induced mRNAs were short-lived.

It was unclear whether cells that had recently recovered from a p53 response were capable of eliciting a second response immediately. When HT29-tsp53 cells were incubated in the permissive temperature for 16h, moved to the restrictive temperature for 6h, and then placed back in the permissive temperature to elicit a second p53 response,

the response of most p53-regulated mRNAs was similar between both p53 responses (Figure 4a)(Cabrita *et al.,* unpublished data). However, the response of MDM2 and BTG2 was attenuated during the second p53 response when compared to the first (Figure 4a). Cabrita *et al.* assessed the appearance of putative miRNA consensus sequences in p53-regulated mRNAs, and found the short-lived p53-regulated transcripts contained sequences recognized by p53-induced miRNAs such as miR-34a-5p and miR-143-3p/miR-145-5p (Cabrita *et al.,* unpublished data). MDM2 and BTG2, in particular, contained multiple putative p53-regulated miRNA consensus sequences. When analyzed using the same experimental conditions as above, the p53-regulated miRNAs were far more stable than the p53-regulated mRNAs during the 6h recovery period, and therefore their levels remained high during the second p53 response (Figure 4b). In absence of the second p53 response, the p53-regulated miRNAs had half-lives >24h. It is possible that the highly stable p53-regulated miRNAs are the cause of the attenuation seen in MDM2 and BTG2 during the second p53 response. This attenuation can also be seen at the protein level. Using the same experimental procedure as above, MDM2 protein levels were seen to be attenuated during the second p53 response in comparison to the first (Figure 4c).

During the initial 16h induction, mRNA and miRNA levels rise in the HT29-tsp53 cells. When returned to the restrictive temperature, mRNA levels plummet, whereas miRNA levels remain high. When cells were once again incubated at the permissive temperature, mRNAs are once again transcribed, and miRNA levels (which were already raised) continued to rise or remained elevated (Cabrita *et al.,* unpublished data). Accordingly, it is presumed that the presence of large quantities of miRNAs

Figure 4: p53-regulated protein, mRNA, and miRNA expression in HT29-tsp53 cells. a) p53-regulated mRNA expression in HT29-tsp53 cells in response to two consecutive p53 responses as induced by incubation at 32^{o}C for 16h, separated by a 6h recovery period at 38^{o}C. MDM2 and BTG2 exhibit attenuated mRNA expression during the second p53 response compared to the first. b) p53-regulated miRNA expression in HT29-tsp53 cells in response to two consecutive p53 responses as induced by incubation at 32^{o}C for 16h, separated by a 6h recovery period at 38^{o}C. p53-regulated miRNAs were substantially induced during the first p53 response, remained stable during the recovery period, and remained elevated through the second response (solid symbols). Open symbols represent miRNA expression in HT29-tsp53 cells which were only subjected to the first p53 response. miRNA levels in these cells decreased only slightly during the following 24h. Individual points in (a) and (b) represent the mean (+/- the standard error of the mean (SEM)) of 3 independent experiments. c) Immunoblot assays of protein concentrations in HT29-tsp53 cells subjected to two p53 responses as described in (a). MDM2 protein levels are clearly attenuated during the second p53 response, while GDF15 protein exhibited similar increases in protein levels during both p53 responses. Data is presented here with permission and modified from Cabrita *et al.* (unpublished).


a
BTG2
CDKN1A
DDB2
FAS
GDF15
MDM2
Expression (%)
200
100
0
0 16 32
Time (hours)
b
miR-143-3p
miR-145-5p
miR-34a-5p
Expression (%)
200
100
0
0 16 32
Time (hours)
c
p53 response
1st
2 nd
Time (h) 0 2 4 6 16 0 2 4 6 16
Mdm2
GDF15
actin

remaining from the initial induction phase feedback onto emerging mRNA targets during the second p53 response, and it has already been described that MDM2 is a target for miR-143/miR-145.

The p53 in HT29-tsp53 cells is not regulated through normal means, and therefore it became of interest to see if the same response can be found in cells harboring wild type p53. Therefore, one of the goals of this project was to identify the response of p53 to repeated and reversible activation in HCT116 cells without changes in temperature, as this constitutes a cellular stress that activates p53. In order to accomplish this, Nutlin-3a was to be used to disrupt p53-MDM2 interaction and thereby activate p53 transactivation. As was previously mentioned, although HCT116 cells harbor wild type p53, they do not respond to Nutlin with apoptosis, which allowed the analysis of their response to consecutive p53 responses without dramatic cell loss.

1.2 Acute myeloid leukemia

1.2.1 Overview

Leukemia is a category of cancer which typically affect the progenitor cells of blood. The type of leukemia diagnosed is based on which progenitor cell is affected. According to the National Cancer Institute (NIH) SEER website, all leukemias will likely represent 3.6% of all new cancer cases in 2016, and 4.1% of all cancer-related deaths (NIH SEER, http://seer.cancer.gov/statfacts/html/leuks.html). Acute myeloid leukemia (AML) is a subcategory of leukemia, and it is estimated that in 2016, 19950 people will be diagnosed with AML, and 10430 people will die from it (NIH SEER,

http://seer.cancer.gov/statfacts/html/amyl.html). With 4.1 new cases per year per 100000 people, and 2.8 deaths as a result of AML per 100000 people per year, AML represents just 1.2% of all new cancer cases.

Acute myeloid leukemia is a subcategory of leukemia, and includes a wide variety of subgroups differentiated based on cellular morphology, cytochemistry, immunophenotype, genetics, and clinical features (reviewed in Vardiman *et al.,* 2008; Dohner *et al.,* 2010; Dombret and Gardin, 2016). According to the World Health Organization (WHO), "myeloid" refers to any cells commonly derived from the common myeloid progenitor (CMP): granulocytes (neutrophils, eosinophils, basophils), monocytes/macrophages, erythrocytes, megakaryocytes, and mast cells (reviewed in Vardiman *et al.,* 2008). According to WHO, patients with greater than 20% neoplastic cells ("blast" cells) in peripheral blood or bone marrow samples are considered to have acute myeloid leukemia, although the diagnosis can be made with lower blast percentage in the event of identification of specific genetic contributions. These blast cells are identified based on morphology according to guidelines, as opposed to identification based on cell markers such as CD34 (discussed later), as not all patients develop blasts which express these markers. Multiparameter flow cytometry analyzing blood cells based on cell differentiation (CD) markers, however, can be useful for identifying blast lineage.

Considering the vast array of diseases that can be categorized under "acute myeloid leukemia" of some form, prognosis for patients is entirely dependent on their individual circumstances. Factors that play a role in prognosis fall into two categories: those that are related to the state of the patient, and those that relate to the state of the specific AML they have (reviewed in Dohner *et al.,* 2010). One criteria that should be

defined here is what constitutes "complete remission" (CR). According to Dohner *et al.*, complete remission of AML in patients is indicated by <5% blasts in the bone marrow, absence of extramedullary disease, absolute neutrophil count >$1.0x10^9$/L, platelet count of >$100x10^9$/L, and independence from red cell transfusions (reviewed in Dohner *et al.,* 2010).

Treatment of many leukemias, including AML, usually involves intense induction therapy using either anthracyclines or cytarabine. These function well in AML in particular, as these drugs generate DNA damage, and AML cases commonly retain wild-type p53 (Hu *et al.,* 1992; Nakano *et al.,* 2000). In young patients, this approach has been shown to produce CR with rates of >80% (Dohner *et al.,* 2010; Dombret and Gardin, 2016). Following remission, younger patients are commonly placed on a regimen of high-dose cytarabine (HiDAC). However, in the event that it is deemed relatively low-risk, patients can be considered for autologous stem cell transplantation. Allogenic transplantation, alternatively, is associated with the lowest rate of recurrence of AML, but is also far more dangerous, with a much higher morbidity and mortality risk. To determine if the potential benefits of transplantation outweigh the potential risks of death, many variables need to be considered, such as patient age, disease stage, donor type, and the time interval between the initial diagnosis and the transplantation event (Dohner *et al.,* 2010; Dombret and Gardin, 2016). Because of this, allogenic transplantation is usually reserved for highly unfavorable AML prognoses and for second remission of other AML cases.

1.2.2 Hematopoiesis

One segment of the project being presented was the handling and analysis of blood samples retrieved from AML patients during the course of their treatment, and therefore it is necessary here to briefly discuss the cells of blood and how they are made.

Hematopoiesis is the process of proliferation and differentiation of hematopoietic stem cells (HSCs) to generate all the cells of blood. This differentiation process is divided up into multiple steps involving a gradual reduction of multipotency (see Figure 5)(reviewed in Orkin and Zon, 2008; Widmaier *et al.,* 2008; Rieger and Schroeder, 2012). Blood can largely be classified into two or three categories: myeloid and lymphoid cells, or erythrocytes, leukocytes, and megakaryocytes/platelets. Erythrocytes are red blood cells, whose purpose is to carry oxygen to the cells and remove carbon dioxide. Leukocytes form a larger category of cells generally termed "white blood cells", and includes granulocytes (neutrophils, eosinophils, and basophils), monocytes (commonly associated with macrophages), and lymphocytes (T cells, B cells, natural killer (NK) cells) (reviewed in Widmaier *et al.,* 2008; Rieger and Schroeder, 2012).

These cells, as mentioned, can also be classified as myeloid or lymphoid based on their differentiation process (Figure 5). Myeloid cells are those derived from what is termed a "common myeloid progenitor" (CMP) and include neutrophils, basophils, eosinophils, monocytes, megakaryocytes, and erythrocytes (although megakaryocytes and erythrocytes can develop through a megakaryocyte-erythrocyte progenitor (MEP), as well). Lymphoid cells include B cells, T cells, and NK cells (reviewed in Widmaier *et al.,* 2008; Rieger and Schroeder, 2012). Refer to Table 1 for general functions of these cells. In the context of AML, the neoplastic cells are those derived from the CMP.

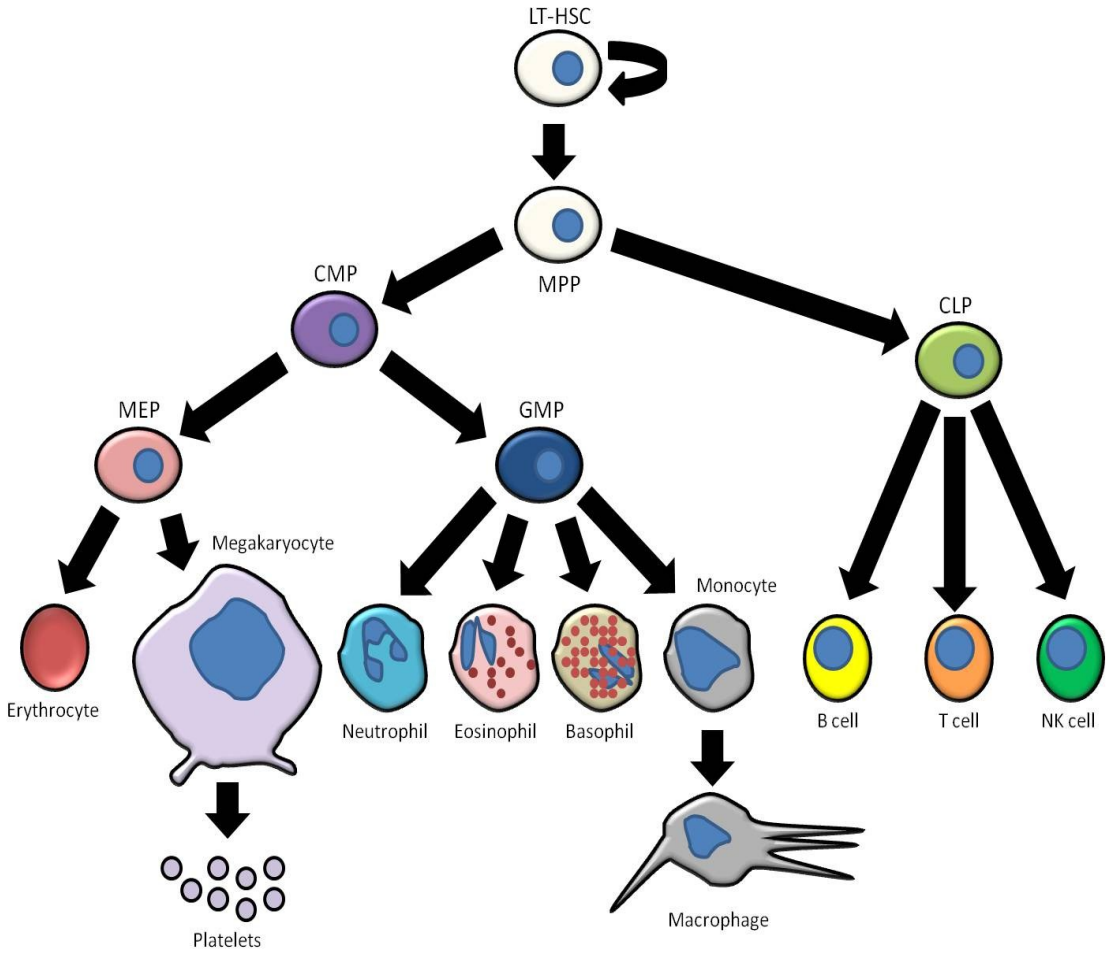


Figure 5: Overview of hematopoietic lineages.

Upon dividing, daughters of hematopoietic stem cells can either continue to proliferate to maintain the population of stem cells, or undergo differentiation. This differentiation results in a gradual loss of multipotency, eventually committing to one of multiple fates. LT-HSC: long-term renewing hematopoietic stem cell. MPP: multipotent progenitor. CMP: common myeloid progenitor. CLP: common lymphoid progenitor. MEP: megakaryocyte/erythrocyte progenitor. GMP: granulocyte/macrophage progenitor (modified from Orkin and Zon, 2008; Rieger and Schroeder, 2012).

Cell	Function
Erythrocytes	Transportation of oxygen to cells and removal of carbon dioxide
Neutrophils	Phagocytosis of foreign substances and release of inflammatory agents
Basophils	Release of inflammatory agents
Eosinophils	Participate in hypersensitivity (allergic) reactions, and assist in the destruction of parasites
Monocyte/Macrophage	Phagocytosis of foreign substances, secretion of toxic substances, present antigens to T cells, and secretion of cytokines which mediate inflammation and maturation of T cells
B cells	Generate and secrete antibodies in response to activation by antigen recognition (as a plasma cell), present antigens to T cells
T cells (CD4+)	Secretion of cytokines to activate other cells which mediate immune response such as B cells, CD8+ T cells, and NK cells.
T cells (CD8+)	Binding of antigens on targeted cells and cause their destruction by releasing cytotoxins
NK cells	Kill virus-infected and cancerous cells, along with killing cells targeted by antibody-production from B cells
Platelets	Blood clotting

Table 1: Major cell groups of blood and their major functions.

(modified from Widmaier *et al.,* 2008).

1.2.4 p53 and AML

It was discussed previously that one of the goals of this project was to identify the response of p53 to repeated and reversible activation through the use of Nutlin-3a. As cancer therapies are generally administered in repeated fractions, the behaviour of p53 in response to repeated activators is important clinically. Our collaborator, Dr. Mitchell Sabloff, at the Ottawa Hospital is conducting clinical trials on phase IV (metastatic) AML patients treated with total body ionizing radiation (TBI) and subsequent allogenic stem cell transplant. This trial was conducted with ethics approval from the Ottawa Hospital, and our participation was further approved by the CUREB-B.

Four phase IV AML patients underwent TBI over the course of 4 days on average, with a total irradiation of 18Gy. Patients were irradiated once in the morning, and once in the afternoon, and between these treatments blood samples and buccal swabs were harvested. Blood samples and buccal swabs were received frozen, with the blood samples having been separated with Ficoll-Pacque®. The fraction of blood received contained the peripheral blood mononuclear cells (PBMCs), which includes T cells, B cells, NK cells, and monocytes.

The portion of this project associated with the patient samples had multiple goals. Firstly, considering that p53 generates major changes in cells following irradiation, it was hypothesized that the patient samples would provide an excellent model for the investigation of repeated p53 activation. The buccal swab samples presented a non-invasive source of normal, non-diseased RNA. Therefore, the buccal swab samples were

expected to present an example of repetitive p53 activation in an actual human system, and this was to be compared to previous results from the HT29-tsp53 cells (data unpublished), as well as those from the HCT116 portion of this project. The blood samples were expected to be somewhat more complicated, as they would contain both normal blood and leukemic cells. The buccal swabs and blood samples were to be compared and compiled in order to provide a clearer picture of the body's response to daily doses of ionizing radiation.

One final aspect of the patient samples is the identification of markers for use in determining prognosis of treatment while treatment is ongoing. As only 4 patients are represented in this project, this aspect of the project does not present a large enough sample size to adequately determine the presence or absence of any such markers. It does, however, serve as a pilot project, as clinical trials are ongoing, and more patient samples will be analyzed in the future.

1.3 Rationale and Hypotheses

The p53 protein and its associated targets clearly present integral components of a cell's ability to suppress tumorigenesis. Many therapeutic agents in use today to combat cancer activate p53 through DNA damage, such as the anthracyclines used in the treatment of leukemias, and radiation used in the transplant setting. These treatments are commonly given on a daily basis, leading to repetitive activation of the p53 network. Although much time and effort has been devoted to illuminating the mechanisms of p53 activation and its consequences, little work has been done on the analysis of repeated p53 activation.

Based on previous work, we hypothesized that treatment of HCT116 cells with Nutlin-3a in a reversible and repeated manner would lead to up-regulation of a variety of target mRNAs and miRNAs, but that the miRNA response would modulate subsequent response(s) at the mRNA and protein level. We also proposed that these patterns would be apparent in the analysis of RNA samples from buccal swabs and blood samples taken from AML patients during TBI treatments. Finally, we hoped to find some trend in gene expression in the human samples which could be further investigated in the future to find RNA marker(s) to denote patient prognosis mid-treatment.

2. Methods:

2.1 Cell culture:

HCT116 cell lines were cultured in McCoy's 5A Media (Hyclone, Logan, UT) with 11% serum in a 3:1 ratio of heat-inactivated newborn calf serum (Gibco, Mississauga, ON): fetal bovine serum (Gibco, Mississauga, ON), with 90units/mL penicillin (Hyclone, Ottawa, ON), and 90µg/mL streptomycin (Hyclone, Ottawa, ON). Cultures were maintained at a subconfluent level by routine subculturing at a 1:4 ratio. During subculturing, cells were washed using 1X phosphate buffered saline (PBS), and were detached from the dish using 0.25% trypsin (Hyclone, Ottawa, ON). Cells were maintained in a humidified incubator for 1 minute at 37^{o}C, 100% humidity and 5% CO_2.

Where needed, Nutlin-3a (Sigma-Aldritch, St.Louis, Mo) treatment media was made by adding 250µL-1000µL of 0.1µmol/mL Nutlin-3a to media to result in a final volume of 10mL, with final concentrations of drug being 2.5-10µmol/L.

2.2 RNA extraction:

Treatment of cultured cells prior to RNA extraction required the removal of any media from the dishes, followed by two washes with 3mL of PBS. Following this, extraction continued in accordance with the mirVana miRNA isolation protocol outlined below.

RNA extraction of samples was conducted using the mirVana miRNA isolation kit (Invitrogen, Fisher Scientific Ltd., Ottawa, ON) as per the manufacturer's instructions. In brief, samples were treated with 300µL (buccal swabs) or 600µL of Lysis Buffer (blood samples and cultured cells), and then collected into 2mL conical tubes. Samples

were briefly vortexed, then treated with 1/10 volume of miRNA Homogenate Solution, vortexed again, and left on ice for 10 minutes. 1 volume of Acid Phenol: Chloroform was added in accordance with the quantity of Lysis Buffer added. Solutions were inverted, vortexed for 1 minute, then centrifuged at 10000rcf for 5 minutes. Upon completion, the aqueous phase was removed and transferred to a new 2mL conical tube. 1.25 volumes of ethanol were added according to the volume of aqueous phase removed, and the resultant solution was mixed thoroughly. 700µL of this solution were transferred to a filter separation column and centrifuged at 10000rcf for 15 seconds, whereupon the eluant was discarded. This was repeated until the total volume of sample was passed through the column. Columns were subsequently rinsed with 700µL of miRNA Wash Solution 1 and centrifuged at 10000rcf for 15 seconds, discarding the eluant. This was repeated twice with 500µL of miRNA Wash Solution 2/3. Columns were then centrifuged at 10000rcf for 1 minute to remove any remaining moisture on the filter. Filters were then transferred to a new 2mL collection tube, and 50-100µL of 90^{o}C RNase-free water was added to the filter, which was centrifuged at 10000rcf for 30 seconds, resulting in the final RNA extract.

In the case of blood samples, 1mL of provided samples were transferred into new 2mL conical tubes and centrifuged at 317xG for 5 minutes, supernatant was removed from the pellet, and 1mL PBS was added. The sample was briefly vortexed to wash the sample, and then centrifuged as before. Following the removal of the supernatant, extraction was carried out following the mirVana miRNA isolation kit protocol outlined above.

Initial treatment of patient buccal swabs prior to RNA extraction required the transfer of swabs from the provided container into a new 2mL conical tube, whereupon 300µL of Lysis Buffer was added. Samples were then vortexed for 1 minute, and left to sit for 5 minutes. Swabs were then removed with forceps, and extraction continued as per the mirVana miRNA isolation outlined above, continuing with the addition of miRNA Homogenate Solution.

2.3 Reverse Transcription (RT):

Prior to reverse transcription, samples were quantified using a DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, DE) and sample added to the following reverse transcription protocol were normalized to the lowest RNA concentration such that equal amounts of RNA were used for the RT reaction. Those samples which required less sample to achieve the base RNA content had the remainder replaced with RNase-free distilled water.

Reverse transcription for conversion of mRNA to cDNA was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Fisher Scientific Ltd., Ottawa, ON) according to the manufacturer's guidelines. In brief, 5.8µL of a master mix containing 2µL 10X reverse transcription buffer, 0.8µL 100mM dNTPs (with dTTP), 2µL 10X reverse transcription random primers, and 1µL MultiScribe™ reverse transcriptase (50U/µL) were added to each 200µL PCR tube. Following this, 4.2µL RNase-free water was added to each tube, and a total of 10µL of sample was added (normalized as above). Samples were subsequently vortexed briefly, and placed into a GeneAmp PCR System 9700 (Applied Biosystems, Fisher Scientific Ltd., Ottawa, ON)

where they were cycled at 25°C for 10minutes, then 37°C for 2 hours, and finally 85°C for 5 minutes. Resultant cDNA was stored at -20°C until needed.

Reverse transcription for conversion of miRNA to cDNA was conducted using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Fisher Scientific Ltd., Ottawa, ON) according to the manufacturer's guidelines. In brief, 2.84µL of master mix containing 0.15µL 100mM dNTPs, 1µL MultiScribe™ reverse transcriptase (50U/µL), 1.5µL 10X reverse transcription buffer, and 0.19µL RNase Inhibitor (20U/µL) were added to each 200µL PCR tube. Following this, 4.16µL RNase-free water was added to each tube, and a total of 5µL of sample was added (normalized as above). Samples were vortexed briefly, and placed into a GeneAmp PCR System 9700 where they were cycled at 16°C for 30 minutes, 42°C for 30 minutes, and finally 85°C for 5 minutes. Resultant cDNA was stored at -20°C until needed.

2.4 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR):

qRT-PCR was completed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Fisher Scientific Ltd., Ottawa, ON) with the StepOne Software v2.3 (Applied Biosystems, Fisher Scientific Ltd., Ottawa, ON).

qRT-PCR for cDNA generated from mRNA was conducted using TaqMan® Gene Expression Assays (Applied Biosystems, Fisher Scientific Ltd., Ottawa, ON) following the manufacturer's guidelines. In brief, 10µL TaqMan® Universal Master Mix II, with UNG (Applied Biosystems, Fisher Scientific Ltd., Ottawa, ON), were added to each MicroAmp® Fast Reaction Tube (Applied Biosystems, Fisher Scientific Ltd., Ottawa, ON). Following this, 1µL of the TaqMan® Gene Expression Assays primer

being used was added to the tubes, followed by 9µL of the appropriate sample. Tubes were briefly vortexed prior to being placed in the StepOnePlus Real-Time PCR System, and were then cycled at 50°C for 2 minutes, at 95°C for 10 minutes, and then underwent 40 cycles comprised of 95°C for 15 seconds followed by 60°C for 1 minute.

qRT-PCR for cDNA generated from miRNA was conducted using TaqMan® MicroRNA Assays (Applied Biosystems, Fisher Scientific Ltd., Ottawa, ON) following the manufacturer's guidelines. In brief, 10µL TaqMan® Universal Master Mix II, with UNG, were added to each MicroAmp® Fast Reaction Tube. Following this, 7.67µL RNase-free water were added, along with 1µL of the TaqMan® MicroRNA Assays primer being used, and finally 1.33µL of the appropriate sample. Tubes were then briefly vortexed prior to being placed in the StepOnePlus Real-Time PCR System, where they underwent the same cycling conditions as previously described.

Any qRT-PCR conducted on samples harvested from patient buccal swabs underwent 50 cycles of the StepOnePlus Real-Time PCR System instead of 40 due to extremely low RNA concentrations.

2.5 Immunoblot analysis:

Cell cultures first had their media removed, and were then rinsed twice with 3mL PBS. Cells were then treated with 600µL of RIPA buffer (Sigma-Aldritch, St.Louis, MO) and collected in a 1.5mL microcentrifuge tube. Cell lysates were stored at -20°C until needed, whereupon they were sonicated using an XL-2000 series sonicator at setting 7 for roughly 12 seconds (Qsonica, LLC., Newtown, CT). Cell lysates were then quantified by comparing absorbance readings of samples to those of a dilution series BSA standard

using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Mississauga, ON) read with a Bio-Rad iMark^{TM} Microplate Reader at a wavelength of 595nm(Bio-Rad, Mississauga, ON). Equal amounts of protein were run through a NuPage 10% Bis-Tris precast polyacrylamide gel (Invitrogen, Carlsbad, CA) at 200V and subsequently transferred onto a nitrocellulose membrane (Bio-Rad, Mississauga, ON) for 1h at 30V. Membranes were blocked in 5% milk in TBST for 1h, washed four times with TBST for 5min each, and treated with primary antibody for 1.5h. Primary antibodies were: Ab-6 p53 (Calbiochem, Billerica, MA), Ab-1 p21 (Calbiochem, Billerica, MA), SMP14 MDM2 (Santa Cruz Biotechnology, Dallas, TX), and actin (Sigma-Aldritch, St.Louis, MO). All primary antibodies were suspended in 0.5% milk in TBST. Membranes were then washed with TBST four times for 5min each, and treated with secondary antibody for 1h. Secondary antibody was goat anti-mouse conjugated to Horse Radish Peroxidase (Santa Cruz Biotechnology, Dallas, TX). Membranes were subsequently washed with TBST four times for 5min each. 1mL of Super Signal Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Ottawa, ON) was added to membranes, incubated for 5min, and then imaged.

3. Results:

3.1 HCT116 cells

3.1.1 Nutlin-3a activation of p53 transactivation in HCT116 cells is reversible

One of the primary goals of this project was to characterize the response of p53 and downstream targets to consecutive Nutlin-3a induced p53 responses separated by a period of recovery. First it was necessary to ensure that the p53 activation by Nutlin-3a treatment led to an increase in p53 expression and activity and that these were reversible upon removal of the drug. To this end, HCT116 cells were treated with 2.5μM or 5μM Nutlin-3a in growth media (see Methods) for 6h. Cells were then subsequently rinsed with PBS and then incubated in fresh growth media for 24h. Protein samples were harvested, and western blots were run using antibodies against p53, MDM2, p21, and actin (Figure 6a). p53 protein levels were clearly induced following 6h of treatment with both 2.5μM and 5μM Nutlin-3a. p53 protein then decreased rapidly, reaching baseline levels within 1h under both treatment conditions. The p53-regulated MDM2 and p21 proteins increased within the 6h of treatment as well. Both MDM2 and p21 protein levels remained high for 3h following the removal of Nutlin-3a, although they did decrease to baseline levels sometime between 3 and 24h following removal of the drug. These results indicate that the p53 induction following Nutlin-3a treatment is rapidly reversible in HCT116 cells while the recovery of downstream targets is delayed in comparison.

mRNA levels were determined under the same conditions as proteins above. Briefly, HCT116 cells were treated with different concentrations of Nutlin-3a for 6h. Cells were then rinsed with PBS, and total RNA was isolated, reverse transcribed and subjected to quantitative PCR analysis (qRT-PCR). TP53 mRNA levels remained

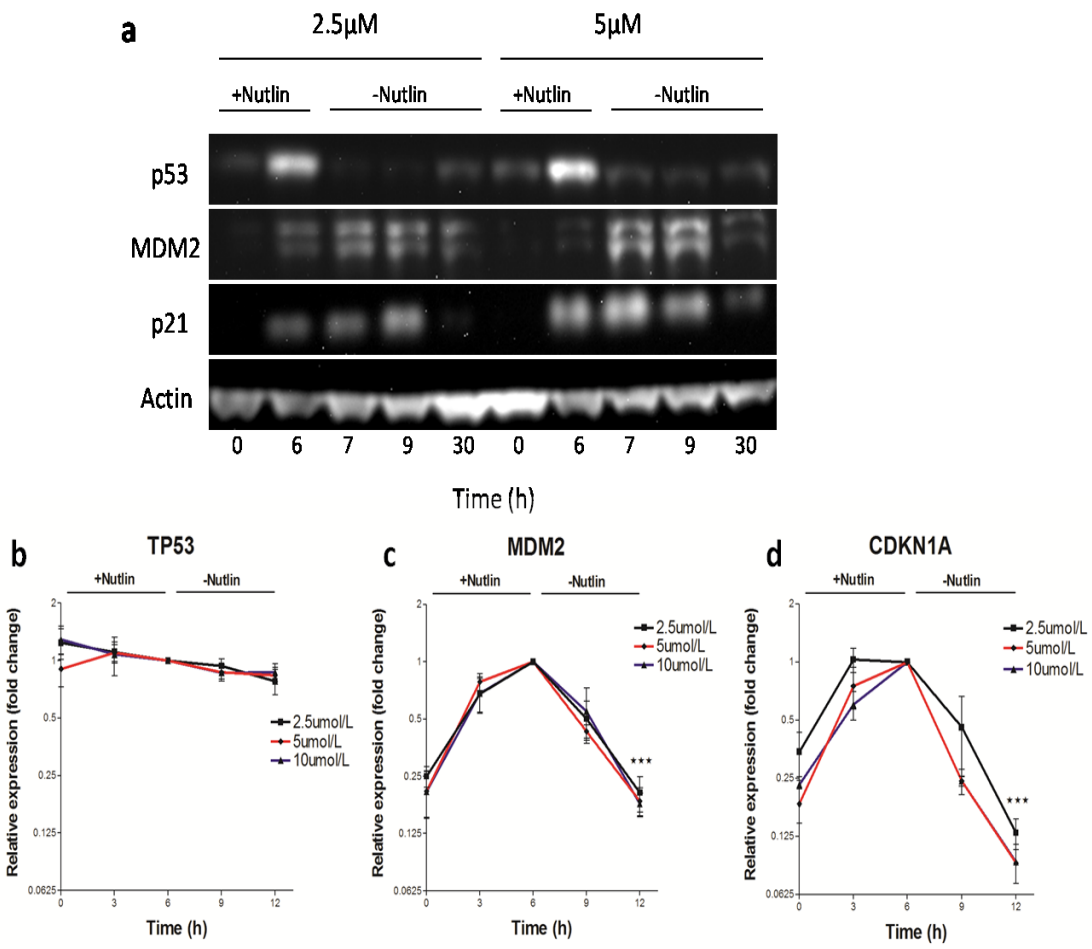


Figure 6: p53 induction in HCT116 cells by Nutlin-3a is reversible.

a) Western blots of HCT116 cells treated with 2.5µM or 5µM Nutlin-3a for 6h, washed with PBS, and incubated in growth media for 24h. b-d) HCT116 cells were exposed to the indicated concentration of Nutlin-3a for 6h, were washed in PBS and were incubated in fresh media without drug for a further 6h. TP53, MDM2 and CDKN1A mRNA was assessed by qRT-PCR. Individual points (b-d) represents the mean (+/- standard error of the mean (SEM)) determined from 3 independent experiments. *** indicates that the value is significantly different from 1 ($p<0.001$) by single sample t test.

relatively constant throughout treatment (Figure 6b), as expected, because p53 activation is primarily achieved by post-translational modification. MDM2 and CDKN1A mRNAs increased in the presence of Nutlin-3a to a similar extent regardless of concentration (Figure 6c, d). After washout, the levels of MDM2 and CDKN1A mRNA decreased just as quickly (Figure 6c, d). The rapid decrease of these transcript levels supports previous work, suggesting that the p53 response is dominated by short-lived transcripts (Melanson *et al.,* 2011). Taken together, Nutlin-3a activates p53 and induces p53-dependent mRNAs in HCT116 cells, and this is readily reversible.

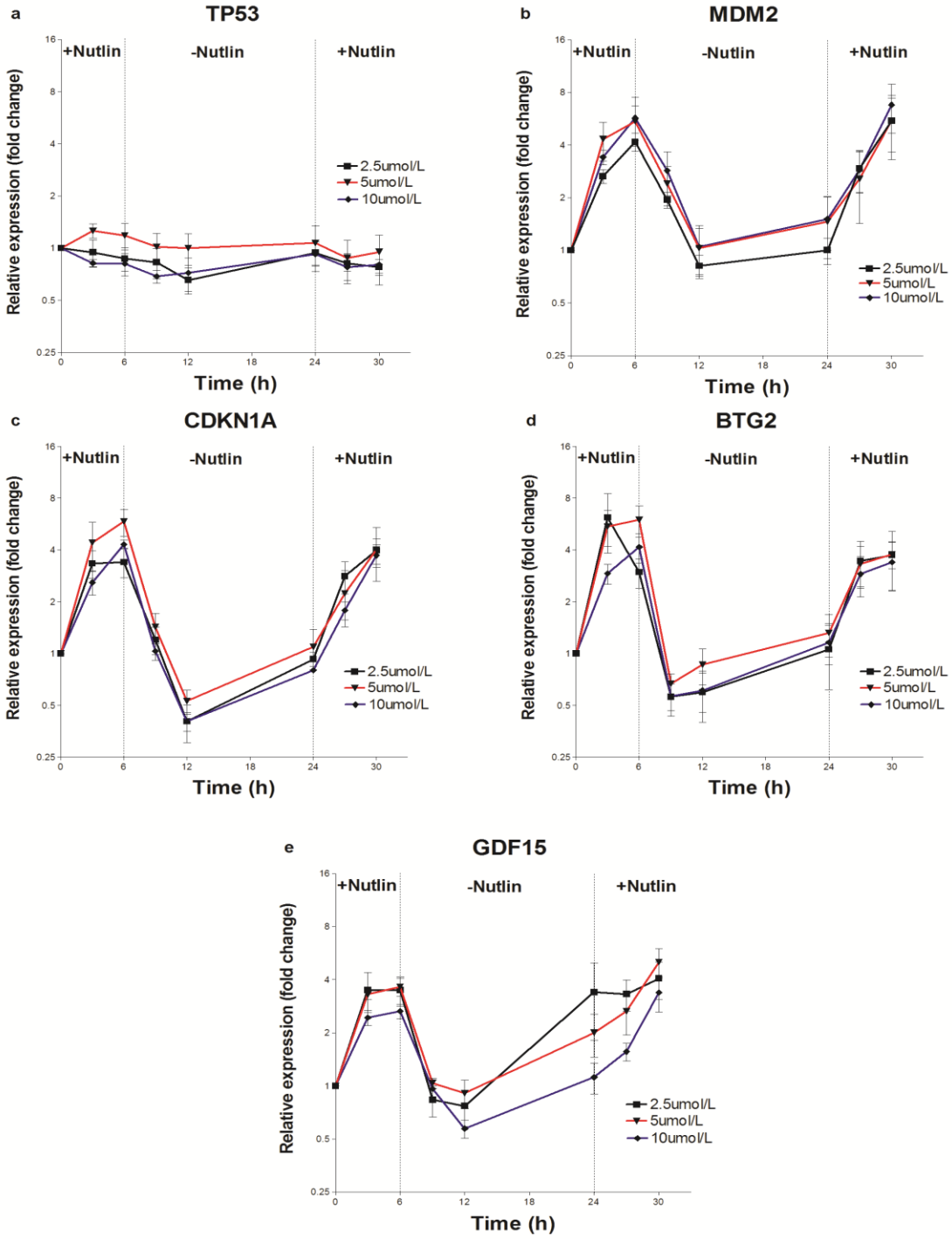
3.1.2 Consecutive p53 activations appear to be independent

The results above indicate that Nutlin-3a is reversible in HCT116 cells. Therefore, this model system allowed us to activate p53 again after recovery, as we have done using the temperature sensitive variant of p53 (Cabrita *et al.,* unpublished). HCT116 cells were treated with 2.5μM, 5μM, or 10μM Nutlin-3a for 6h, rinsed with PBS, and incubated in fresh growth media for 18h. The cells were then treated with the same concentration of Nutlin-3a for another 6h. Total RNA was harvested at various times and analyzed by qRT-PCR.

Once again, TP53 mRNA levels stayed relatively constant throughout the experiment, regardless of the concentration of Nutlin-3a used (Figure 7a). The other p53-regulated transcripts increased in the presence of Nutlin-3a similarly at all concentrations tested (Figure 7b-e). MDM2, CDKN1A, BTG2, and GDF15 transcript levels decreased rapidly following the removal of Nutlin-3a, such that their levels had decreased to baseline or below by 6h after the removal of Nutlin-3a. When Nutlin-3a was added the

Figure 7: mRNA expression of HCT116 cells treated twice with Nutlin-3a on consecutive days.

Cells were treated for 6h with Nutlin-3a, washed with PBS and incubated in fresh growth media for 18h, then they were treated with Nutlin-3a for another 6h. Dotted lines at 6h and 24h represent removal and addition of Nutlin-3a, respectively. The expression of the indicated mRNA was normalized to GAPDH. Individual points represent the mean (+/- SEM) determined from 3 independent experiments. No significant difference is detectable between Nutlin-3a concentrations by Two-way ANOVA.


a
TP53
+Nutlin
-Nutlin
+Nutlin
Relative expression (fold change)
Time (h)
2.5umol/L
5umol/L
10umol/L
b
MDM2
c
CDKN1A
d
BTG2
e
GDF15

second time, MDM2, CDKN1A, BTG2, and GDF15 mRNAs increased again to a similar extent, regardless of Nutlin-3a concentration. Therefore, the p53 responses appeared to be similar in magnitude. This was unexpected, because we previously reported that the increases in p53-regulated mRNAs in response to p53 induction were selectively attenuated by a previous p53 response using a temperature sensitive variant of p53 that permitted similar reversible control of p53 activity.

MicroRNAs can inhibit gene expression at the translational and post-transcriptional levels; therefore, it was important to assess protein expression as well. HCT116 cells were subjected to the same experimental procedure and cell lysates were collected for immunoblot analysis (Figure 8). Once again, p53 levels increased within the first 3h of Nutlin-3a treatment, and decreased rapidly falling to levels similar to baseline within the first 3h in absence of Nutlin-3a. p53 protein levels then rose within 3h during the second induction to levels comparable to the first response. Similarly, MDM2 and p21 levels increased in the presence of Nutlin-3a, and then decreased to baseline levels after the removal of Nutlin-3a. Both proteins were induced when exposed to Nutlin-3a again. These results are consistent with the results obtained from the mRNA expression analysis, and suggest that these two p53 responses separated by a period of recovery were similar. Therefore, the present analysis does not support specific parts of our previous work (Cabrita *et al.,* unpublished data).

In this previous work, the attenuation of the second p53 response was attributed to a mechanism involving p53-induced miRNA-mediated feedback (Cabrita *et al.,* unpublished data). Specifically miR-34a-5p, miR-143-3p, and miR-145-5p appeared to target the 3'UTRs of MDM2 and BTG2 preventing their increase in response to a

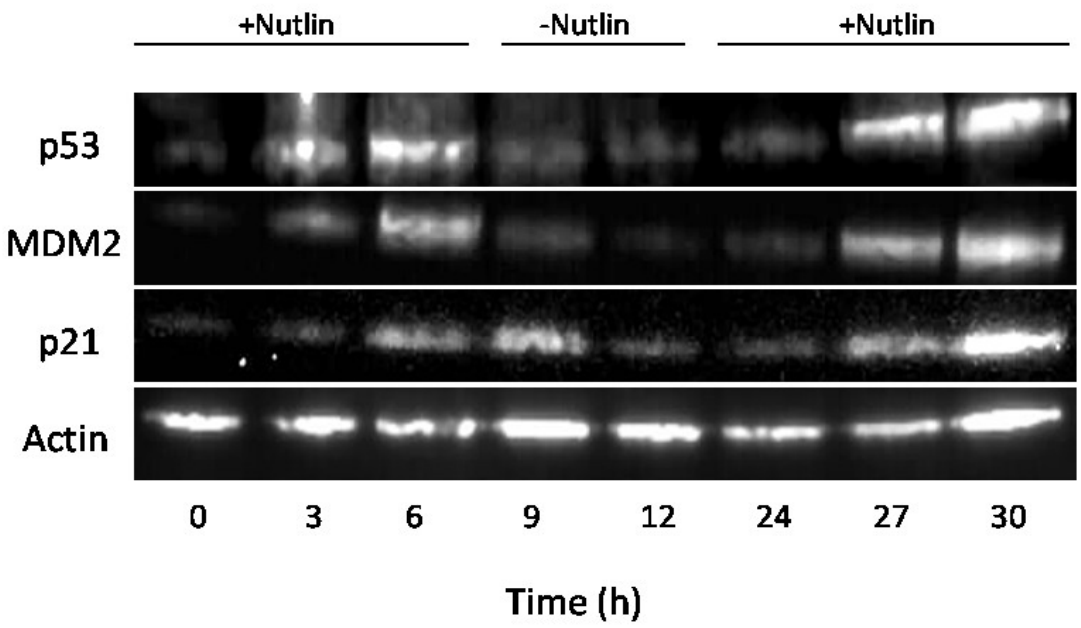


Figure 8: Immunoblot assay of protein in HCT116 cells treated with Nutlin-3a. HCT116 cells treated with 2.5μM Nutlin-3a for 6h, washed with PBS, incubated in growth media for 18h, then treated with Nutlin-3a for another 6h to activate p53 again. Whole cell lysates were collected for immunoblot analysis of the indicated proteins. Similar results were obtained in 4 similar experiments.

secondary p53 activation. Therefore, the expression of these miRNAs was analyzed throughout the course of similar Nutlin-3a experiments (Figure 9). Surprisingly, miR-34a-5p, miR-143-3p, and miR-145-5p were not strongly induced by Nutlin-3a treatment in HCT116 cells, failing to increase more than 2-fold above baseline. This is drastically lower than miRNA induction detected (10 to 50 fold) in the other model system (Cabrita *et al.,*unpublished data). The absence of a prominent p53-dependent miRNA induction in HCT116 cells exposed to Nutlin-3a likely explains the disparity between studies.

3.2 AML patient samples

3.2.1 The use of buccal swabs to identify normal tissue response to irradiation is hampered by low RNA yield and heterogeneity in the sampling population

In order to assess the effects of total body radiation therapy on the normal tissues of patients, buccal epithelial cells were harvested from patients daily. Patients were treated with 2.5Gy of ionizing radiation twice daily, once in the morning and once in the afternoon. Buccal epithelial cells were harvested roughly 4 hours after the start of their morning treatment for analysis of total RNA as described in the methods. Both mRNA and miRNA expression was assessed by qRT-PCR (Figures 10 and 11). It is important to note that not all samples amplified during qRT-PCR, and that limited sample and extremely low sample RNA concentration meant that only a limited number of mRNAs could be assessed.

MDM2 and CDKN1A mRNAs were used as indicators of p53 activity. MDM2 (Figure 10a) and CDKN1A (Figure 10b) levels generally decreased throughout treatment for all patients, although MDM2 and CDKN1A levels recovered to baseline levels in

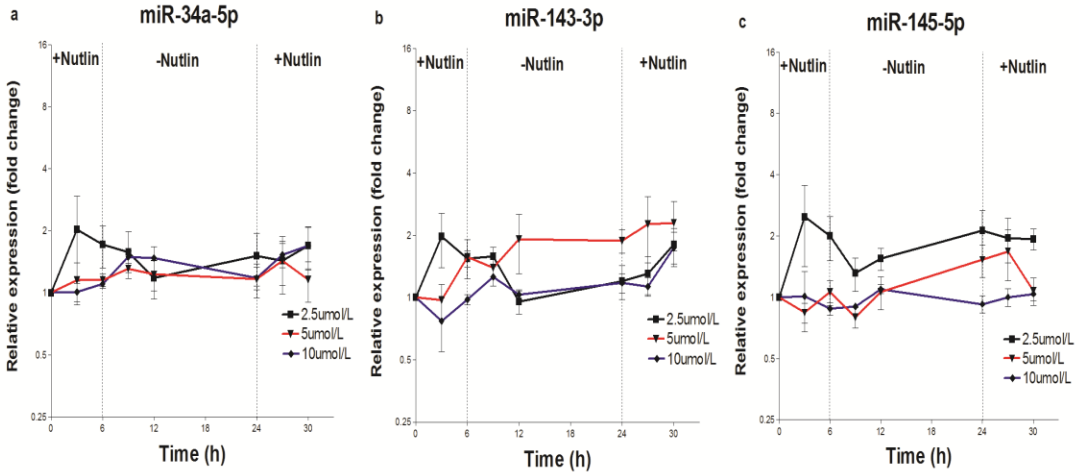


Figure 9: miRNA expression in HCT116 cells treated twice with Nutlin-3a. HCT116 cells treated for 6h with 2.5µM, 5µM, or 10µM Nutlin-3a (square, inverted triangle, and circle symbols, respectively) to stimulate p53 activation were washed with PBS and incubated in fresh growth media for 18h, then treated with the same concentration of Nutlin-3a for another 6h. Dotted lines at 6h and 24h represent removal and addition of Nutlin-3a, respectively. miRNA levels are normalized to miR-103. Individual points represent the mean (+/- SEM) determined from 3 independent experiments. No significant difference is detectable between Nutlin-3a concentrations by Two-way ANOVA.

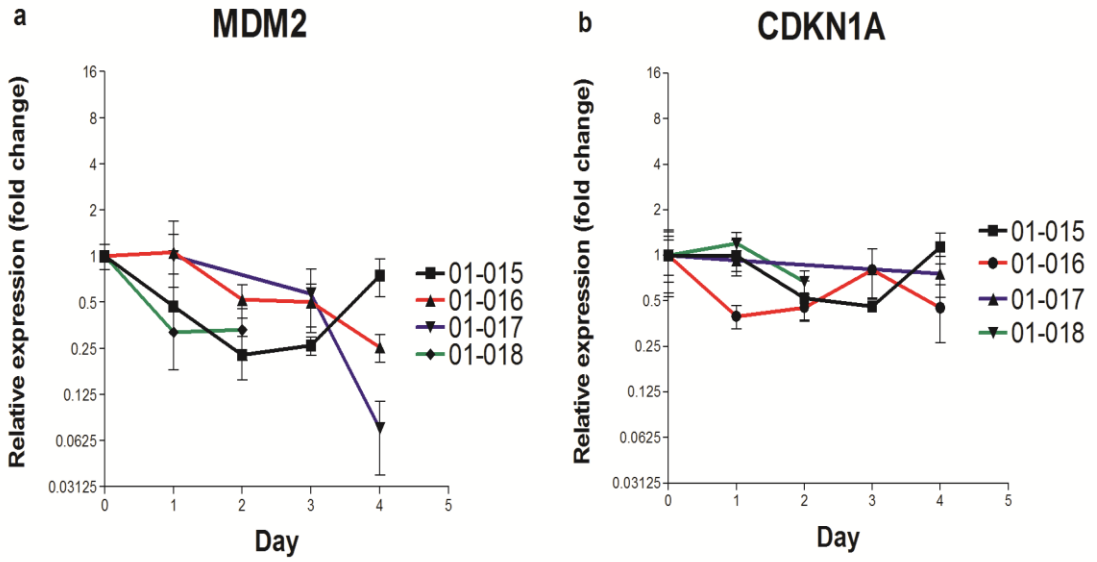


Figure 10: mRNA expression of patient buccal epithelium samples during TBI treatment. Patient samples were harvested daily between 2.5Gy morning and afternoon treatments, with a baseline sample (Day 0) harvested roughly a month prior to the beginning of treatment. The expression of the indicated mRNA was normalized to GAPDH. Individual points represent the mean (+/- SEM) of a minimum of 2 technical replicates.

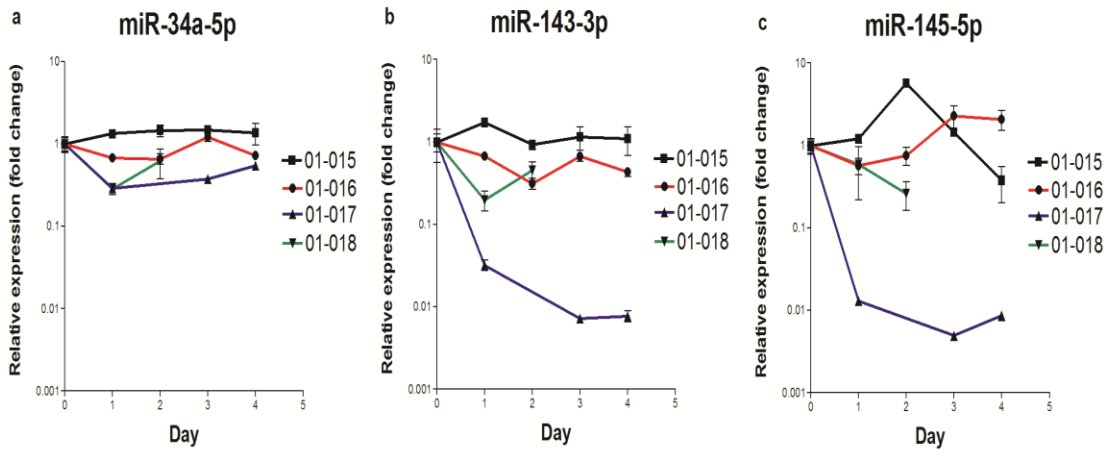


Figure 11: miRNA expression of patient buccal epithelium samples during TBI treatment. Patient samples were harvested daily between 2.5Gy morning and afternoon treatments, with a baseline sample harvested as described in Figure 10. miRNA levels are normalized to miR-103. Individual points represent the mean (+/- SEM) of a minimum of 2 technical replicates.

patient 01-015 samples by day 4.The decrease in p53-responsive gene expression was in complete contrast to what was expected, as ionizing radiation leads to p53 activation and buccal swabs were supposed to contain a normal population of cells with wild type p53. Similarly, the p53-inducible miRNAs assessed (miR-34a-5p, miR-143-3p, and miR-145-5p) were not increased during the course of radiation treatment (Figure 11). miR-145-5p levels increased in patients 01-015 and 01-016, although this was only transient in patient 01-015. Also, miR-143-3p and miR-145-5p levels in patient 01-017 decreased dramatically throughout treatment. Combining the mRNA and miRNA responses, there was no clear p53 response detected in the RNA population obtained from these patient samples.

The buccal mucosa contains multiple layers which can be categorized based on expression of integral cytoskeletal elements termed cytokeratins (reviewed in Squier and Kremer, 2001; Presland and Jurevic, 2002). The proliferative basal layer of the buccal mucosa maintains its population while also providing cells to the suprabasal layers, which undergo a process of terminal differentiation to populate the superficial layer. During this differentiation, the cytokeratins expressed in the cells change, and so the origin of a cell or the relative proportions of cells in a population can be identified based on cytokeratin expression patterns. KRT13 can be used as a marker for non-proliferative, suprabasal cells in the buccal mucosa, and KRT14 is expressed in the proliferative cells in the basal layer.

We reasoned that the p53 response could be absent in terminally differentiated cells, such as those in the superficial layers of the buccal mucosa which are most accessible to buccal swabs, and that the ratio of KRT14+ to KRT13+ cells may change

during the course of treatment. To test this with available samples, qRT-PCR analysis was performed using primers specific for cytokeratin 13(KRT13) and cytokeratin 14(KRT14), as well as TP53 (Figure 12). KRT13 expression remained stable, or increased throughout treatment (Figure 12b). In contrast, KRT14 expression decreased in 2 of the 3 informative patient samples (Figure 12c). Therefore, the proportions of these cytokeratins changed in the patient samples throughout treatment, likely indicating changes in the proportion of these cell types in individual samples.

It is hypothesized that the major function of p53 in the response to irradiation and DNA damage is to prevent the fixation of mutation. This would be necessary in proliferative cells, and less important in non-proliferative, differentiated cells, like those found in the suprabasal layers of the buccal mucosa. By extension, increases in p53-regulated mRNAs in response to irradiation may be best represented by those cells which express KRT14 rather than those that express KRT13 in the buccal mucosa. TP53 mRNA levels and KRT14 mRNA correlate well in the single informative patient(Figure 12a, c), which is consistent with p53 predominantly being expressed in the proliferative, KRT14+ population.

If p53 expression likely plays a more important role in proliferative (KRT14+) cells than in non-proliferative (KRT13+) cells, then normalization to KRT14 would be anticipated to unmask increases in p53-regulated mRNAs during treatment. Interestingly, normalization to KRT14 unmasks slight increases in p53-regulated mRNAs and miRNAs in both patients 01-015 and 01-016 (Figure 13). The increases in mRNA levels of MDM2 are quite small (Figure 13a), although CDKN1A levels increase more substantially throughout treatment relative to baseline (Figure 13b). Interestingly, the induction of p53-

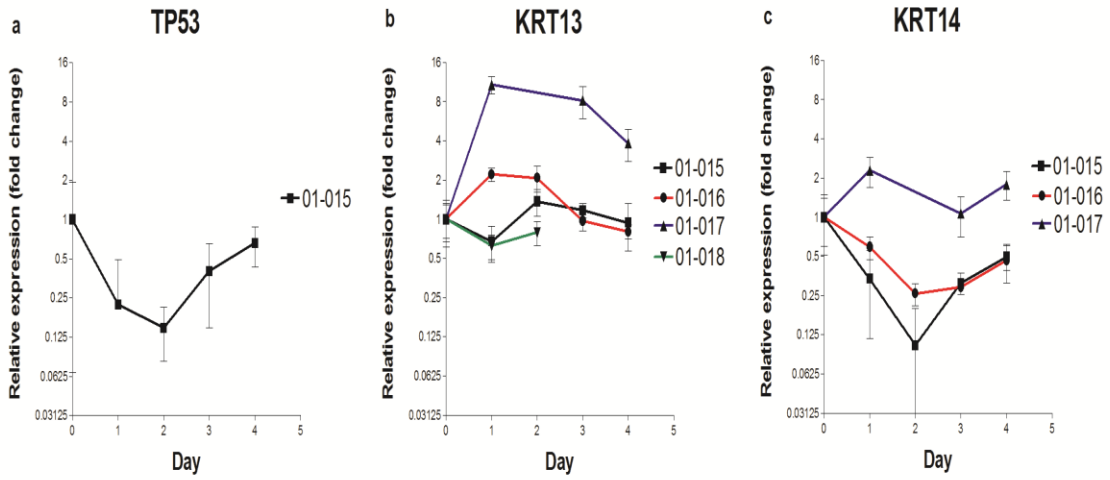
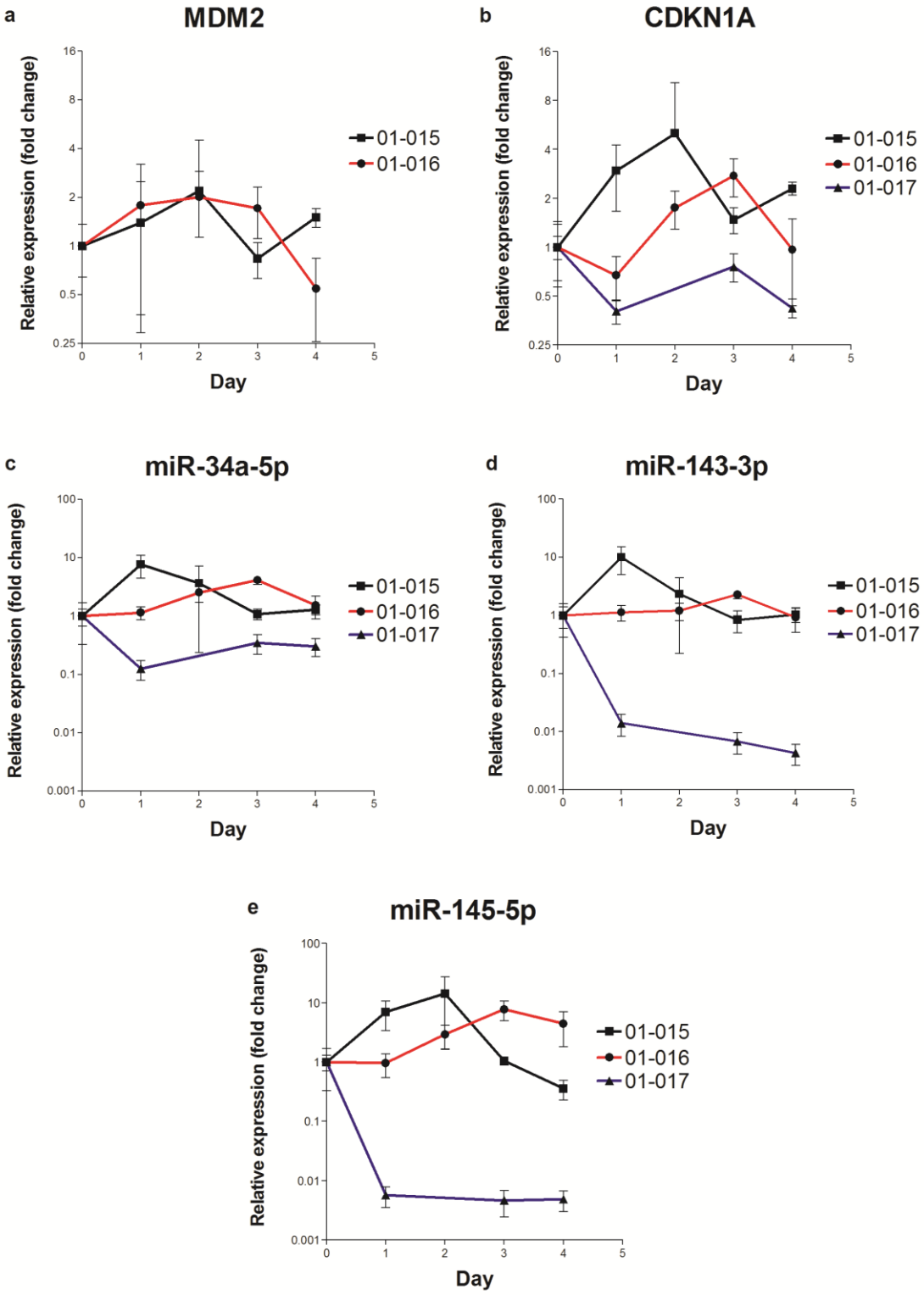


Figure 12: TP53 and cytokeratin mRNA expression in patient buccal epithelial samples during TBI treatment.

Patient samples were harvested daily between 2.5Gy morning and afternoon treatments, with a baseline sample harvested as described in Figure 10. Expression of the indicated mRNA was normalized to GAPDH. Individual points represent the mean (+/- SEM) of a minimum of 2 technical replicates.

Figure 13: mRNA and miRNA expression of patient buccal epithelial samples normalized to KRT14 expression.

Patient samples were harvested daily between 2.5Gy morning and afternoon treatments, with a baseline sample harvested as described in Figure 10. Here the expression of the indicated mRNA was normalized to KRT14, and miRNAs are normalized to both miR-103 and then expressed relative KRT14. Individual points represent the mean (+/- SEM) of a minimum of 2 technical replicates.


a
MDM2
Relative expression (fold change)
Day
01-015
01-016
b
CDKN1A
Relative expression (fold change)
Day
01-015
01-016
01-017
c
miR-34a-5p
Relative expression (fold change)
Day
01-015
01-016
01-017
d
miR-143-3p
Relative expression (fold change)
Day
01-015
01-016
01-017
e
miR-145-5p
Relative expression (fold change)
Day
01-015
01-016
01-017

regulated miRNAs in patient 01-015 appears to be transient, and is slower to develop in patient 01-016 (Figure 13c-e). These results are consistent with a small p53 response generated from a subset of KRT14+ cells. However, levels of mRNA and miRNA in patient 01-017 continued to decrease throughout treatment compared to baseline, and a lack of amplification of KRT14 in the samples of patient 01-018 made it impossible to assess changes upon KRT14 normalization. It is clear that, although activation of p53 and induction of p53-regulated mRNAs is likely occurring in the buccal mucosa of these patients in response to TBI it is incredibly difficult to demonstrate reliably. This is likely because of radiation induced decreases in the KRT14+ population coupled with the higher accessibility of KRT13+ cells. This difficulty is compounded by incredibly low RNA yield from buccal swab RNA isolation which made it difficult to detect measurable amplification of qPCR products.

3.2.2 AML patient blood sample cell heterogeneity obscures the p53 response, but can be alleviated by normalization to TP53.

As it was important to analyze the effects of TBI on diseased cells as well as normal tissues to form a full picture of the human body's response, blood samples were also harvested from phase IV AML patients. These samples were taken from the same patients who provided the buccal swabs, and were taken at roughly the same time, as described above. The blood samples were analyzed based on morphology to provide the information in Table 2. Blood samples were then separated with Ficoll-Pacque®, and the PBMCs were harvested and frozen prior to analysis. The PBMC fraction of blood contains predominantly lymphocytes, monocytes, and circulating blast cells, although the

Patient	Day	WBC	Blasts	%Blasts	Lymphocytes	%Lymphocytes
01-015	Baseline	50	41.2	82.4	3.5	7.0
	1	17.2	13.2	76.7	2.1	12.2
	2	6.9	3.7	53.6	0.8	12.8
	3	2.3	0.7	30.4	0.1	4.3
	4	1	0.2	20.0	0.1	10.0
01-016	Baseline	2.3	1.4	60.9	0.8	34.8
	1	5.8	3.5	60.3	2.1	36.2
	2	1.9	1	52.6	0.7	36.8
	3	1.9	1	52.6	0.9	47.4
	4	1.3	1.1	84.6	0.2	15.4
01-017	Baseline	7.1	1.8	25.4	0.7	9.9
	1	9.4	2.9	30.9	0.6	6.4
	2	3.2	0.4	12.5	0.5	15.6
	3	1.4	0.2	14.3	0.1	7.1
	4	0.8	0.04	5.0	0.02	2.5
01-018	Baseline	5.2	3.5	67.3	-	-
	1	33.3	-	-	-	-
	2	49.2	44.9	91.3	4.3	8.7
	3	39.3	-	-	-	-
	4	30.0	26.7	89.0	3.0	10.0

Table 2: Phase IV AML patient blood sample cell counts.

Note that there are almost always more blast cells than lymphocytes. %Blasts and %lymphocytes indicate the percentage of overall white blood cells that belong to that category. WBC: white blood cells.

process of harvesting the PBMCs can lead to contamination with platelets. Total RNA was isolated from patient blood samples as described in the methods. Patient samples were analyzed by qRT-PCR for p53-regulated mRNA and miRNA expression, as with the buccal swab samples.

Analysis of patient blood prior to Ficoll separation based on cell morphology produced the blood cell counts seen in Table 2. Interestingly, there are almost always more blast cells in the patient blood samples than lymphocytes. Also, based purely on the decline in the blast cell population throughout treatment, it appears that both patient 01-015 and 01-017 responded favorably to treatment (Table 2). However, patient 01-015 responded best clinically to TBI treatment out of the four patients tested in this project and was the only patient who was disease free at follow up.

Similarly to the buccal mucosa, blood is a heterogenous mixture of a variety of blood cell types, as discussed earlier. These blood cells can be differentiated based on cluster of differentiation (CD) markers, also referred to as cell differentiation markers (reviewed in Lai *et al.*, 1998). These markers are expressed in specific cell types and are therefore highly useful in identifying cells, and were therefore used here to identify relative proportions of cells in the patient blood samples, similar to the use of cytokeratins when analyzing buccal swabs. CD3 is expressed in T cells, CD19 is expressed in B cells, and CD41 is expressed in platelets. CD34 is commonly used to identify stem cells in blood, and is most often found in bone marrow, but the AML patients who provided samples for this project were all tested and express CD34 in their blast cells. Therefore, CD34 mRNA was used to estimate changes in the proportion of blast cells in the patient blood samples.

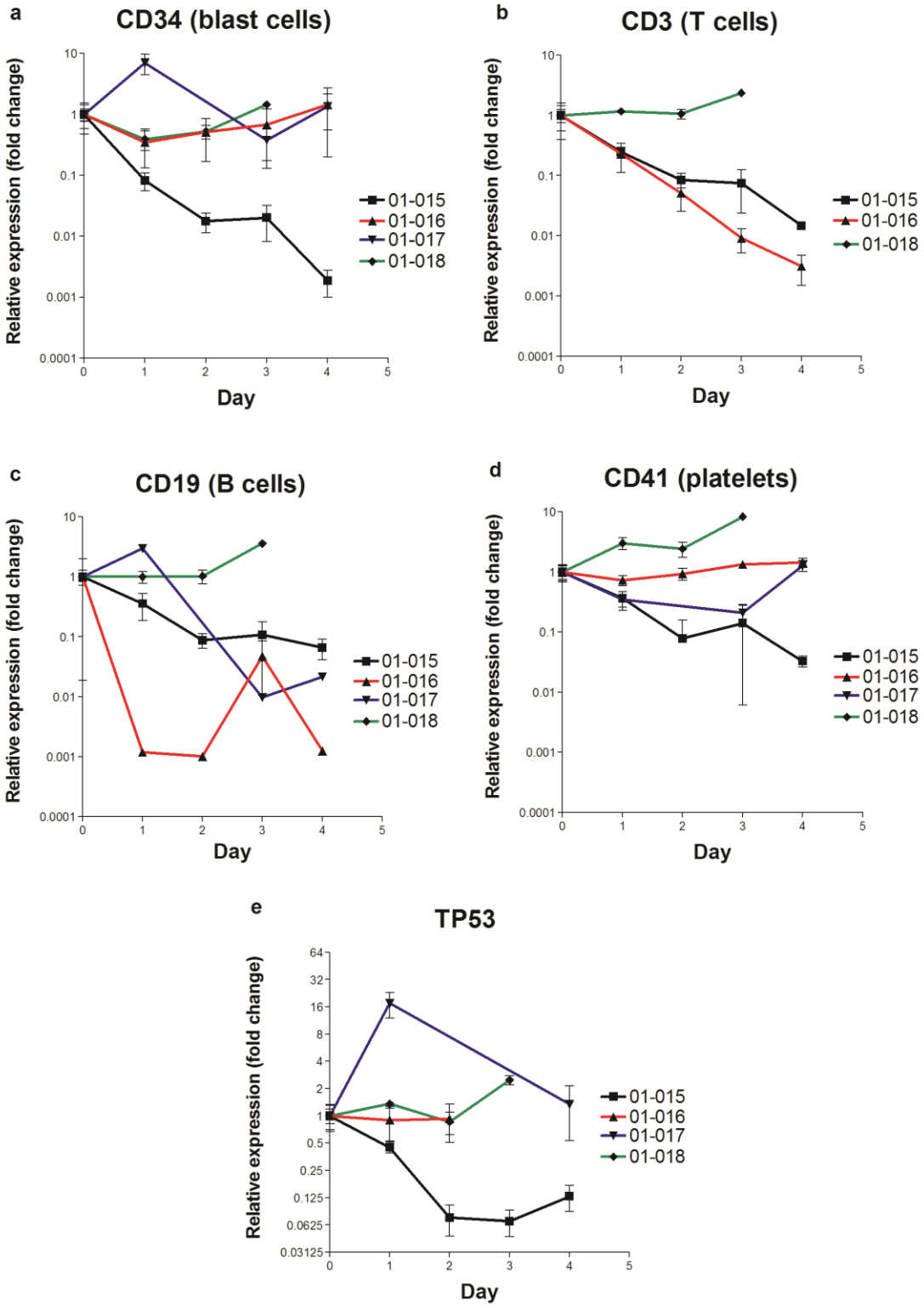
Expression of CD34 decreased 1000-fold by day 4 in patient 01-015 (Figure 14a), whereas CD34 levels increased transiently in patient 01-017 samples, and remained stable throughout treatment in patients 01-016 and 01-018. This outcome is consistent with patient 01-015 responding the best to treatment, although the transient increase in CD34 expression in patient 01-017 is at odds with the observed blast cell count in Table 2. There was no significant change in CD34 expression in patients 01-016 or 01-018, consistent with their blast counts. Together, CD34 expression correlated well with blast counts and therapeutic response in 3 of 4 patients while CD34 levels did not correlate in patient 01-017.

CD3 (T-cell marker) levels decreased throughout treatment similarly in patients 01-015 and 01-016, but remained very stable in patient 01-018 and was not amplified in patient 01-017 (Figure 14b). Similarly, CD19 (B-cell marker) expression decreased through treatment for all patients except 01-018 whose expression remained stable (Figure 14c). CD41 (platelet marker) expression differed for all patients throughout treatment (Figure 14d). CD41 expression increased in patient 01-018, but decreased in patient 01-015. Taken together, all cell types were among the most sensitive in patient 01-015 while all cell types appeared to be radiation-resistant in patient 01-018.

TP53 levels were also analyzed to determine if p53 mRNA expression prediced responses in patients. Its expression was remarkably similar to CD34 expression, suggesting that p53 was expressed predominantly in these CD34+ cells (Figure 14a, e). This may reflect a similar predominance for the p53 response to exist primarily in proliferative cells, much like we found in the buccal mucosa. The changes in multiple CD marker expressions throughout treatment are consistent with the clinical outcomes of the

Figure 14: CD marker and TP53 mRNA expression of patient blood samples during TBI treatment.

Patient samples were harvested daily between 2.5Gy morning and afternoon treatments, with a baseline sample harvested as described in Figure 10. The expression of the indicated mRNA was normalized to GAPDH. Individual points represent the mean (+/- SEM) of a minimum of 2 technical replicates.


a
CD34 (blast cells)
Relative expression (fold change)
Day
01-015
01-016
01-017
01-018
b
CD3 (T cells)
Relative expression (fold change)
Day
01-015
01-016
01-018
c
CD19 (B cells)
Relative expression (fold change)
Day
01-015
01-016
01-017
01-018
d
CD41 (platelets)
Relative expression (fold change)
Day
01-015
01-016
01-017
01-018
e
TP53
Relative expression (fold change)
Day
01-015
01-016
01-017
01-018

patients. Although the levels of CD3 and CD19 expression decreased during treatment in multiple patients, CD34 expression decreased solely in the samples taken from patient 01-015, who had the only complete remission(Figure 14a-c).

Expression of a variety of p53-regulated mRNAs was measured using the same patient RNA samples (Figure 15). Only CDKN1A increased consistently during treatment. This was apparent in all patient samples except 01-016 (Figure 15b). Changes in MDM2 and BTG2 varied more among patients (Figure 15a, c). Nonetheless, MDM2 levels appeared to increase in the same patients with induced CDKN1A (Figure 15a). Also, patient 01-016 was unique in that there was no evidence of increased expression of any of these p53-regulated transcripts.

The blood samples of patient 01-017 typically presented very similar patterns of mRNA expression among most of the mRNAs assessed. This pattern consisted of substantially increased expression on day 1, which then decreased slightly by day 3, and increased again on day 4 (Figure 14a, c, e, Figure 15a-c). This, along with the similarity of expression patterns of TP53 and CD34 mRNAs, lead us to believe that changes in the proportion of specific cells could be affecting our data, similar to the buccal mucosa cells. Samples were subsequently normalized to TP53 expression. Upon normalization of mRNAs to TP53 levels, a far more robust increase in p53-regulated mRNAs was seen in all patients, with the exception of patient 01-016 (Figure 16). Patient 01-015 clearly showed the largest increase in p53-regulated mRNAs during treatment, which is consistent with their clinical outcome, having responded best to treatment of all four patients.

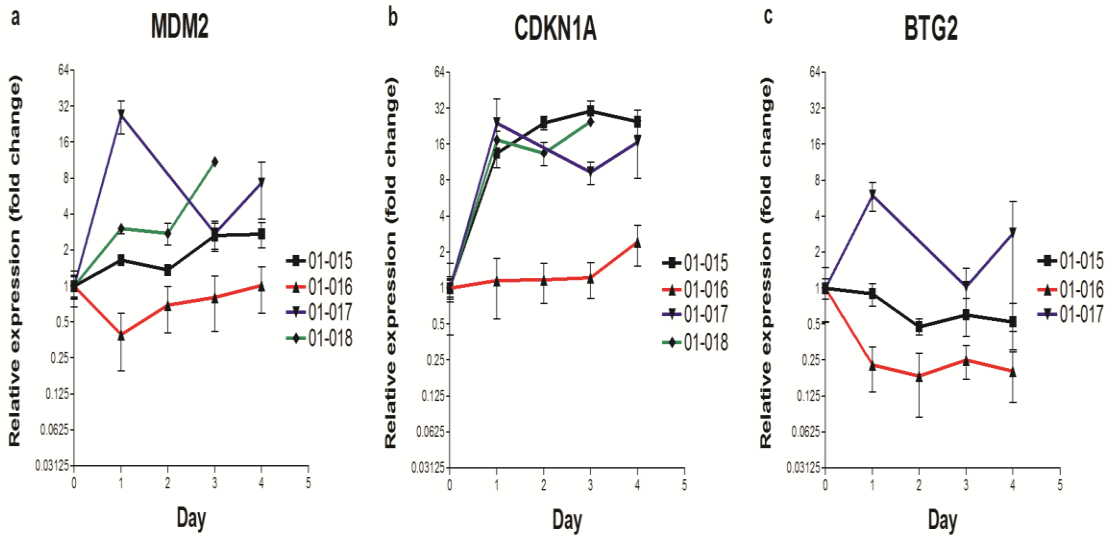


Figure 15: mRNA expression in patient blood samples during TBI treatment. Patient samples were harvested daily between 2.5Gy morning and afternoon treatments, with a baseline sample harvested as described in Figure 10. The expression of the indicated mRNA was normalized to GAPDH. Individual points represent the mean (+/- SEM) of a minimum of 2 technical replicates.

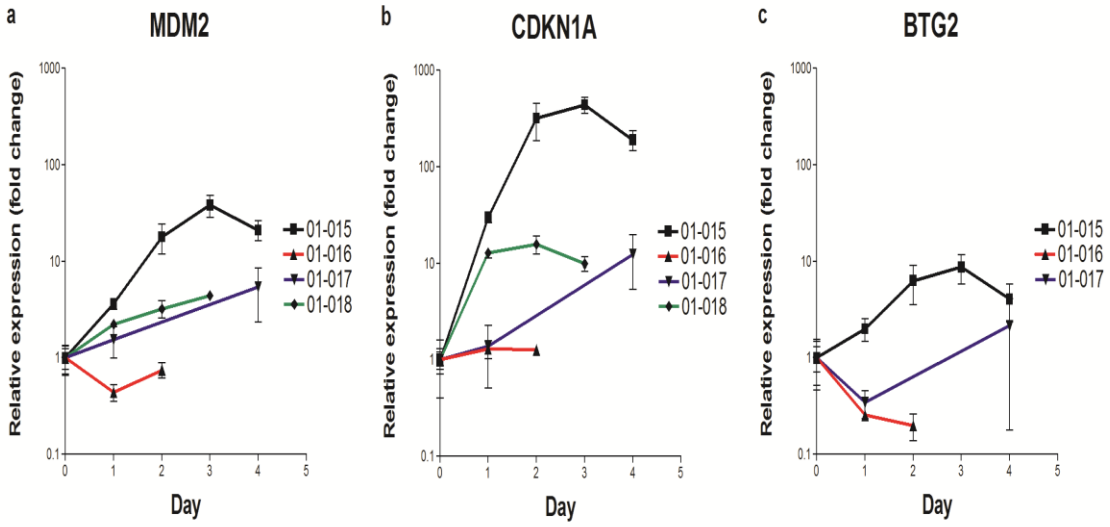


Figure 16: mRNA expression in patient blood samples during TBI treatment normalized to TP53.

Patient samples were harvested daily between 2.5Gy morning and afternoon treatments, with a baseline sample harvested as described in Figure 10. The expression of the indicated mRNA was expressed relative to TP53 expression. Individual points represent the mean (+/- SEM) of a minimum of 2 technical replicates.

p53-responsive miRNAs were also analyzed (Figure 17). miRNA levels did not change much throughout treatment, except in patient 01-015. All patients showed minor miR-34a-5p induction up until day 4, whereas miR-34a-5p levels in patients 01-015 and 01-016 increase further (Figure 17a). miR-143-3p levels increased during treatment in patients 01-015 and 01-018, but decreased in patients 01-016 and 01-017 (Figure 17b), and miR-145-5p levels only increased in patient 01-015 during treatment (Figure 17c). When normalized to TP53, the expression of all three p53-regulated miRNAs increased in samples obtained from patient 01-015 (Figure 18). miRNA levels in patients 01-016 and 01-018 were relatively stable during treatment when normalized to TP53, while miRNAs in patient 01-017 decreased transiently before returning to baseline levels. miRNA induction only appearing in samples from patient 01-015 when normalized to TP53 is consistent with the clinical outcome of the patients, where only patient 01-015 responded favorably. Taken together, there is a clear p53 response in these clinical samples when the loss of p53 expressing cell populations is taken into account. Also, the p53 response correlates with favorable therapeutic outcomes and may distinguish between favorable outcomes better than direct cell counts (i.e. patients 01-015 and 01-017).

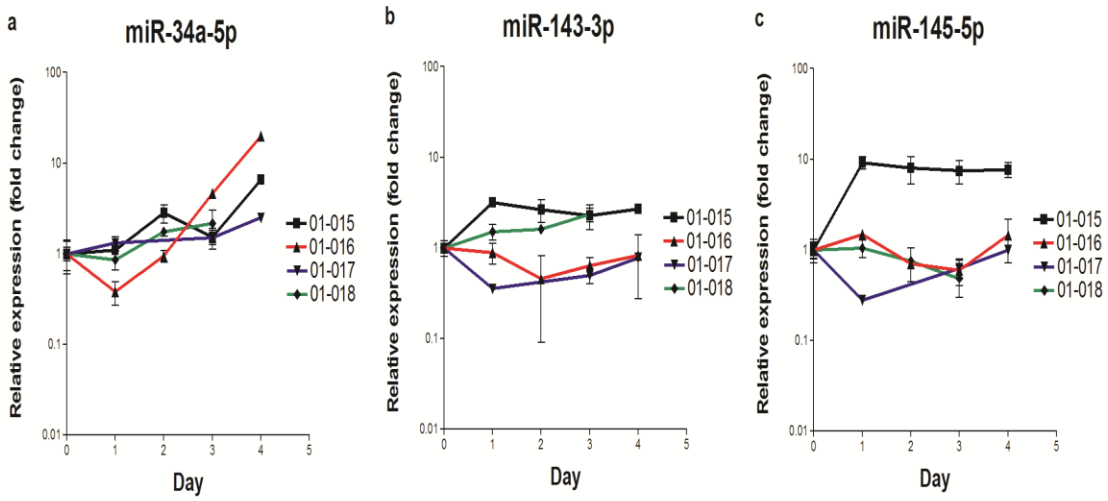


Figure 17: miRNA expression in patient blood samples during TBI treatment. Patient samples were harvested daily between 2.5Gy morning and afternoon treatments, with a baseline sample harvested as described in Figure 10. The expression of the indicated miRNA was normalized to miR-103. Individual points represent the mean (+/- SEM) of a minimum of 2 technical replicates.

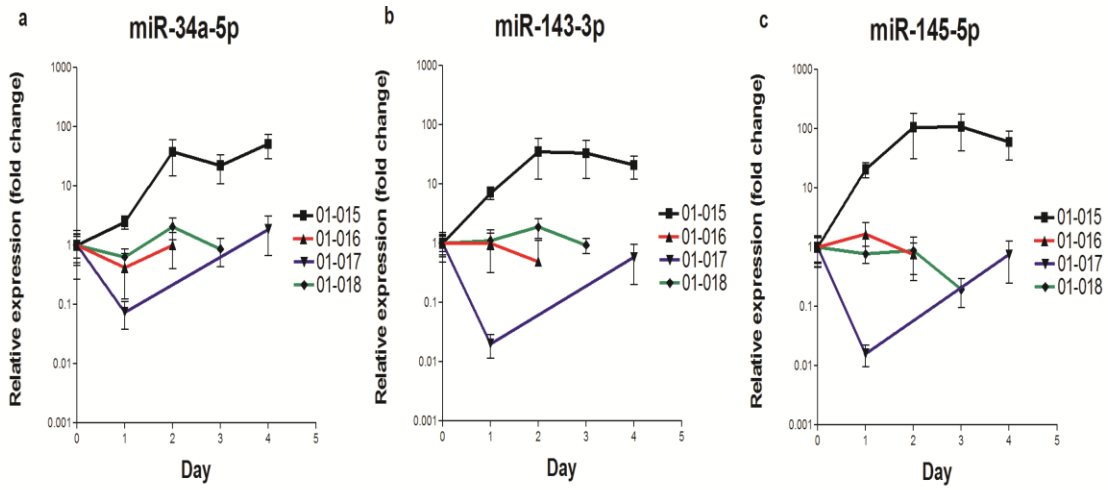


Figure 18: miRNA expression in patient blood samples during TBI treatment normalized to TP53.

Patient samples were harvested daily between 2.5Gy morning and afternoon treatments, with a baseline sample harvested as described in Figure 10. The expression of the indicated miRNA was normalized to miR-103 and expressed relative to TP53 expression. Individual points represent the mean (+/- SEM) of a minimum of 2 technical replicates.

4. Discussion:

4.1 HCT116 cells

4.1.1 The p53 response elicited by Nutlin-3a treatment is reversible in HCT116 cells.

It was previously shown in our lab that HT29-tsp53 cells, expressing a temperature sensitive variant of p53, could be used to modulate p53 activity in a reversible manner. Cells placed at the permissive temperature elicited a typical p53 response. A rapid attenuation of p53 transcriptional activity was observed when cells were returned to the restrictive temperature and this was associated with the rapid decay of over 70% of the induced mRNAs (Melanson *et al.,* 2011). Using the same experimental design, it was later reported that miRNAs induced by p53 remain remarkably stable compared to mRNAs (Cabrita *et al.,* data unpublished). When cells were transferred to the restrictive temperature a second time, the p53 response was not identical (Cabrita *et al.,* data unpublished). At least 2 mRNAs, MDM2 and BTG2, were not induced to the same extent while the p53-regulated miRNAs remained elevated. The 3'UTR MDM2 and BTG2 mRNAs contained sequences corresponding to several p53-regulated miRNA binding sites. These results led us to propose that the attenuation of MDM2 and BTG2 during the second p53 response was a result of the highly stable, elevated levels of miRNAs which had been transcribed during the first response. These miRNAs left a legacy of activation to essentially "prime" cells to respond differently to further stresses. One could speculate that this alteration in the p53 response could affect the outcome of p53 in chronic or fractionated settings. These findings spawned this project.

We sought to test this model in a different cell system, independent of temperature change. It was necessary to determine if Nutlin-3a-dependent p53 activation was reversible upon removal of the drug. Nutlin-3a led to increased p53 protein and this was reversed within 1h following washout without affecting p53 mRNA levels (Figure 6a,b). Nutlin-3a also led to increased expression of the p53-regulated proteins, MDM2 and p21, and this was again reversible but with delayed kinetics despite the rapid loss of MDM2 and CDKN1A (encoding p21) mRNA expression. Although the speed at which these transcripts decreased was slower than what was seen by Melanson *et al.* in HT29-tsp53 cells, all of the p53-responsive transcripts tested had decreased to ≤25% of the induced level within 6 hours of the removal of Nutlin-3a (Melanson *et al.,* 2011). These results indicate that the Nutlin-3a-mediated p53 response was reversible and this work confirms the findings of Melanson *et al.* that the p53 response decays rapidly following the loss of p53 activity.

It would make thematic sense for important p53-responsive mRNAs to be unstable, as the proteins encoded by these transcripts can generate major changes in the outcome of the cell, and therefore need to be tightly regulated to ensure the provoked p53 response does not get out of control. As an example, CDKN1A/p21 activation is well known to induce a G_1 cell cycle arrest (el-Deiry *et al.,* 1993; Deng *et al.,* 1995; Waldman *et al.,* 1995; reviewed in Meek, 2015). MDM2 up-regulation is well known as an inhibitor of the overall p53 response, and is integral for cell survival (Jones *et al.,* 1995; Montes de Oca Luna *et al.,* 1995; reviewed in Meek, 2015). Evidently, proteins with such extreme impacts on the overall state of the cell need to be kept in check. Beyond the extensive protein feedback loops involved in maintaining appropriate activity of these

proteins and p53 itself, as well as their associated mRNAs, Melanson *et al*., and now we, have suggested that further regulation of the p53 response comes in the form of highly unstable p53-responsive mRNAs (Melanson *et al.,* 2011).

4.1.2 HCT116 cells do not respond to consecutive p53 responses with attenuation of p53-responsive mRNAs.

In previous results from our laboratory using a temperature sensitive variant of p53 to study consecutive p53 responses separated by a recovery period, we found that there was the selective attenuation of BTG2 and MDM2 induction during the second p53 response (Cabrita *et al.,* unpublished data). This was attributed to miRNA-mediated feedback involving miR-34a-5p, miR-143-3p and miR-145-5p (Cabrita *et al.,* unpublished data). In conceptually similar experiments using Nutlin-3a treatment to induce two p53 responses separated by a period of recovery, p53-responsive transcripts increased similarly with each challenge. The similarity in consecutive p53 responses was also observed at the protein level, p53, Mdm2, and p21 protein levels increased similarly in both the first and second p53 responses (Figure 8).

In addition, p53-responsive miRNA levels changed very little during Nutlin-3a treatments in HCT116 cells. The similarity of the consecutive p53 responses and the absence of a pronounced p53-miRNA response is in contrast to what was seen previously in our lab in HT29-tsp53 cells, where p53-responsive miRNAs increased substantially in response to incubation at the permissive temperature (Cabrita *et al.,* 2016; Cabrita *et al.,* unpublished data). If the attenuation of mRNAs seen previously was a result of p53-responsive miRNA feedback, then the lack of miRNA induction found here in HCT116 cells would explain the absence of any statistically significant attenuation in the p53-

responsive mRNA levels. The lack of miRNA induction found here may also contribute to the discrepancy in the stability of p53-regulated mRNAs detected by Melanson *et al.* and ourselves. miRNA expression affects mRNA stability so it is possible that the instability of p53-induced mRNAs in our previous work is at least in part dependent on miRNA expression. Thus the lack of p53-regulated miRNA induction found in HCT116 cells in response to Nutlin-3a treatment could result in an increase in stability of the target mRNAs in this model system.

It is possible that, if we had achieved comparable induction of p53-responsive miRNAs then we may have detected similar attenuation of p53-dependent MDM2 and BTG2 expression. However, in order to achieve similar increases in these miRNAs using Nutlin-3a, the cells would have to be treated with Nutlin for upwards of 24h (Kumamoto *et al.,* 2008). This would pose a challenge to perform meaningful experiments because sustained p53 and miR-34a-5p expression would likely lead to irreversible changes in these cells, including senescence (Kumamoto *et al.,* 2008), as this p53 response would no longer be reversible. Therefore, we were unable to fully test the miRNA feedback model.

It was particularly interesting that there appeared to be little difference in mRNA or miRNA responses to different concentrations of Nutlin-3a. This would suggest that the cells are saturated even at the lowest Nutlin-3a concentrations used here. However, other evidence suggests that varying concentrations of Nutlin-3a in HCT116 cells can alter Mdm2 and p21 protein levels and CDKN1A mRNA induction (Vassilev *et al.,* 2004; Carvajal *et al.,* 2005). Vassilev *et al.* provided immunoblot evidence of dose-dependent increases in protein levels of MDM2 and p21 in response to 8h Nutlin-3a treatment in HCT116 cells, with concentrations of Nutlin-3a between 0-8μM (Vassilev *et al.,* 2004).

They also demonstrated similar results for p21 mRNA using qRT-PCR, with Nutlin-3a concentrations between 0-18μM. Carvajal *et al.* also provided qRT-PCR evidence of dose-dependent increases in p21 mRNA after 24h of Nutlin-3a treatment using concentrations between 0-10μM. These other results involved longer treatment periods, but the source of this difference remains unclear.

4.2 AML patient samples

4.2.1 Changes in cell population during treatment occlude the p53 response in buccal epithelium cells.

Acute myeloid leukemia (AML) is a subcategory of blood-related cancers (i.e. leukemias) which is characterized by neoplastic progenitor cells (blast cells) descended from the myeloid lineage of hematopoiesis (reviewed in Vardiman *et al.,* 2008; Dohner *et al.,* 2010; Dombret and Gardin, 2016). AML patients are commonly treated with chemotherapeutics such as anthracyclines which cause DNA damage, and this is often effective in AML patients because of the low incidence of p53 mutation seen in AML cases (Hu *et al.,* 1992; Nakano *et al.,* 2000). However, when necessary, patients can undergo allogenic stem cell transplants in an effort to replace their diseased blood cells with healthy cells generated from the healthy stem cells. The patients included in this study were phase IV AML patients who underwent total body irradiation (TBI) with ionizing radiation prior to receiving an allogenic stem cell transplant. The TBI treatment was intended to ablate the blast cells in the blood of the patients to give them the best chance at repopulating their blood with donor cells. The radiotherapy was conducted over multiple days, with treatments of 2.5Gy in the morning and afternoon, to a total of 18Gy.

Relatively little research has been conducted on the effects of total body irradiation in humans (Wang *et al.,* 2015). Samples from these patients provided an excellent resource to investigate the effects of irradiation on both normal and diseased tissues of the human body. To this end, buccal epithelial cells and blood samples were analyzed for expression of p53 and p53-regulated mRNAs and miRNAs in response to ionizing radiation-induced DNA double-strand breaks.

It was assumed that the epithelial cells contained in the buccal swabs obtained from patients would display a typical p53 response because the buccal epithelium in these patients represents normal, non-diseased tissue. However, analysis of p53-responsive mRNAs and miRNAs in the patient buccal swabs did not increase as anticipated. It was proposed that the buccal swabs could be sampling cells from multiple cell layers, each of which would express different levels of p53 and/or could be differentially susceptible to p53 activation in response to DNA double strand breaks induced by ionizing radiation.

The buccal mucosa is a form of stratified squamous epithelium referred to as non-keratinized epithelium (reviewed in Squier and Kremer, 2001; Presland and Jurevic, 2002). The buccal mucosa itself is comprised of the basement membrane, the basal layer, and the suprabasal layers (Figure 19). The basal layer is attached to the basement membrane, and is comprised of a layer of cuboidal or columnar keratinocytes which are mitotically active (reviewed in Squier and Kremer, 2001; Presland and Jurevic, 2002). This proliferative layer is necessary to replenish cells that are removed from the most superficial layer of the epithelium, although proliferation can be stimulated in more superficial layers in response to injury and disease by inflammatory cytokines or growth factors. Following mitosis of progenitor cells, daughters can either continue to proliferate

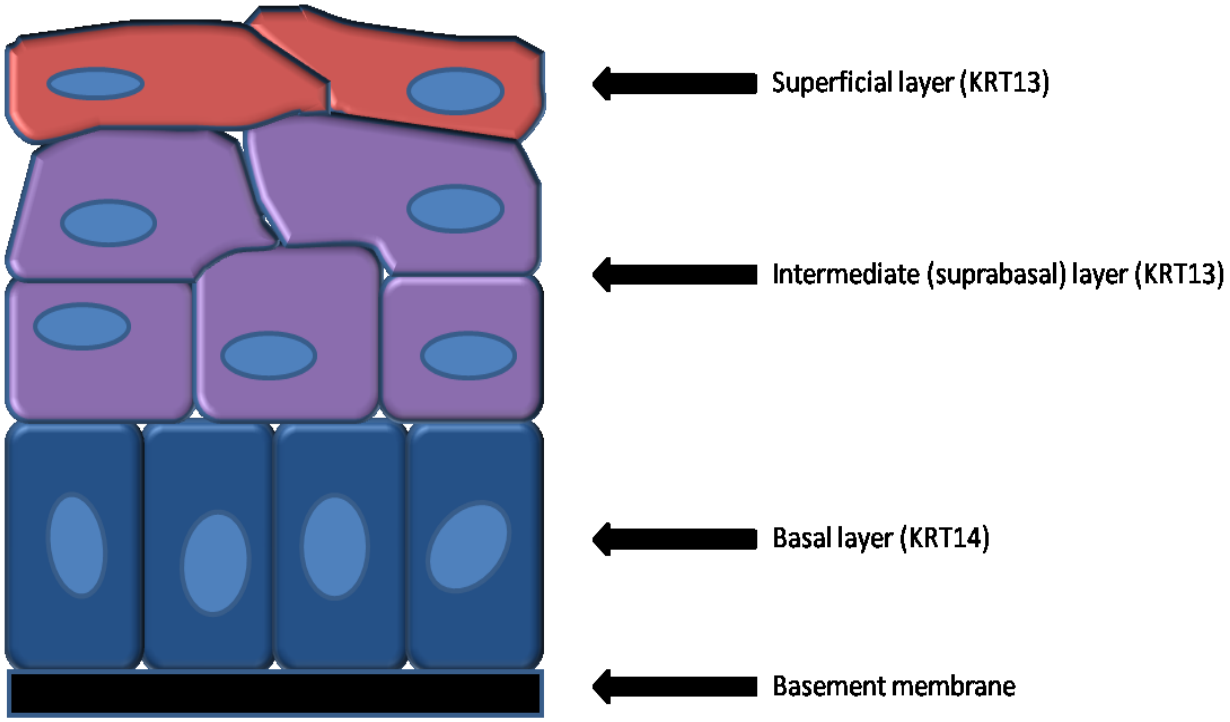


Figure 19: Simple depiction of the buccal mucosa.

The buccal mucosa is an example of a non-keratinized epithelium. The basement membrane is formed of a fibrous connective tissue, to which the basal layer is attached. Cells in the basal layer are proliferative, and daughter cells either maintain the basal layer population, or proceed through differentiation in the suprabasal layers. Differentiation involves a gradual enlargement and flattening of cells (not as pronounced as in keratinized epithelium), and alterations in the expression of proteins such as cytokeratins. Fatty granules referred to as cored granules eventually aggregate to the apical aspect of the superficial layer, forming an intercellular permeability barrier. Cytokeratins 5 and 14 are expressed in the basal layer, whereas those that are undergoing differentiation express cytokeratins 4 and 13. Modified from Squier and Kremer, 2001; Presland and Jurevic, 2002.

in the basal layer, or begin maturing by entering the suprabasal region. During this time, the differentiating cells alter their pattern of cytokeratin production. The population from which keratinocytes are collected can be identified by the particular expression patterns of cytokeratins (reviewed in Squier and Kremer, 2001; Presland and Jurevic, 2002). Cells in the proliferative basal layer express cytokeratins 5 and 14, whereas maturing cells in non-keratinized epithelium express keratins 4 and 13.

KRT13 and KRT14 mRNA were analyzed in the patient buccal swabs to identify the relative proportions of cells that were being harvested, and p53 mRNA levels were assessed to determine the effect of population changes on overall p53 levels. Not surprisingly, KRT13 and KRT14 expression in the patient buccal swabs displayed a roughly inverse relationship. What was surprising was that the p53 expression in patient 01-015 buccal swabs closely follows the expression of KRT14 (Figure 12a, c). There is evidence that KRT14 expression is important for proliferation of cells in the buccal mucosa, and the p53 response may be activated more readily in proliferating cells. It is also intriguing that KRT14 expression initially decreased in patient 01-015 and 01-016, but then subsequently increased (Figure 12c). The initial decrease in the proportion of KRT14+ cells can be attributed to the death of the proliferative cell population in response to the ionizing radiation and the associated p53-dependent apoptotic response. This would lead to a relative increase in KRT13+ cells due to changes in the relative proportion of cells in the buccal epithelium. However, the eventual increase in KRT14 expression by day 3 and 4 of treatment may be a result of KRT14 expression emerging in the suprabasal layers of the buccal mucosa in response to trauma or mucositis, which is a

common result of head and neck irradiation (Bonan *et al.,* 2006; Nobusawa *et al.,* 2014). This spread of KRT14 expression and associated proliferative status would likely explain the eventual increase in p53 expression, as well (Figure 12a). Alternatively, changes in the sampled population could simply be a result of differences in the harvesting methodology, i.e. differences in how hard the nurses swabbed the patients, particularly when the buccal epithelium becomes inflamed and sensitive.

We hypothesized that the KRT14+ cells would be most sensitive to irradiation and most likely to respond to radiation by eliciting a p53 response. When the p53-responsive mRNAs and miRNAs were normalized to KRT14 levels in the patient buccal swabs to adjust for changes in these cell populations, a small p53 response in patients 01-015 and 01-016 was apparent. Surprisingly, patient 01-017 continued to show a complete absence of p53 activation. It is possible that this result reflects prior activation of the p53 response by therapies received prior to the transplant conditioning protocol, as patients have received a variety of therapies prior to their inclusion in the study. Alternatively, the swabs contained primarily KRT13+ cells and this dominated the qRT-PCR signal.

Based on our preliminary analysis, it is clear that buccal swabs are unlikely to present a clear view of the response of normal human tissue to total body ionizing radiation. The differences in response of a heterogenous cell population, coupled with the extremely low sample size and resultant RNA yield, simply were not conducive to extracting statistically sound information.

4.2.2 Cell differentiation marker expression presents a higher resolution image of treatment progression than morphological analysis

It is generally accepted by those in the field of hematology and immunology that cell differentiation markers can be used to identify cell types in the blood (reviewed in Lai *et al.*, 1998). However, primary analysis of abnormal blood samples often still consists of morphological identification, as it is an extremely low-cost method, and more reliably identifies abnormal blood cells than automated counting. If a person were to look only at the blood counts presented in Table 2, which are based on cell morphology, it would appear that both patients 01-015 and 01-017 responded favorably to treatment, as they both display significant declines in the blast population (Table 2). However, the actual clinical outcome of the patients does not agree with this assessment, as it was identified after treatment that only patient 01-015 responded favorably. In contrast, the clinical outcomes of the patients do match the CD marker expression patterns seen in Figure 14, where only patient 01-015 saw a substantial decrease in CD34 expression.

It is interesting that the radiation-induced changes in the expression of TP53 in the patient blood samples are similar to those of CD34 expression. Analysis of CD markers show decreased expression of CD3 and CD19 in patients, with the exception of patient 01-018. This would indicate decreases in the proportions of CD3+ and CD19+ cells in the blood samples. There was no compensatory increase in any of the CD markers examined, so it is unclear which cell population gives rise to the bulk of the remaining mRNA in these CD34- CD3- and CD19-depleted samples. It is possible that monocytes, which are also purified in the PBMC fraction of Ficoll, contribute mRNA in these depleted samples, but that remains to be tested. Nonetheless, the similarity of the TP53

expression to CD34 would suggest that whatever cell type is increasing in proportion in these samples does not provide substantial p53 activity beyond what is being contributed by the CD34+, CD3+, CD19+, and CD41+ cells.

4.2.3 The p53 response in human blood samples in response to radiotherapy can be revealed by normalization to p53 mRNA

Initial analysis of the p53-responsive mRNAs demonstrated p53 responses in all patients except for patient 01-016. However, as we had already determined that p53 levels varied during treatment in response to changes in the cell population, we normalized the p53-regulated mRNAs to p53 mRNA expression in hopes of extracting the 'real' p53 response in these samples. This revealed a far more robust induction of p53-regulated mRNA in patient 01-015, which is consistent with their clinical outcome (Figure 16). However, normalization to p53 levels did not increase the apparent response in patients 01-017 or 01-018 and decreased the response in patient 01-016.

Similarly, p53-responsive miRNAs showed a relatively small p53 response to radiotherapy, with the exception of patient 01-015. However, normalization to p53 mRNA levels magnified this difference, increasing the apparent size of the p53 response in patient 01-015, and diminishing the response in all other patients. As p53 normalization distinguished patient 01-015 as the only patient to display an induction of p53-responsive miRNAs, and patient 01-015 displayed the only favorable clinical outcome, p53 mRNA levels coupled with analysis of p53-responsive mRNAs and miRNAs may be useful markers of therapeutic efficacy.

Patient	Outcome	Clinical Response		p53 Response			
				Buccal Swabs		Blood Samples	
		Cell Count	CD Marker	mRNA	miRNA	mRNA	miRNA
01-015	+	+	+	+/-	+/-	+	+
01-016	-	-	-	+/-	+/-	-	-
01-017	-	+	-	-	-	+	-
01-018	-	-	-	N/A	N/A	+	-

Table 3: Compiled patient sample outcomes.

A table comparing the results from all analyses of the patient samples to the final outcome of each patient. Outcome refers to the final outcome of the patient: although patients 01-015 and 01-017 both responded favorably to treatment, patient 01-017 relapsed, and so only patient 01-015 had a positive final outcome. Clinical response refers to the predicted patient outcome by morphological analysis (cell count) and CD marker analysis. Results for the buccal swabs are regarding the KRT14-normalized data, and results for the blood samples are in reference to the TP53-normalized data. + and - denote positive and negative results, respectively. +/- denotes a minor, non-statistically significant result. N/A denotes results which were unavailable.

Conclusions

The response of cells to consecutive p53 responses was assessed in cultured cells as well as biological samples from patients receiving total body irradiation as part of the conditioning regiment in preparation for bone marrow transplantation. In the *in* vitro portion of the presented work, we confirmed that the p53 response is rapidly reversed due to rapid mRNA decay. However, we did not detect any apparent miRNA-mediated attenuation of p53-regulated mRNAs in response to consecutive p53 responses induced by treatment with Nutlin-3a in HCT116 cells. The lack of p53-induced miRNA induction likely explains this discrepancy from our previous work. In patient samples, the p53 response could not be reliably identified in patient buccal epithelium cells, likely because of heterogeneity in the sampled cell population and extremely low sample size and resultant RNA yield. A p53 response could be identified in blood samples harvested from patient 01-015, as defined by substantial increases in both p53-regulated mRNAs and miRNAs. The discovery that p53-regulated miRNAs only increased during treatment in patient 01-015, who responded most favorably to treatment, warrants further investigation to determine if p53 mRNA expression coupled with p53-regulated mRNA and miRNA induction may be a potential marker for treatment progression.

References

Aloni-Grinstein R, Shetzer Y, Kaufman T, Rotter V. (2014) p53: the barrier to cancer stem cell formation. *FEBS Lett.* **588**(16): 2580-9.

Andreeff M, Kelly KR, Yee K, Assouline S, Strair R, Popplewell L, Bowen D, Martinelli G, Drummond MW, Vyas P, Kirschbaum M, Iyer SP, Ruvolo V, Gonzalez GM, Huang X, Chen G, Graves B, Blotner S, Bridge P, Jukofsky L, Middleton S, Reckner M, Rueger R, Zhi J, Nichols G, Kojima K. (2016) Results of the phase I trial of RG7112, a small-molecule MDM2 antagonist in leukemia. *Clin Cancer Res.* **22**(4): 868-76.

Avantaggiati ML, Ogryzko V, Gardner K, Giordano A, Levine AS, Kelly K. (1997) Recruitment of p300/CBP in p53-dependent signal pathways. *Cell* **89**(7): 1175-84.

Ayed A, Mulder FA, Yi GS, Lu Y, Kay LE, Arrowsmith CH. (2001) Latent and active p53 are identical in conformation. *Nat Struct Biol.* **8**(9): 756-60.

Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, vanTuinen P, Ledbetter DH, Barker DF, Nakamura Y, White R, Vogelstein B. (1989) Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* **244**(4901): 217-21.

Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. (1990) Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* **249**(4971): 912-5.

Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y, Ziv Y. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* **281**(5383): 1674-7.

Baptiste N, Friedlander P, Chen X, Prives C. (2002) The proline-rich domain of p53 is required for cooperation with anti-neoplastic agents to promote apoptosis of tumor cells. *Oncogene* **21**(1): 9-21.

Bargonetti J, Friedman PN, Kern SE, Vogelstein B, Prives C. (1991) Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. *Cell* **65**(6): 1083-91.

Barlev NA, Liu L, Chehab NH, Mansfield K, Harris KG, Halazonetis TD, Berger SL. (2001) Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. *Mol Cell.* **8**(6): 1243-54.

Berger M, Vogt Sionov R, Levine AJ, Haupt Y. (2001) A role for the polyproline domain of p53 in its regulation by Mdm2. *J Biol Chem.* **276**(6): 3785-90.

Bonan PR, Kaminagakura E, Pires FR, Vargas PA, Almeida OP. (2006) Cytokeratin expression in initial oral mucositis of head and neck irradiated patients. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* **101**(2): 205-11.

Bond J, Haughton M, Blaydes J, Gire V, Wynford-Thomas D, Wyllie F. (1996) Evidence that transcriptional activation by p53 plays a direct role in the induction of cellular senescence. *Oncogene* **13**(10): 2097-104.

Böttger V, Böttger A, Garcia-Echeverria C, Ramos YF, van der Eb AJ, Jochemsen AG, Lane DP. (1999) Comparative study of the p53-mdm2 and p53-MDMX interfaces. *Oncogene* **18**(1): 189-99.

Brady CA, Jiang D, Mello SS, Johnson TM, Jarvis LA, Kozak MM, Kenzelmann Broz D, Basak S, Park EJ, McLaughlin ME, Karnezis AN, Attardi LD. (2011) Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell* **145**(4): 571-83.

Burch LR, Midgley CA, Currie RA, Lane DP, Hupp TR. (2000) Mdm2 binding to a conformationally sensitive domain on p53 can be modulated by RNA. *FEBS Lett.* **472**(1): 93-8.

Campbell HG, Mehta R, Neumann AA, Rubio C, Baird M, Slatter TL, Braithwaite AW. (2013) Activation of p53 following ionizing radiation, but not other stressors, is dependent on the proline-rich domain (PRD). *Oncogene* **32**(7): 827-36.

Canman CE, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB, Siliciano JD. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* **281**(5383): 1677-9.

Carvajal D, Tovar C, Yang H, Vu BT, Heimbrook DC, Vassilev LT. (2005) Activation of p53 by MDM2 antagonists ca protect proliferating cells from mitotic inhibitors. *Cancer Res.* **65**(5): 1918-24.

Chen X, Ko LJ, Jayaraman L, Prives C. (1996) p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev.* **10**(19): 2438-51.

Chen L, Agrawal S, Zhou W, Zhang R, Chen J. (1998) Synergistic activation of p53 by inhibition of MDM2 expression and DNA damage. *Proc Natl Acad Sci USA* **95**(1): 195-200.

Chen L and Chen J. (2003) Mdm2-ARF complex regulates p53 sumoylation. *Oncogene* **22**(34): 5348-57.

Chène P, Fuchs J, Bohn J, Garcia-Echeverria C, Furet P, Fabbro D. (2000) A small synthetic peptide, which inhibits the p53-hdm2 interaction, stimulates the p53 pathway in tumour cell lines. *J Mol Biol.* **299**(1): 245-53.

Chi SW, Lee SH, Kim DH, Ahn MJ, Kim JS, Woo JY, Torizawa T, Kainosho M, Han KH. (2005) Structural details on Mdm2-p53 interaction. *J Biol Chem.* **280**(46): 38795-802.

Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD, Green DR. (2005) PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. *Science* **309**(5741): 1732-5.

Chuikov S, Kurash JK, Wilson JR, Xiao B, Justin N, Ivanov GS, McKinney K, Tempst P, Prives C, Gamblin SJ, Barlev NA, Reinberg D. (2004) Regulation of p53 activity through lysine methylation. *Nature* **432**(7015): 353-60.

Clore GM, Omichinski JG, Sakaguchi K, Zambrano N, Sakamoto H, Appella E, Gronenborn AM. (1994) High-resolution structure of the oligomerization domain of p53 by multidimensional NMR. *Science* **265**(5170): 386-91.

Craig AL, Burch L, Vojtesek B, Mikutowska J, Thompson A, Hupp TR. (1999) Novel phosphorylation sites of human tumour suppressor protein p53 at Ser20 and Thr18 that disrupt the binding of mdm2 (mouse double minute 2) protein are modified in human cancers. *Biochem J.* **342**(Pt1): 133-41.

Deng C, Zhang P, Harper JW, Elledge SJ, Leder P. (1995) Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**(4): 675-84.

Diller L, Kassel J, Nelson CE, Gryka MA, Litwak G, Gebhardt M, Bressac B, Ozturk M, Baker SJ, Vogelstein B, Friend SH. (1990) p53 functions as a cell cycle control protein in osteosarcomas. *Mol Cell Biol.* **10**(11): 5772-81.

Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK, Dombret H, Fenaux P, Grimwade D, Larson RA, Lo-Coco F, Naoe T, Niederwieser D, Ossenkoppele GJ, Sanz MA, Sierra J, Tallman MS, Lowenberg B, Bloomfield CD; European LeukemiaNet. (2010) Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **115**(3): 453-74.

Dombret H, Gardin C. (2016) An update of current treatments for adult acute myeloid leukemia. *Blood* **127**(1): 53-61.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature* **356**(6366): 215-21.

Dumaz N and Meek DW. (1999) Serine 15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. *EMBO J.* **18**(24): 7002-10.

Dumaz N, Milne DM, Meek DW. (1999) Protein kinase CK1 is a p53-threonine 18 kinase which requires prior phosphorylation of serine 15. *FEBS Lett.* **463**(3): 312-6.

El-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. (1992) Definition of a consensus binding site for p53. *Nat Genet.* **1**(1): 45-9.

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**(4): 817-25.

Eliyahu D, Raz A, Gruss P, Givol D, Oren M. (1984) Participation of p53 cellular tumor antigen in transformation of normal embryonic cells. *Nature* **312**(5995): 646-9.

Eliyahu D, Goldfinger N, Pinhasi-Kimhi O, Shaulsky G, Skurnik Y, Arai N, Rotter V, Oren M. (1988) Meth A fibrosarcoma cells express two transforming mutant p53 species. *Oncogene* **3**(3): 313-21.

Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi-Kimhi O, Oren M. (1989) Wild-type p53 can inhibit oncogene-mediated focus formation. *Proc Natl Acad Sci USA* **86**(22): 8763-67.

Fields S and Jang SK. (1990) Presence of a potent transcription activating sequence in the p53 protein. *Science* **249**(4972): 1046-9.

Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M, Levine AJ. (1988) Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol Cell Biol.* **8**(2): 531-9.

Finlay CA, Hinds PW, Levine AJ. (1989) The p53 proto-oncogene can act as a suppressor of transformation. *Cell* **57**(7): 1083-93.

Fiscella M, Ullrich SJ, Zambrano N, Shields MT, Lin D, Lees-Miller SP, Anderson CW, Mercer WE, Appella E. (1993) Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression. *Oncogene* **8**(6): 1519-28.

Foord OS, Bhattacharya P, Reich Z, Rotter V. (1991) A DNA binding domain is contained in the C-terminus of wild-type p53 protein. *Nucleic Acids Res.* **19**(19): 5191-8.

Friedler A, Veprintsev DB, Freund SM, von Glos KI, Fersht AR. (2005) Modulation of binding of DNA to the C-terminal domain of p53 by acetylation. *Structure* **13**(4): 629-36.

Gannon JV, Lane DP. (1991) Protein synthesis required to anchor a mutant p53 protein which is temperature-sensitive for nuclear transport. *Nature* **349**(6312): 802-6.

Garcia PB and Attardi LD. (2014) Illuminating p53 function in cancer with genetically engineered mouse models. *Semin Cell Dev Biol.* **27**: 74-85.

Green DR, Kroemer G. (2009) Cytoplasmic functions of the tumour suppressor p53. *Nature* **458**(7242): 1127-30.

Gu W and Roeder RG. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90**(4): 595-606.

Gu W, Shi XL, Roeder RG. (1997) Synergistic activation of transcription by CBP and p53. *Nature* **387**(6635): 819-23.

Guihard S, Ramolu L, Macabre C, Wasylyk B, Noël G, Abecassis J, Jung AC. (2012) The NEDD8 conjugation pathway regulates p53 transcriptional activity and head and neck cancer cell sensitivity to ionizing radiation. *Int J Oncol.* **41**(4): 1531-40.

Ha JH, Won EY, Shin JS, Jang M, Ryu KS, Bae KH, Park SG, Park BC, Yoon HS, Chi SW. (2011) Molecular mimicry-based repositioning of nutlin-3 to anti-apoptotic Bcl-2 family proteins. *J Am Chem Soc.* **133**(5): 1244-7.

Haaland I, Opsahl JA, Berven FS, Reikvam H, Fredly HK, Haugse R, Thiede B, McCormack E, Lain S, Bruserud O, Gjertsen BT. (2014) Molecular mechanisms of nutlin-3 involve acetylation of p53, histones and heat shock proteins in acute myeloid leukemia. *Mol Cancer* **13**: 116.

Haupt Y, Maya R, Kazaz A, Oren M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387**(6630): 296-9.

Hermeking H. (2007) p53 enters the microRNA world. *Cancer Cell* **12**(5): 414-8.
Hollstein M, Sidransky D, Vogelstein B, Harris CC. (1991) p53 mutations in human cancers. *Science* **253**(5015): 49-53.

Honda R, Tanaka H, Yasuda H. (1997) Oncoprotein Mdm2 is a ubiquitin ligase E3 for tumour suppressor p53. *FEBS Lett.* **420**(1): 25-7.

Hu G, Zhang W, Deisseroth AB. (1992) P53 gene mutations in acute myelogenous leukaemia. *Br J Haematol.* **81**(4): 489-94.

Hu MC, Qiu WR, Wang YP. (1995) JNK1, JNK2 and JNK3 are p53 N-terminal serine 34 kinases. *Oncogene* **15**(19): 2277-87.

Hu W, Feng Z, Levine AJ. (2012) The regulation of multiple p53 stress responses is mediated through Mdm2. *Genes Cancer* **3**(3-4): 199-208.

Hupp TR and Lane DP. (1994) Allosteric activation of latent p53 tetramers. *Curr Biol.* **4**(10): 865-75.

International Agency for Research on Cancer. IARC TP53 Database. Accessed July 21, 2016. http://p53.iarc.fr/TP53SomaticMutations.aspx

Ito A, Kawaguchi Y, Lai CH, Kovacs JJ, Higashimoto Y, Appella E, Yao TP. (2002) MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. *EMBO J.* **21**(22): 6236-45.

Jeffrey PD, Gorina S, Pavletich NP. (1995) Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms. *Science* **267**(5203): 1498-502.

Jenkins JR, Rudge K, Currie GA. (1984) Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. *Nature* **312**(5995): 651-4.

Jenkins LM, Durell SR, Mazur SJ, Appella E. (2012) p53 N-terminal phosphorylation: a defining layer of complex regulation. *Carinogenesis* **33**(8): 1441-9.

Joerger AC and Fersht AR. (2008) Structural biology of the tumor suppressor p53. *Annu Rev Biochem.* **77**: 557-82.

Jones SN, Roe AE, Donehower LA, Bradley A. (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* **378**(6553): 206-8.

Kawai H, Lopez-Pajares V, Kim MM, Wiederschain D, Yuan ZM. (2007) RING domain-mediated interaction is a requirement for MDM2's E3 ligase activity. *Cancer Res.* **67**(13): 6026-30.

Kern SE, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C, Vogelstein B. (1991) Identification of p53 as a sequence-specific DNA-binding protein. *Science* **252**(5013): 1708-11.

Komarova EA, Krivokrysenko V, Wang K, Neznanov N, Chernov MV, Komarov PG, Brennan ML, Golovkina TV, Rokhlin OW, Kuprash DV, Nedospasov SA, Hazen SL, Feinstein E, Gudkov AV. (2005) p53 is a suppressor of inflammatory response in mice. *FASEB J.* **19**(8): 1030-2.

Kraiss S, Quaiser A, Oren M, Montenarh M. (1988) Oligomerization of oncoprotein p53. *J Virol.* **62**(12): 4737-44.

Kress M, May E, Cassingena R, May P. (1979) Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. *J Virol.* **31**(2): 472-83.

Krummel KA, Lee CJ, Toledo F, Wahl GM. (2005) The C-terminal lysines fine-tune P53 stress responses in a mouse model but are not required for stability control or transactivation. *Proc Natl Acad Sci USA* **102**(29): 10188-93.

Kulikov R, Winter M, Blattner C. (2006) Binding of p53 to the central domain of Mdm2 is regulated by phosphorylation. *J Biol Chem.* **281**(39): 28575-83.

Kubbutat MH, Ludwig RL, Ashcroft M, Vousden KH. (1998) Regulation of Mdm2-directed degradation by the C terminus of p53. *Mol Cell Biol.* **18**(10): 5690-8.

Kumamoto K, Spillare EA, Fujita K, Horikawa I, Yamashita T, Appella E, Nagashima M, Takenoshita S, Yokota J, Harris CC. (2008) Nutlin-3a activates p53 to both down-regulate inhibitor of growth 2 and up-regulate mir-34a, mir-34b, and mir-34c expression, and induce senescence. *Cancer Res.***68**(9): 3193-203.

Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ, Pavletich NP. (1996) Structure of the Mdm2 oncoprotein bound to the p53 tumour suppressor transactivation domain. *Science* **274**(5289): 948-53.

Lai L, Alaverdi N, Maltais L, Morse HC 3rd. (1998) Mouse cell surface antigens: nomenclature and immunophenotyping. *J Immunol.* **160**(8): 3861-8.

Lambert PF, Kashanchi F, Radonovich MF, Shiekhattar R, Brady JN. (1998) Phosphorylation of p53 serine 15 increases interaction with CBP. *J Biol Chem.* **273**(49): 33048-53.

Lane DP and Crawford LV. (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278**(5701): 261-3.

Lees-Miller SP, Sakaguchi K, Ullrich SJ, Appella E, Anderson CW. (1992) Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Mol Cell Biol.* **12**(11): 5041-9.

Levine AJ and Oren M. (2009) The first 30 years of p53: growing ever more complex. *Nat Rev Cancer* **9**(10): 749-58.

Li M, Brooks CL, Wu-Baer F, Chen D, Baer R, Gu W. (2003) Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science* **302**(5652): 1972-5.

Lill NL, Grossman SR, Ginsberg D, DeCaprio J, Livingston DM. (1997) Binding and modulation of p53 by p300/CBP coactivators. *Nature* **387**(6635): 823-7.

Lin J, Chen J, Elenbaas B, Levine AJ. (1994) Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. *Genes Dev.* **8**(10): 1235-46.

Linzer DI, Levine AJ. (1979) Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17**(1): 43-52.

Liu G, Park YJ, Tsuruta Y, Lorne E, Abraham E. (2009) p53 attenuates lipopolysaccharide-induced NF-kappaB activation and acute lung injury. *J Immunol.* **182**(8): 5063-71.

Liu WL, Midgley C, Stephan C, Saville M, Lane DP. (2001) Biological significance of a small highly conserved region in the N terminus of the p53 tumour suppressor protein. *J Mol Biol.* **313**(4): 711-31.

Lu H and Levine AJ. (1995) Human TAFII31 protein is a transcriptional coactivator of the p53 protein. *Proc Natl Acad Sci USA* **92**(11): 5154-8.

Maltzman W and Czyzyk L. (1984) UV irradiation stimulates levels of p53 cellular tumour antigen in nontransformed mouse cells. *Mol Cell Biol.* **4**(9): 1689-94.

Marine JC, Jochemsen AG. (2005) Mdmx as an essential regulator of p53 activity. *Biochem Biophys Res Commun.* **331**(3): 750-60.

May P, May E. (1999) Twenty years of p53 research: structural and functional aspects of the p53 protein. *Oncogene* **18**(53): 7621-36.

Maya R, Balass M, Kim ST, Shkedy D, Leal JF, Shifman O, Moas M, Buschmann T, Ronai Z, Shiloh Y, Kastan MB, Katzir E, Oren M. (2001) ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev.* **15**(9): 1067-77.

Mayo LD, Turchi JJ, Berberich SJ. (1997) Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res.* **57**(22): 5013-6.

McCurrach ME, Connor TM, Knudson CM, Korsmeyer SJ, Lowe SW. (1997) bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. *Proc Natl Acad Sci USA* **94**(6): 2345-9.

McKinney K, Mattia M, Gottifredi V, Prives C. (2004) p53 linear diffusion along DNA requires its C terminus. *Mol Cell.* **16**(3): 413-24.

Meek DW (2015). Regulation of the p53 response and its relationship to cancer. *Biochem J.* **469**(3): 325-46.

Meek DW and Eckhart W. (1988) Phosphorylation of p53 in normal and simian virus 40-trasformed NIH 3T3 cells. *Mol Cell Biol.* **8**(1): 461-5.

Melanson BD, Bose R, Hamill JD, Marcellus KA, Pan EF, McKay BC. (2011) The role of mRNA decay in p53-induced gene expression. *RNA* **17**(12): 2222-34.

Melero JA, Stitt DT, Mangel WF, Carroll RB. (1979) Identification of new polypeptide species (48-55K) immunoprecipitable by antiserum to purified large T antigen and present in SV40-infected and -transformed cells. *Virology* **93**(2): 466-80.

Michalovitz D, Halevy O, Oren M. (1990) Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell* **62**(4): 671-80.

Momand J, Zambetti GP, Olson DC, George D, Levine AJ. (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**(7): 1237-45.

Momand J, Jung D, Wilczynski S, Niland J. (1998) The MDM2 gene amplification database. *Nucleic Acids Res.* **26**(15): 3453-9.

Montes de Oca Luna R, Wagner DS, Lozano G. (1995) Rescue of early embryonic lethality in Mdm2-deficient mice by deletion of p53. *Nature* **378**(6553): 203-6.

Mowat M, Cheng A, Kimura N, Bernstein A, Benchimol S. (1985) Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus. *Nature* **314**(6012): 633-6.

Mujtaba S, He Y, Zeng L, Yan S, Plotnikova O, Sachchidanand, Sanchez R, Zeleznik-Le NJ, Ronai Z, Zhou MM. (2004) Structural mechanism of the bromodomain of the coactivator CBP in p53 transcriptional activation. *Mol Cell* **13**(2): 251-63.

Nakano K, Vousden KH. (2001) PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* **7**(3): 683-94.

Nakano Y, Naoe T, Kiyoi H, Kitamura K, Minami S, Miyawaki S, Asou N, Kuriyama K, Kusumoto S, Shimazaki C, Akiyama H, Saito K, Nishimura M, Motoji T, Shinagawa K, Saito H, Ohno R. (2000) Prognostic value of p53 gene mutations and the product expression in de novo acute myeloid leukemia. *Eur J Haematol.* **65**(1): 23-31.

National Cancer Institute Surveillance, Epidemiology, and End Results Program. SEER stat fact sheets: acute myeloid leukemia (AML). Accessed July 5, 2016. http://seer.cancer.gov/statfacts/html/amyl.html

National Cancer Institute Surveillance, Epidemiology, and End Results Program. SEER stat fact sheets: leukemia. Accessed July 5, 2016. http://seer.cancer.gov/statfacts/html/leuks.html.

Nobusawa A, Sano T, Negishi A, Yokoo S, Oyama T. (2014) Immunohistochemical staining patterns of cytokeratins 13, 14, and 17 in oral epithelial dysplasia including orthokeratotic dysplasia. *Pathol Int.* **64**(1): 20-7.

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**(5468): 1053-8.

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. (1993) Oncoprotein Mdm2 conceals the activation domain of tumour suppressor p53. *Nature* **362**(6423): 857-60.

Oren M and Levine AJ. (1983) Molecular cloning of a cDNA specific for the murine p53 cellular tumor antigen. *Proc Natl Acad Sci USA* **80**(1): 56-9.

Orkin SH, Zon LI. (2008) Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* **132**(4): 631-44.

O'Rourke RW, Miller CW, Kato GJ, Simon KJ, Chen DL, Dang CV, Koeffler HP. (1990) A potential transcriptional activation element in the p53 protein. *Oncogene* **5**(12): 1829-32.

Parada LF, Land H, Weinberg RA, Wolf D, Rotter V. (1984) Cooperation between gene encoding p53 tumor antigen and ras in cellular transformation. *Nature* **312**(5995): 649-51.

Pavletich NP, Chambers KA, Pabo CO. (1993) The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. *Genes Dev.* **7**(12B): 2556-64.

Pereg Y, Shkedy D, de Graaf P, Meulmeester E, Edelson-Averbukh M, Salek M, Biton S, Teunisse AF, Lehmann WD, Jochemsen AG, Shiloh Y. (2005) Phosphorylation of Hdmx mediates its Hdm2- and ATM-dependent degradation in response to DNA damage. *Proc Natl Acad Sci USA* **102**(14): 5056-61.

Pietenpol JA, Tokino T, Thiagalingam S, El-Deiry WS, Kinzler KW, Vogelstein B. (1994) Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc Natl Acad Sci USA* **91**(6): 1998-2002.

Pise-Masison CA, Radonovich M, Sakaguchi K, Appella E, Brady JN. (1998) Phosphorylation of p53: a novel pathway for p53 inactivation in human T-cell lymphotropic virus type 1-transformed cells. *J Virol.* **72**(8): 6348-55.

Presland RB, Jurevic RJ. (2002) Making sense of the epithelial barrier: what molecular biology and genetics tell us about the functions of oral mucosal and epidermal tissues. *J Dent Educ.* **66**(4): 564-74.

Raycroft L, Wu HY, Lozano G. (1990) Transcription activation by wild-type but not transforming mutants of the p53 anti-oncogene. *Science* **249**(4972): 1049-51.

Reed SM, Quelle DE. (2014) p53 acetylation: regulation and consequences. *Cancers* **7**(1): 30-69.

Rieger MA, Schroeder T. (2012) Hematopoiesis. *Cold Spring Harb Perspect Biol.* **4**(12): pii: a008250.

Rodriguez MS, Desterro JM, Lain S, Lane DP, Hay RT. (2000) Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. *Mol Cell Biol.* **20**(22): 8458-67.

Rokavec M, Li H, Jiang L, Hermeking H. (2014) The p53/miR-34 axis in development and disease. *J Mol Cell Biol.* **6**(3): 214-30.

Rotter V. (1979) p53, a transformation-related cellular-encoded protein, can be used as a biochemical marker for the detection of primary mouse tumor cells. *Proc Natl Acad Sci USA* **80**(9): 2613-7.

Sabbatini P, McCormick F. (2002) MDMX inhibits the p300/CBP-mediated acetylation of p53. *DNA Cell Biol.* **21**(7): 519-25.

Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, Anderson CW, Appella E. (1998) DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev.* **12**(18): 2831-41.

Sakuramo D, Sabbatini P, White E, Prendergast GC. (1997) The polyproline region of p53 is required to activate apoptosis but not growth arrest. *Oncogene* **15**(8): 887-98.

Samad A, Anderson CW, Carroll RB. (1986) Mapping of phosphomonoester and apparent phosphodiester bonds of the oncogene product p53 from simian virus 40-transformed 3T3 cells. *Proc Natl Acad Sci USA* **83**(4): 897-901.

Scolnick DM, Chehab NH, Stavridi ES, Lien MC, Caruso L, Moran E, Berger SL, Halazonetis TD. (1997) CREB-binding protein and p300/CBP-associated factor are transcriptional coactivators of the p53 tumor suppressor protein. *Cancer Res.* **57**(17): 3693-6.

Shaulsky G, Goldfinger N, Ben-Ze'ev A, Rotter V. (1990) Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. *Mol Cell Biol.* **10**(12): 6565-77.

Shieh SY, Ikeda M, Taya Y, Prives C. (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by Mdm2. *Cell* **91**(3): 325-34.

Shieh SY, Taya Y, Prives C. (1999) DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. *EMBO J.* **18**(7): 1815-23.

Shimizu H, Burch LR, Smith AJ, Dornan D, Wallace M, Ball KL, Hupp TR. (2002) The conformationally flexible S9-S10 linker region in the core domain of p53 contains a novel Mdm2 binding site whose mutation increases ubiquitination *in vivo*. *J Biol Chem.* **277**(32): 28446-58.

Shin JS, Ha JH, He F, Muto Y, Ryu KS, Yoon HS, Kang S, Park SG, Park BC, Choi SU, Chi CW. (2012) Structural insights into the dual-targeting mechanism of Nutlin-3. *Biochem Biophys Res Commun.* **420**(1): 48-53.

Shvarts A, Steegenga WT, Riteco N, van Laar T, Dekker P, Bazuine M, van Ham RC, van der Houven van Oordt W, Hateboer G, van der Eb AJ, Jochemsen AG. (1996) MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J.* **15**(19): 5349-57.

Shvarts A, Bazuine M, Dekker P, Ramos YF, Steegenga WT, Merckx G, van Ham RC, van der Houven van Oordt W, van der Eb AJ, Jochemsen AG. (1997) Isolation and identification of the human homolog of a new p53-binding protein, Mdmx. *Genomics* **43**(1): 34-42.

Siliciano JD, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan MB. (1997) DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev.* **11**(24): 3471-81.

Smith AE, Smith R, Paucha E. (1979) Characterization of different tumor antigens present in cells transformed by simian virus 40. *Cell* **18**(2): 335–46.

Smith JM, Stubbert LJ, Hamill JD, McKay BC. (2007) The contribution of transactivation subdomains 1 and 2 to p53-induced gene expression is heterogenous but not subdomain-specific. *Neoplasia* **9**(12): 1057-65.

Soussi T, Caron de Fromentel C, Méchali M, May P, Kress M. (1987) Cloning and characterization of a cDNA from Xenopus laevis coding for a protein homologous to human and murine p53. *Oncogene* **1**(1): 71-8.

Soussi T, Caron de Fromentel C, May P. (1990) Structural aspects of the p53 protein in relation to gene evolution. *Oncogene* **5**(7): 945-52.

Squier CA and Kremer MJ. (2001) Biology of oral mucosa and esophagus. *J Natl Cancer Inst Monogr.* (29): 7-15.

Srivastava S, Zou ZQ, Pirollo K, Blattner W, Chang EH. (1990) Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature* **348**(6303): 747-9.

Stindt MH, Carter S, Vigneron AM, Ryan KM, Vousden KH. (2011) Mdm2 promotes SUMO-2/3 modification of p53 to modulate transcriptional activity. *Cell Cycle* **10**(18): 3176-88.

Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K. (2009) Modulation of microRNA processing by p53. *Nature* **460**(7254): 529-33.

Tanimura S, Ohtsuka S, Mitsui K, Shirouzu K, Yoshimura A, Ohtsubo M. (1999) MDM2 interacts with MDMX through their RING finger domains. *FEBS Lett.* **447**(1): 5-9.

Tebaldi T, Zaccara S, Alessandrini F, Bisio A, Ciribilli Y, Inga A. (2015) Whole-genome cartography of p53 response elements ranked on transactivation potential. *BMC Genomics* **16**: 464. doi: 10.1186/s12864-015-1643-9.

Thut CJ, Chen JL, Klemm R, Tijan R. (1995) p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Science* **267**(5194): 100-4.

Tortora G, Caputo R, Damiano V, Bianco R, Chen J, Agrawal S, Bianco AR, Ciardiello F. (2000) A novel MDM2 anti-sense oligonucleotide has anti-tumor activity and potentiates cytotoxic drugs acting by different mechanisms in human colon cancer. *Int J Cancer* **88**(5): 804-9.

Tovar C, Rosinski J, Filipovic Z, Higgins B, Kolinsky K, Hilton H, Zhao X, Vu BT, Qing W, Packman K, Myklebost O, Heimbrook DC, Vassilev LT. (2006) Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. *Proc Natl Acad Sci USA* **103**(6): 1888-93.

Tyner SD, Venkatachalam S, Choi J, Jones S, Ghebranious N, Igelmann H, Lu X, Soron G, Cooper B, Brayton C, Park SH, Thompson T, Karsenty G, Bradley A, Donehower LA. (2002) p53 mutant mice that display early ageing-associated phenotypes. *Nature* **415**(6867): 45-53.

Unger T, Nau MM, Segal S, Minna JD. (1992) p53: a transdominant regulator of transcription whose function is ablated by mutations occurring in human cancer. *EMBO J.* **11**(4): 1383-90.

Vaseva AV, Moll UM. (2009) The mitochondrial p53 pathway. *Biochim Biophys Acta.* **1787**(5): 414-20.

Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303**(5659): 844-8.

Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, Harris NL, Le Beau MM, Hellstrom-Lindberg E, Tefferi A, Bloomfield CD. (2008) The 2008 revision

of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* **114**(5): 937-51.

Venot C, Maratrat M, Dureuil C, Conseiller E, Bracco L, Debussche L. (1998) The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression. *EMBO J.* **17**(16): 4668-79.

Vu B, Wovkulich P, Pizzolato G, Lovey A, Ding Q, Jiang N, Liu JJ, Zhao C, Glenn K, Wen Y, Tovar C, Packman K, Vassilev L, Graves B. (2013) Discovery of RG7112: A small-molecule MDM2 inhibitor in clinical development. *ACS Med Chem Lett.* **4**(5): 466-9.

Waldman T, Kinzler KW, Vogelstein B. (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res.* **55**(22): 5187-90.

Walker KK and Levine AJ. (1996) Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc Natl Acad Sci USA* **93**(26): 15335-40.

Wallace M, Worrall E, Pettersson S, Hupp TR, Ball KL. (2006) Dual-site regulation of Mdm2 E3-ligase activity. *Mol Cell.* **23**(2): 251-63.

Wang J, Shao L, Hendrickson HP, Liu L, Chang J, Luo Y, Seng J, Pouliot M, Authier S, Zhou D, Allaben W, Hauer-Jensen M. (2015) Total body irradiation in the "hematopoietic" dose range induces substantial intestinal injury in non-human primates. *Radiat Res.* **184**(5): 545-53.

Wang X, Wang J, Jiang X. (2011) MdmX protein is essential for Mdm2 protein-mediated p53 polyubiquitination. *J Biol Chem.* **286**(27): 23725-34.

Wasylyk C, Salvi R, Argentini M, Dureuil C, Delumeau I, Abecassis J, Debussche L, Wasylyk B. (1999) p53 mediated death of cell overexpressing MDM2 by an inhibitor of MDM2 interaction with p53. *Oncogene* **18**(11): 1921-34.

Wei CL, Wu Q, Vega VB, Chiu KP, Ng P, Zhang T, Shahab A, Yong HC, Fu Y, Weng Z, Liu J, Zhao XD, Chew JL, Lee YL, Kuznetsov VA, Sung WK, Miller LD, Lim B, Liu ET, Yu Q, Ng HH, Ruan Y. (2006) A global map of p53 transcription-factor binding sites in the human genome. *Cell* **124**(1): 207-19.

Weinberg RL, Freund SM, Veprintsev DB, Bycroft M, Fersht AR. (2004) Regulation of DNA binding of p53 by its C-terminal domain. *J Mol Biol.* **342**(3): 801-11.

Widmaier EP, Raff H, Strang KT. (2008) 12th ed. Vander's human physiology: the mechanisms of body function. Boston, McGraw-Hill Higher Education.

Wu X, Bayle JH, Olson D, Levine AJ. (1993) The p53-mdm-2 autoregulatory feedback loop. *Genes Dev.* **7**(7A): 1126-32

Xirodimas DP, Saville MK, Bourdon JC, Hay RT, Lane DP. (2004) Mdm2-mediated NEDD8 conjugation of p53 inhibits p53 transcriptional activity. *Cell* **118**(1): 83-97.

Yin C, Knudson CM, Korsmeyer SJ, Van Dyke T. (1997) Bax suppresses tumorigenesis and stimulates apoptosis *in vivo*. *Nature* **385**(6617): 637-40.

Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M. (1991) Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature* **352**(6333): 345-7.

Yu GW, Rudiger S, Veprintsev D, Freund S, Fernandez-Fernandez MR, Fersht AR. (2006) The central region of HDM2 provides a second binding site for p53. *Proc Natl Acad Sci USA* **103**(5): 1227-32.

Zakut-Houri R, Oren M, Bienz B, Lavie V, Hazum S, and Givol D. (1983) A single gene and a psuedogene for the cellular tumor antigen p53. *Nature* **306**(5943): 594-7.

Zauberman A, Barak Y, Ragimov N, Levy N, Oren M. (1993) Sequence-specific DNA binding by p53: identification of target sites and lack of binding to p53-Mdm2 complexes. *EMBO J.* **12**(7): 2799-808.

Zhang J, Sun Q, Zhang Z, Ge S, Han ZG, Chen WT. (2013) Loss of microRNA-143/145 disturbs cellular growth and apoptosis of human epithelial cancers by impairing the MDM2-p53 feedback loop. *Oncogene* **32**(1): 61-9.

Zhu J, Zhou W, Jiang J, Chen X. (1998) Identification of a novel p53 functional domain that is necessary for mediating apoptosis. *J Biol Chem.* **273**(21): 13030-6.