The p53 family interacting pathways in carcinogenesis and cellular response to DNA damage

by

Jodi L. Johnson

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of

Jodi L. Johnson

has been approved

Mentor/Chair-- Molly Kulesz-Martin

Member—Charles Lopez

Member—Rosalie Sears

Member—Hua Lu

Member—Philip Stork

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List of Abbreviations

Ab	Antibody
AMV	Avian Myeloblastosis Virus
Aspp	Apoptosis stimulating protein of p53
Bax	Bcl2 associated protein X
bp	Base pairs
BSA	Bovine serum albumin
C terminal	Carboxy-terminal
Ca^{2+}	Calcium ion
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
cip1	Cyclin dependent kinase interacting protein-1
DAI	DNA affinity immunoblotting
DAI/IP	DNA affinity immunoblotting followed by immunoprecipitation
DBB	DNA binding buffer
DMBA	Dimethylbenz[α]anthracene
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ΔN	Dominant negative deletion of transcriptionally active domain
DWD	Distance weighted discrimination
EBV	Epstein-Barr Virus
EGF	Epidermal growth factor
EMEM	Eagle's minimal essential medium
EMSA	Electrophoretic mobility shift assay
FACS	Florescence activated cell sorting
g	Gauge
Gadd45	Growth arrest and DNA-damage-inducible gene
gDNA	Genomic DNA
GFP	Green fluorescent protein
Gy	Grays

H&E	Hematoxylin and Eosin
HNSCC	Head and neck squamous cell carcinoma
Hrs.	Hours
HRP	Horseradish peroxidase
IB	Immunoblotting
IP	Immunoprecipitation
IR	Ionizing radiation
kb	kilo bases
kD	Kilodalton
Killer/DR5	Killer/Death receptor 5
MAS	Microarray Suite
Mdm2	Mouse double minute 2
MEF	Mouse embryonic fibroblast
Min.	Minutes
N	Normal
N terminal	Amino terminal
NER	Nucleotide excision repair
NHEK	Normal human epidermal keratinocyte
Noxa	Latin for damage
NCI	National Cancer Institute
OCT	Optimal cutting temperature
OHSU	Oregon Health & Science University
Oligo	Oligonucleotide
p53as	Alternatively spliced p53
p53rs	Regularly spliced p53
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pfu	Polymerase isolated from archaeon *Pyrococcus furiosus*
PI	Propidium iodide
PIG3	p53-inducible gene 3

PK	Primary keratinocyte
PRNP	Positional ranking and normalization
Puma	p53 upregulated modulator of apoptosis
Puro	Puromycin
qPCR	Quantitative RT-PCR
RIPA	Radioimmunoprecipitation buffer
RT-PCR	Reverse transcriptase polymerase chain reaction
Rb	Retinoblastoma
RNA	Ribonucleic acid
rpm	Revolutions per minute
SAM	Sterile alpha motif
SCC	Squamous cell carcinoma
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec.	Seconds
si-Con	siRNA control
si-p73	siRNA directed against p73
siRNA	Small inhibitory RNA
SV40	Simian vacuolating virus 40
SYBR	Synergy Brands Inc.
T	Tumor
TA	Transcriptionally active
Taq	Polymerase isolated from bacteria *Thermus aquaticus*
TBS	Tris Buffered Saline
TBS-t	Tris Buffered Saline with 0.05 tween
TPA	tetradecanoyl-phorbol-13-acetate
U	Units
UVB	Ultraviolet B
Waf-1	Wild-type tp53-activated factor
wtp53	Wild-type p53

Abstract:

The objective of this study is to examine, in light of the expression of multiple p53 family member isoforms, the specific role of p73 in malignant conversion, cellular response to DNA damage, and direct or indirect cooperation with other p53 family members in a clonal model of epidermal carcinogenesis. We first focused on the role of p73 in malignant conversion. Whether sporadic or siRNA induced, loss of p73 in initiated $p53^{+/+}$ keratinocytes lead to conversion to squamous cell carcinoma (SCC) *in vivo* which was reversible upon reconstitution of TAp73α but not ΔNp73α. Second, we investigated the cellular response to ionizing radiation (IR) in the presence and absence of p73, showing that loss of p73 at malignant conversion was associated with resistance to IR *in vitro*. The loss of radiation sensitivity and malignant conversion was characterized by reduced steady state DNA binding levels of transcriptionally active p63 isoforms to the p21 promoter, failure to induce specific p53 family transcriptional targets, and failure to arrest in G1. Reconstitution of TAp73α, but not ΔNp73α, increased steady state DNA binding capabilities of TAp63β, TAp63γ, and ΔNp63γ, and steady state levels of p53 family target mRNA, but did not restore cellular sensitivity to IR. We thus uncovered a functional cooperation between TA isoforms of p73 and p63 and showed that p73-mediated DNA damage response was uncoupled from its tumor suppressive role. We observed preferential DNA binding of the inhibitory ΔNp63α isoform both *in vitro* and *in vivo* in SCC suggesting that in the absence of TAp73α a balance is tipped toward DNA binding of the inhibitory isoforms. Third, we studied the role of the p53 family in keratinocyte response to UVB. Tumorigenic cells lacking p73 that were resistant to IR remained sensitive to UVB, accompanied by DNA binding of the TAp63γ isoform,

suggesting that keratinocyte response to UVB is not dependent upon p73 and suggesting a hierarchy of p53 family member responses to DNA damage. Finally, we examined TAp73α interaction with the p53 family inhibitor Mdm2. Mdm2 was in complex with DNA-bound p53 family members in malignant cells, but reconstitution of cells with TAp73α correlated with removal of Mdm2 from the complex, making them more like primary keratinocytes or initiated cells. Like the initiated cells, cells expressing TAp73α were refractory to treatment with the Mdm2-p53 inhibitor Nutlin-3 while cells lacking p73 expression or expressing ΔNp73α were sensitive. Thus, we suggest that p73 may be acting as a molecular shield to keep p53 family member inhibitors, such as ΔNp63α and Mdm2, at bay. Further understanding of p53 family interplay in tumor development and DNA damage response could lead to new therapies or optimization of current therapeutic strategies in solid tumors of epithelium, particularly where deregulation or loss of p63 and p73 expression is associated with increased tumor invasiveness, treatment resistance, and poor patient prognosis.

1. Introduction:

1.1 Brief history of p53 discovery

p53 was discovered more than 25 years ago as a mammalian protein that was targeted for disruption by tumor virus proteins such as SV40 and frequently overexpressed (in a defective form) in human tumors [Crawford et al., 1981; Lane and Crawford, 1979; Linzer and Levine, 1979; Reich and Levine, 1982; Thomas et al., 1983]. Evidence accumulated that wild type p53 acted a tumor suppressor rather than an oncogene [Lane and Benchimol, 1990; Nigro et al., 1989]. Since then, research of p53 activities has been reported in nearly 44,000 journal articles [Horn and Vousden, 2007]. The astounding amount of research conducted on p53 stems from its frequent mutation in human cancers (approximately 50% overall, more than any other single gene) and from its broad spectrum of activities. Besides mutation, inactivation of the p53 pathway also occurs through p53 mislocalization and through misregulation of proteins involved in upstream activation or downstream effects of p53, broadening p53's relevance to nearly all human cancers. The p53 protein acts as a transcription factor to induce target genes involved in cell cycle checkpoints, DNA repair, cell death, and cell senescence, particularly in response to DNA damage [Bond et al., 2005]. As "guardian of the genome", p53 is responsible for ensuring that DNA repair proteins have the opportunity to repair damaged DNA in cells under stress or mediate apoptosis if the damage is extensive. The expression and functional activation of p53 is tightly regulated by multiple cofactor proteins which will be discussed in detail herein. It is still largely unknown how p53 mediates the cellular "decision" to undergo cell cycle arrest or apoptotic cell death following damage.

1.2 Discovery of p63 and p73 as p53 family members

Until recently it was thought that p53 functioned uniquely since no related genes were found during more than 20 years of p53 research. However, two new members of the p53 family, p63 (also called KET, p51A, p51B, p41, p73L, and NBP) and p73, were discovered in 1997 and classified in relation to p53 based on their structural similarity [Kaghad et al., 1997; Yang et al., 1998]. Both p63 and p73 share approximately 25% sequence similarity with p53 in their transcriptional activation domains, approximately 65% homology to p53 in their DNA binding domains, and approximately 35% identity with p53 in their oligomerization domains with p73 being slightly more homologous to p53 than p63 [Davis and Dowdy, 2001; Little and Jochemsen, 2002].

1.3. p53 family gene and protein structure

The p53 family of genes has been shown to have similar features and to code for proteins with similar structures that can act as transcription factors or inhibitors of transcription. The following section will describe the common features of the p53 family member genes and their products as well as discussing some important structural differences between family members. A schematic of the p53 family member isoforms is provided in Figure 1.

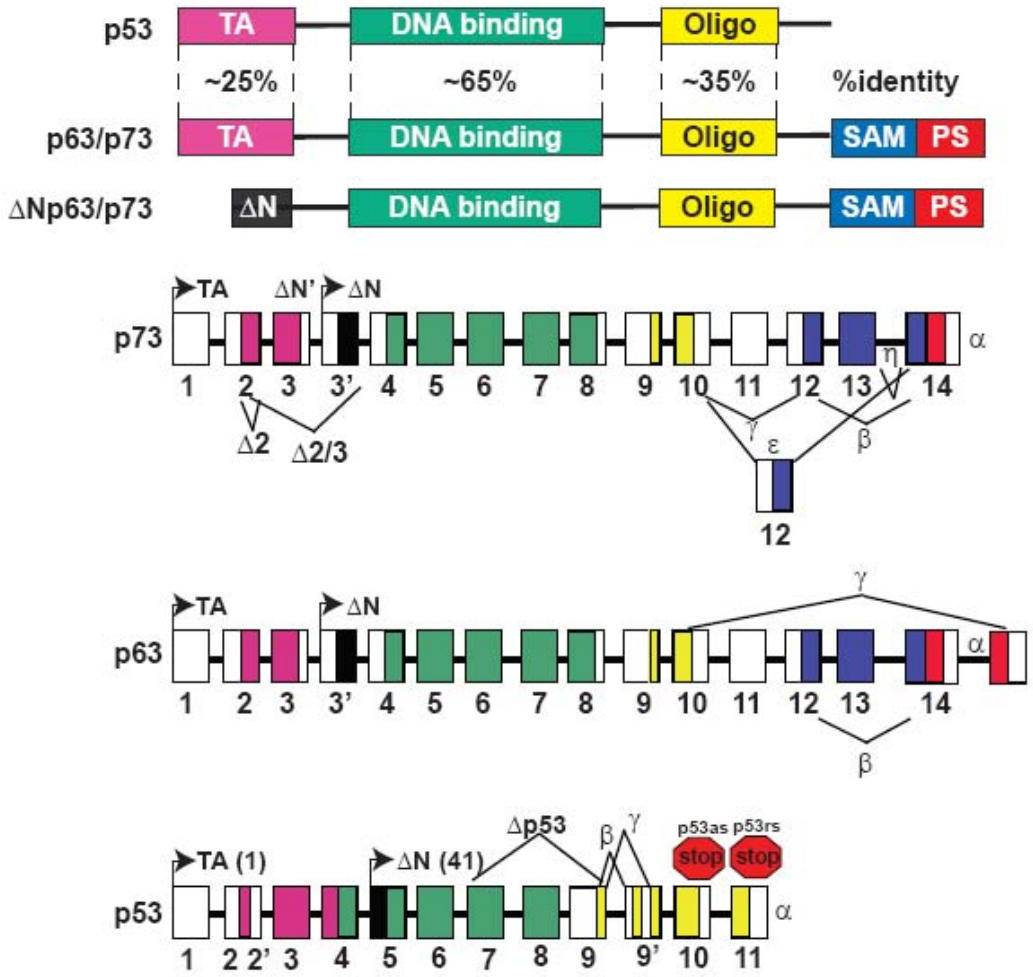

Figure 1: The p53 family and isoforms. Alignment and homology existing between the p53 family of proteins followed by a schematic of the exons of each individual p53 family member gene, indicating where splicing occurs to produce the multiple splice variants. White boxes are non-coding regions, pink boxes are regions encoding the transactivation domain, green boxes encode the DNA binding domain, yellow boxes encode the oligomerization domain, blue boxes encode the SAM domain, and red boxes encode a "post-SAM" (PS) domain. Black boxes are unique to ΔN isoforms of p53 family members. Figure modified from [Bourdon et al., 2005].

1.3.1. TA and ΔN isoforms from different promoters

It was immediately apparent upon discovery that the p63 and p73 genes were capable of being transcribed into multiple splice variants and expressed as multiple proteins varying in their C-termini. It was also discovered that a second transcriptional start site located in the third intron of both p63 and p73 genes coded for N-terminally truncated forms of the proteins (ΔN), often acting as dominant negative inhibitors of the full length proteins because they lack the transcriptional activation (TA) domain and can therefore inhibit the TA isoforms through direct proten:protein interactions and through competition for DNA binding [Melino et al., 2002]. Following discovery of the two transcriptional start sites and multiple splice variants of p63 and p73, the p53 gene structure was reassessed. It was discovered that p53 also made use of a second transcriptional start site to generate ΔN p53 isoforms and that multiple p53 splice variants also existed [Bourdon et al., 2005]. Some of the ΔN proteins have secondary transactivation domains allowing transcriptional control of an alternate subset of genes than those being regulated by the TA isoforms [Dohn et al., 2001; Ghioni et al., 2002; Liu et al., 2004]. The balance of expression of the TA and ΔN p53 family member isoforms is carefully regulated for normal cellular functions in development, cellular differentiation, and DNA damage response and many types of human cancers exhibit deregulation of isoform expression during progression of disease as will be discussed further in subsequent sections.

1.3.2. DNA binding domain homology

The most highly conserved region in the p53 family is the DNA binding domain (65% conserved) [Kaghad et al., 1997] leading to the original speculation that p53, p63,

and p73 may regulate transcription of many of the same downstream targets. Indeed, it was found that all three family members could transactivate many of the same targets (Table 1) including cell cycle arrest response gene *p21*; DNA damage repair gene *Gadd45*; and apoptotic response genes *Bax*, *Noxa*, and *Puma*, [el-Deiry et al., 1993; Kastan et al., 1992; Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda et al., 2000]. However, phenotypes of p63 and p73 null mice and microarray analysis of genes transactivated by each of the family members have shown that each has a distinct physiological role and set of downstream targets in addition to common ones [Fontemaggi et al., 2002; Vigano et al., 2006; Wu et al., 2003; Yang et al., 2002]. The p53 consensus DNA binding site is 5'- RRRCWWGYYY-3' (R=G or A, W= T or A, Y = C or T [el-Deiry et al., 1992] typically found as two tandem half sites separated by 0 to 13 base pairs. Alterations in one or more base pairs making up the consensus sequence can alter the affinity of p53 for the binding site [Weinberg et al, 2005]. The p73 protein binds strongly to this same DNA binding site, but differences in DNA binding affinity and DNA binding domain conformation between p53 and p73 may explain why the two also transactivate different downstream targets [Lokshin et al., 2007]. The p63 protein, although it does bind the canonical p53 binding site, preferentially binds an alternate DNA binding site with sequence RRRCGTGYYY [Osada et al., 2005]. This alternative site allows p63-specific transactivation of targets involved in skin homeostasis and in development. For example, the alternative p63 binding site is found in the promoters of epidermally-expressed genes such as envoplakin (EVPL) that mediates cellular junctions between keratinocytes of the epidermis and esophagus and developmentally-expressed genes such as the SWI/SNF-related, matrix-associated, actin-dependent regulator of

chromatin (SMARCD3). Thus the p53 family members, with highly similar DNA binding domains, transcriptionally control the expression of both common and divergent genes for mediating cellular functions [Harms et al., 2004].

Table 1: p53 family downstream target genes and functions

Gene	Cellular function	Transactivated by p53 family members? p53	p73	p63	GenBank ID	Reference
Apaf1	Apoptosis	+	-	-	317	Fortin, 2001; Moroni, 2001; Robles, 2001
Bax	Apoptosis	+	+	+	581	Miyashita, 1995, Kato, 1999, Flores, 2002
Bid	Apoptosis	+	+	-	637	Sax, 2002; Karpinich, 2006
Fas	Apoptosis	+	-	-	355	Owen-Schaub, 1995
Dram	Apoptosis	+	+	-	55332	Crighton, 2006
Ferredoxin reductase (fdxr)	Apoptosis	+	+	+	2232	Hwang, 2001; Liu, 2002
Igfbp3	Apoptosis	+	+	-	3486	Buckbinder, 1995; Fontemaggi, 2002
Killer/DR5	Apoptosis	+	+	+	8795	Wu, 1997, Dohn, 2001
Noxa	Apoptosis	+	+	+	5366	Oda, 2000; Flores, 2002
p53Aip1	Apoptosis	+	+	-	63970	Oda, 2000
Perp	Apoptosis	+	-	+	64065	Attardi, 2000; Ihrie, 2005
Pig3	Apoptosis	+	+	+	9540	Flatt, 2000; Contente, 2002; Dohn, 2001; Fontemaggi, 2002
Pig8/Ei24	Apoptosis	+	+	-	9538	Lehar, 1996; Gu, 2000; Dohn, 2001
Puma	Apoptosis	+	+	-	27113	Nakano, 2001; Yu, 2001; Melino, 2003
Cdkn1A (p21/Cip1)	Cell cycle	+	+	+	1026	el-Deiry, 1993; Dohn, 2001
14-3-3-sigma	Cell cycle	+	-	+	7532	Hermeking, 1997; Vigano, 2006
gadd45	Cell cycle/DNA repair	+	+	+	1647	Hollander, 1993; Dohn, 2001
p53R2	Cell cycle/DNA repair	+	+	-	50484	Nakano, 2000; Tanaka, 2000
Maspin	Angiogenesis and metastasis	+	-	+	5268	Zou, 2000; Spiesbach, 2005
Mdm2	Autoregulation	+	+	+	4193	Barak, 1993; Wu, 1993; Dohn, 2001; Vigano, 2006
Cop1	Autoregulation	+	+	-	64326	Dornan, 2004; Dulloo, 2005
Pirh2	Autoregulation	+	+	-	25898	Leng, 2003; Dulloo, 2005
TP73	Autoregulation	+	+	+	7161	Chen, 2001; Grob, 2001
CCNG1 (Cyclin G)	Autoregulation	+	+	+	900	Okamoto, 1994; Okamoto, 1996; Ohtsuka, 2003; Wu, 2003
Pedf	neuronal cell survival, angiogenesis inhibitor	-	+	+	76979	Sasaki, 2005
Trim32	E3-ubiquitin ligase	-	+	-	69807	Fontemaggi, 2002
loricrin (Lor)	keratinocyte differentiation	-	+	+	16969	Di Laurenzi, 2000
involucrin	keratinocyte differentiation	-	+	+	3713	Di Laurenzi, 2000

A select list of genes that are transcriptionally regulated by the p53 family members with particular attention to apoptosis, autoregulation, and keratinocyte differentiation. The p53 target genes, p21, Gadd45, Bax, Noxa, and Puma, used in this study have been included.

1.3.3. C terminal splice variants

The p53 family of genes encodes multiple proteins due to mRNA splice variants. The p63 gene codes for six mRNA sequences and six proteins while the p73 gene can code for up to thirty-five mRNA sequences and twenty-eight proteins, only fourteen of which have been described at the protein expression level [Bourdon et al., 2005]. Review of the p53 gene sequence in light of the known p63 and p73 expressed isoforms revealed that p53 was also expressed as up to twelve mRNAs and nine separate proteins [Bourdon et al., 2005; Murray-Zmijewski et al., 2006]. Functionally, TAp63γ is the most transcriptionally active isoform (almost as active as p53) while TAp63α had almost no transcriptional activity [Yang et al., 1998]. Initial analyses of p63 isoform transcriptional activities was performed in Saos2 cells which are p53 null and which express very low or undetectable levels of endogenous p53 family members. The ΔNp63γ and ΔNp63β isoforms had some transactivational activity due to a second transactivation domain [Dohn et al., 2001; Ghioni et al., 2002] and cotransfection of TAp63γ in a 5:1 ratio with ΔNp63γ yielded an increase in transcriptional activity over that seen by TAp63γ alone using a reporter construct driven by a promoter containing multiple p53 consensus binding sites [Yang et al., 1998]. ΔNp63α, however, was a strong suppressor of transactivation by TAp63γ [Yang et al., 1998]. Analysis of the transcriptional activity of transfected p73 isoforms in MCF7 cells (wild type p53) revealed that TAp73β is the most active form, nearly as powerful a transactivator as p53 [Ueda et al., 1999]. TAp73α was transcriptionally inactive, but increased TAp73β transcriptional activity when the two isoforms were cotransfected into p53 null cells and transcriptional activity was tested using a reporter construct driven by a minimal Mdm2 promoter containing the p53

binding site [Alarcon-Vargas et al., 2000]. The p73γ isoform was significantly less efficient in transactivating the p21(Waf1/Cip1) promoter in p53 null Saos2 cells than TAp73β whereas the TAp73δ isoform was intermediate [De Laurenzi et al., 1998]. The physiological reason behind the large number of p53 family protein isoforms is largely unknown as is the tissue specificity of isoform function. Furthermore, the mechanism behind how the p53 family member isoforms cooperate and compete with one another for normal cellular function and how these family relations may go awry in human disease, including carcinogenesis, is only beginning to be explored.

1.3.4. Mdm2 interaction domain

The N terminus of the p53 family of proteins, referred to as an activation domain, is frequently post-translationally modified by phosphorylation for functional control. This N terminal region is where interactions with cofactors such as Mdm2 and p300 occur [Harms and Chen, 2006]. The Mdm2 protein is a negative regulator and p300 is an activator of the p53 family as will be discussed in subsequent sections. Because this N terminal region of p53 family members is not found in the ΔN isoforms, it is thought that Mdm2 can not interact with the ΔN p53 family isoforms. However, a second Mdm2 interaction site in the p53 DNA binding domain, common to all isoforms of the p53 family, suggests that Mdm2 may indeed interact with all isoforms even though this has not been shown [Chi et al., 2005; Shimizu et al., 2002].

1.3.5. Oligomerization domain

High-affinity DNA binding and transcriptional activation of p53 requires that p53 bind DNA as a tetramer [McLure and Lee, 1998]. Tetramerization is regulated by the C terminal tetramerization domain or oligomerization domain, as it will be referred to

subsequently. Though the wild type p53 protein does not hetero-oligomerize with p63 or p73, p63 and p73 isoforms are capable of interacting through the oligomerization domain [Davison et al., 1999; Gaiddon et al., 2001]. Mutant p53 can hetero-oligomerize with wild type p53 and it was shown that three mutant p53 molecules but only one ΔNp53 molecule (construct lacking the first 90 aa of p53) was sufficient to functionally inactivate wild type p53 [Chan et al., 2004]. The TA and ΔN isoforms of p63 and p73 can hetero-oligomerize and this interaction frequently inhibits the transactivation potential of the TA isoforms. However, the ΔN isoforms do not always inhibit the TA isoforms as in the case of ΔNp63γ enhancing the transactivation capacity of TAp63γ [Yang et al., 1998] or when the ΔN isoforms contain secondary transactivation domains [Ghioni et al., 2002]. In general, the p63 and p73 isoforms (both TA and ΔN) containing the C terminal Sterile Alpha Motif (SAM) domain (thus the α isoforms) are less transcriptionally active than isoforms lacking the SAM domain (discussed further in section 1.3.6), with the ΔNp63α and ΔNp73α isoforms being strong repressors of TAp63 and TAp73 isoforms [Ghioni et al., 2002; Harms and Chen, 2006; Liu and Chen, 2005; Ozaki et al., 1999].

1.3.6. Variations in structure between family members

The extreme C terminus of the three p53 family members is structurally divergent. The p53 protein uniquely expresses a C terminal basic domain that spans the last 30 residues and is a crucial regulatory domain for p53 function [Harms and Chen, 2006]. Nearly every residue in this domain has been found to be post-translationally modified by at least one type of modification such as phosphorylation, methylation, acetylation, ubiquitination, neddylation, or sumoylation. The transactivation of downstream target genes by p53 is accelerated by the presence of an intact basic C

terminus that aids in the search for specific DNA targets [Liu and Kulesz-Martin, 2006; Liu, Lagowski et al., 2004]. This basic C terminus is unique to p53, not found in p63 and p73, and therefore most likely puts the p53 protein as the dominant transactivator of genes that all three family members can transactivate. However, during evolution, the p53 protein lost the exons coding for the sterile alpha motif (SAM) domain and the post-SAM (PS) domain that are found at the C termini of the p73α and p63α proteins. The SAM domain is thought to mediate protein-protein interactions, but does not mediate interactions between p63 and p73 themselves. These interactions are mediated through the oligomerization domain. The SAM domain is important for the divergent functions of p63 and p73 in development, and naturally occurring mutations in the SAM domain of p63 are common to ectodermal dysplasia syndromes that will be discussed in subsequent sections. The SAM domain of p73α negatively regulates its transcriptional activity by blocking p73 interaction with p300 and the loss of the SAM domain contributes to the increased transcriptional activity of p73β compared to p73α [Liu and Chen, 2005]. The SAM domain is also thought to be an inhibitory domain for p63 transcriptional activity [Scoumanne et al., 2005]. Thus, the divergence at the C termini of the p53 family of proteins is one reason why the p53 proteins carry out different functions in development and tumor suppression.

1.4. Evolution of p53 family members

The p53, p63 and p73 genes evolved from a common ancestral “p53-like” gene found in simple organisms such as present-day protostomes (*Caenorhabditis elegans, Drosophila,* and mollusks). Figure 2 illustrates the evolution of the p53 family members.

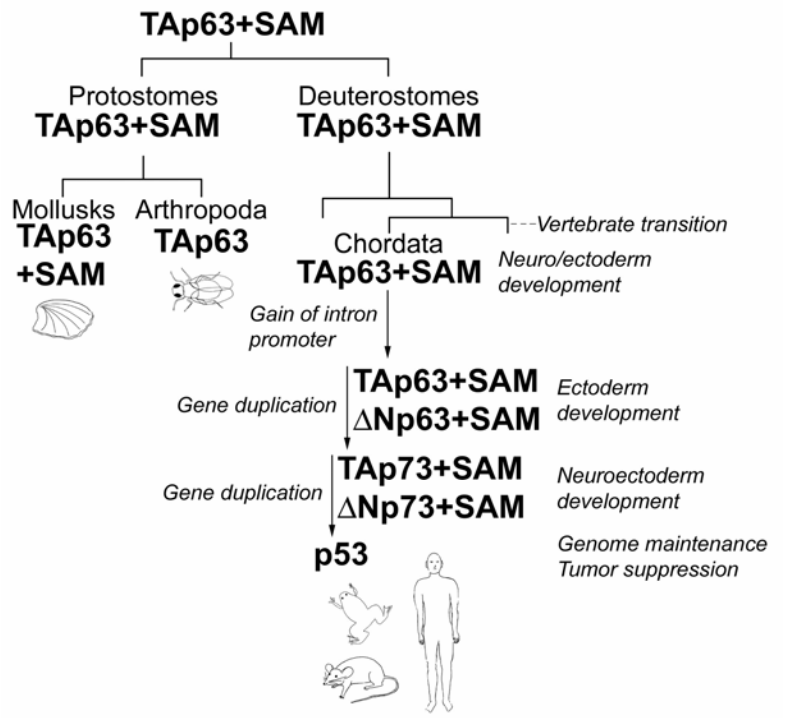

Figure 2: Evolution of the p53 family of genes. The schematic indicates where gene duplications and alterations in protein domains diverged during the evolution of the p53 family. Evolutionary conservation of DNA sequence suggests that TAp63 was the ancestral precursor of the p53 family. Functional specialization among family members evolved as organisms became more complex. A second promoter in intron 3 of p63 and p73 was introduced, producing ΔN products that can act as dominant negative inhibitors of the full length, transcriptionally active forms. The p53 protein arose as a tumor suppressor in higher order organisms with increased life spans. Figure modified from [Johnson et al., 2005; Melino et al., 2002].

In *Drosophila*, the p53-like gene was capable of coding for multiple isoforms showing that the complex gene structure of p53 was evolutionarily conserved [Brodsky et al., 2000; Jin et al., 2000; Ollmann et al., 2000]. The p53-like ancestor most closely resembles full length TAp63 with gene duplication during the evolution of Chordata (*Xenopus*, fish, mouse, human) leading to the production of first p73 and then p53 [Yang et al., 2002]. The role of the p53-like gene in protostomes is predominantly developmental since adults of these species do not undergo tissue renewal and are therefore at low risk for cancer [Blandino and Dobbelstein, 2004]. The ancestral ΔNp63γ-like isoform found in frogs (*Xenopus*) is responsible for both neuroectoderm and ectoderm development, while the more recently evolved p73 protein took on the neuroectoderm development function in mouse and man, leaving ectodermal formation to p63. The roles of p63 and p73 in embryonic development correspond with their primary roles as transcription factors for regulation of apoptosis. Apoptosis, under strict spatial and temporal regulation within discrete cells, is responsible for the sculpting of embryonic tissues. Specific developmental roles of p53 family members will be discussed in detail subsequently. Briefly, mice null for p63 die shortly after birth due to extensive ectodermal defects including loss of skin formation and truncated limbs while p73 null mice live to adulthood, but display significant neuronal defects. The p53 protein, in addition to being a tumor suppressor protein, also has a role in coordinating developmental apoptosis specifically in neurons. Thus all three p53 family members play a role in development with p53 having the most tissue specifically confined developmental role but the most ubiquitous tumor suppressive role. While functions of mammalian p53, p63, and p73 cannot be completely separated (all three proteins play a

role in cell cycle arrest as well as apoptosis) it is interesting to speculate on their order of evolution and why all three are still needed. Potential cooperative interactions among the three family members in response to DNA damage and tumor suppression provide one explanation for why all three have been evolutionarily maintained.

1.5. Developmental and tissue specific activities of p73, p63, p53 protein: lessons from mice and men

The three p53 family proteins each have distinct functions during development, and knock-out mice have been informative for elucidation of these unique functions as illustrated in Figure 3. Knowledge gained from p53, p63, and p73 knock-out mice have also brought to light the detrimental effects caused by germ line mutations in the p53 family members in humans.

1.5.1. p63 in epidermal formation

Mice lacking p63 expression have severe developmental defects including truncated limbs and epidermis that lacks stratification and does not express differentiation markers [Mills et al., 1999]. The mice die shortly after birth due to dehydration. Epidermal adnexal structures that also depend upon epidermal-mesenchymal interactions, such as teeth, hair follicles, and mammary glands, are absent in p63-deficient mice. These striking developmental defects in mice bore resemblance to human developmental disorders with symptoms including combinations and differing severities of facial abnormalities (thin upper lip or clefting), failure to separate fingers (ectrodactyly of the hands and feet, i.e. “lobster claw anomaly”), nasolacrimal duct obstruction, cleft strands connecting eyelids (i.e. ankyloblepharon), and cup shaped ears. For these autosomal dominant disorders; ectrodactyly-ectodermal dysplasia [Celli et al., 1999],

ankyloblepharon-ectodermal dysplasia clefting (Hay-Wells Syndrome) [Fomenkov et al., 2003], acro-dermatoungual-lacrimal-tooth (ADULT) syndrome [Duijf et al., 2002], limb-mammary syndrome [van Bokhoven et al., 2001], Rapp-Hodgkin syndrome [Chan et al., 2005], and split hand-split foot malformation syndrome [van Bokhoven et al., 2001], a thorough genetic analysis of human p63 was performed and mutant forms of p63 were found in all of them [Brunner et al., 2002]. Thus the p63 protein is crucial for epidermal development and its mutation has been linked to known human developmental diseases.

1.5.2. p73 in neural formation

Knock out of p73 genes in mice results in severe abnormalities of the nervous system and runting [Yang et al., 2002; Yang, Walker et al., 2000]. p73 is required for normal development of the hippocampus, a region central to learning and memory that continues to develop throughout adulthood [Abraham and Meyer, 2003]. Mice lacking p73 exhibit abnormal reproductive and social behavior due to defects in pheromone detection, attributable to a dysfunction of a nasal organ that normally expresses high levels of p73. The tissue specificity of the p73 deficient phenotype (concentrated to the brain and related structures) may be associated with variable patterns of monoallelic expression in brain, vs. biallelic expression of p73 in all other tissues [Hu et al., 2000]. In p73 null mice, neurons in the superior cervical ganglion (SCG) were depleted by 75% due to p53-dependent apoptosis compared to littermates expressing p73, while ΔNp73 inhibited apoptosis [Lee et al., 2004]. To date, there are no human developmental syndromes associated with germ line p73 mutations. A systematic sequencing of p73 genes in familial disorders in humans, guided by some of the features seen in the p73 knock-out mice, could elucidate underlying p73 mutations that are likely to be present.

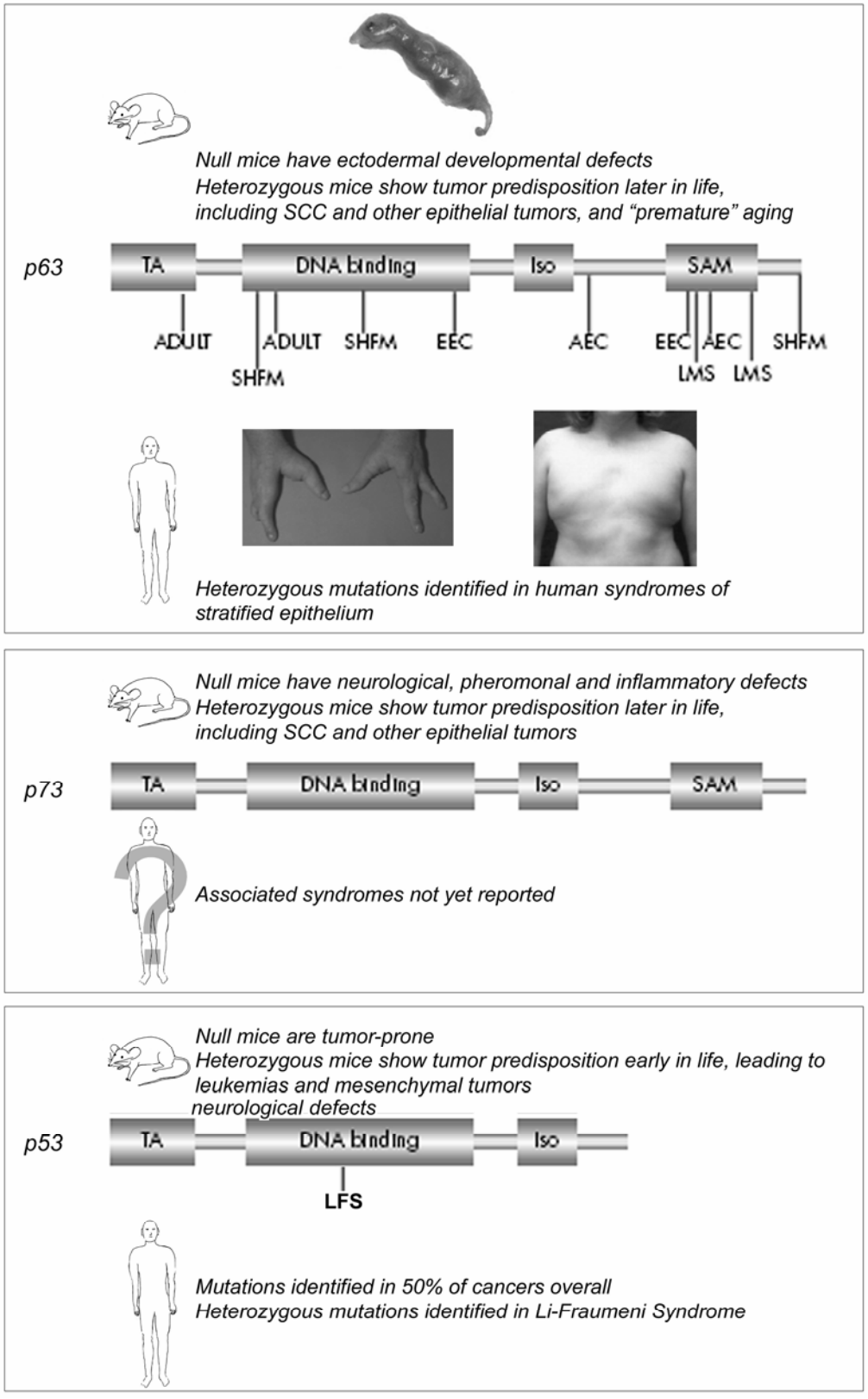

Figure 3: The p53 family in mouse models and human disease

Figure 3: The p53 family in mouse models and human disease. Phenotypic defects in mice either lacking respective p53 family members or heterozygous for mutations in the p53 family members. Where relevant, comparisons have been made to the human diseases linked to mutations in each p53 family member. Defects for p63 null mice include severely truncated limbs, skin lacking stratification, and absence of structures that depend upon epidermal/mesenchymal interactions, such as teeth, hair follicles, and mammary glands. The null mouse image is reprinted with permission from Nature Publishing Group from [Yang et al., 1999]. Defects for p73 and p53 mice are as noted. Indicated below each p53 family member protein structure are associated human hereditary disorders linked to specific protein functional domains. Germ line mutations in human p63 lead to the following disorders: Acro-dermato-ungual-lacrimal-tooth (ADULT), split hand-split foot malformation (SHFM), ectrodactyly-ectodermal dysplasia (EEC), ankyloblepharon-ectodermal dysplasia clefting (AEC - Hay-Wells Syndrome), limb-mammary syndrome (LMS), and Rapp Hodgkin syndrome. Clinical examples of ectrodactyly and mammary gland hypoplasia, phenotypes common to these disorders, are shown (reprint with permission from Elsivier, [van Bokhoven and McKeon, 2002]). As yet, human disorders have not been linked to germ line p73 mutations. Heterozygous mutations in germ line p53 lead to Li-Fraumeni syndrome (LFS), a predisposition to tumorigenesis.

1.5.3. p53 in tumor suppression

The p53 protein is at the hub of the cell's response to DNA damage from multiple sources and responds to this stress by inducing cell cycle arrest, DNA damage repair, cellular senescence, differentiation, and apoptosis, leading to the protein being named the "guardian of the genome" [Lane, 1992]. However, it was recently shown that the DNA damage responses carried out by p53 could be uncoupled from its tumor suppressive function [Christophorou et al., 2006]. Immediately following treatment with IR, p53 mediated an acute DNA damage response, but this response did not lead to suppression of tumors. Rather, p53 mediated its tumor suppressive function through induction of the $p19^{ARF}$ pathway only in select cells. The p53 gene is the most frequently mutated gene in human cancer [Hollstein et al., 1994] and is functionally inactivated in up to 90% of human tumors [Bykov and Wiman, 2003; Hainaut and Hollstein, 2000]. Knocking out p53 expression in mice lead to early demise from the types of cancers (involving tissues such as blood, muscle and bone) that also develop early in human patients with Li Fraumeni syndrome, the rare human disease caused by germ line mutation of p53 [Donehower et al., 1992; Jacks et al., 1994; Olivier et al., 2003]. However, it is thought that the p53 protein was evolutionarily maintained not because of its role in tumor suppression but for its involvement in apoptosis during development. While most p53 null mice lived to adulthood, long enough to develop spontaneous tumors faster than wild type mice, a subset of p53 null mice developed craniofacial abnormalities during development and died from overproduction of neural tissue and inability to achieve neural tube closure [Aranda-Anzaldo and Dent, 2007; Armstrong et al., 1995; Sah et al.,

1995]. Thus p53, like its family members, does play a very limited, tissue specific role in development.

1.5.4. The p53 family in skin

Both p63 and p73 are expressed along with p53 in normal human epidermis and in epidermal keratinocytes (NHEK) in cell culture. When NHEKs are induced to differentiate by high calcium ion exposure *in vitro* for up to four days, the TAp73δ isoform expression increases and all isoforms of p63 decrease [De Laurenzi, Rossi et al., 2000]. The TAp73γ, TAp73δ and TAp63 isoforms transactivate the promoters of loricrin and involucrin, two differentiation-associated molecules critical to epidermal barrier function. p73 can be detected in basal cells of the epidermis, in columnar basal cells in the hair follicle and peripheral cells, and in sebaceous glands and meibomian glands (specialized sebaceous gland found in the eyelid) [Kamiya et al., 2004]. TAp73 isoforms are reported to have roles in differentiation of neuroblastoma (both retinoic acid–induced and spontaneous) [De Laurenzi, Raschella et al., 2000], of normal myeloid cells [Tschan et al., 2000], and, together with p63, in terminal differentiation of human keratinocytes [De Laurenzi, Rossi et al., 2000]. A schematic of p53 family protein expression in skin and during wound healing is provided in Figure 4.

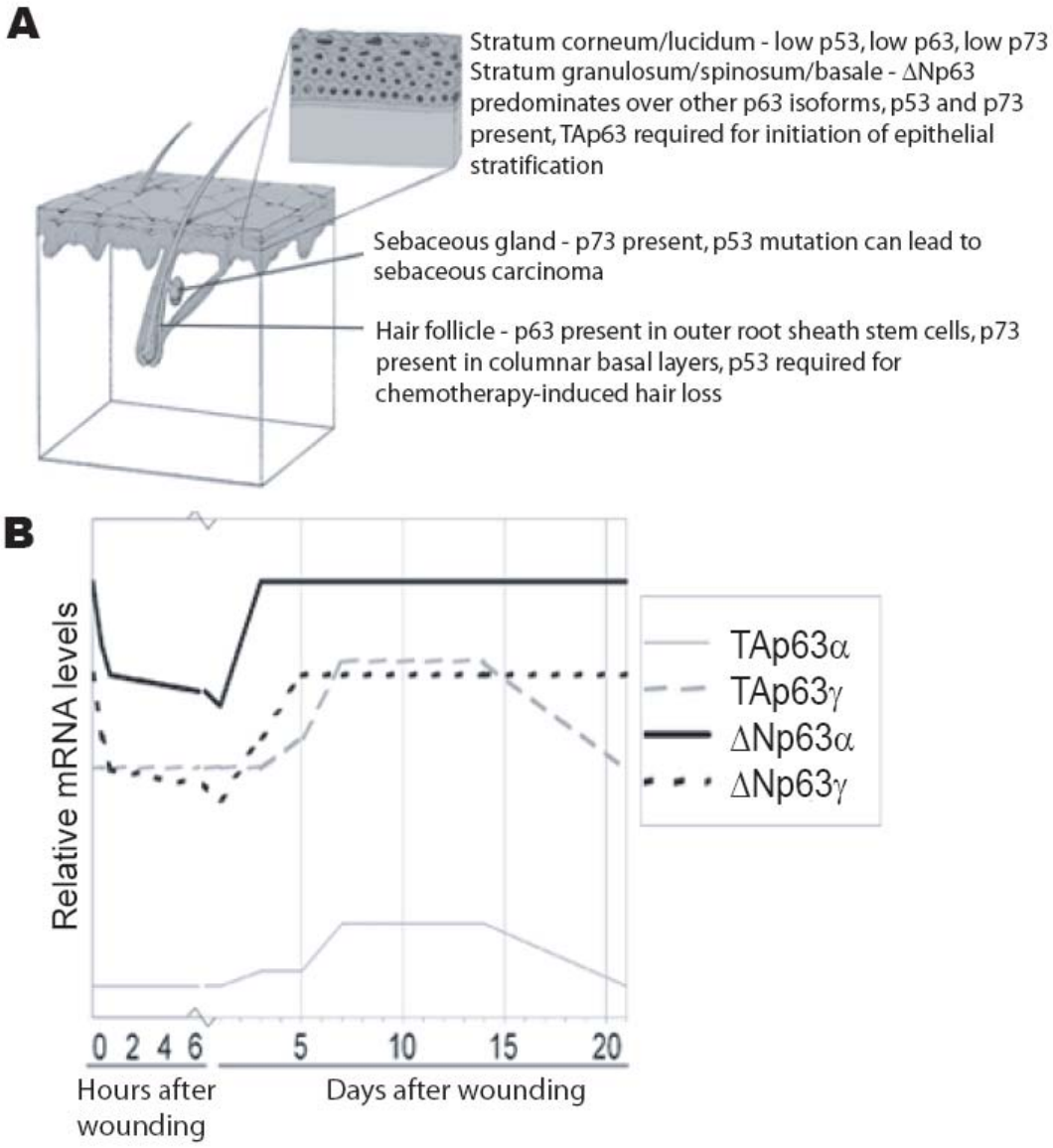

Figure 4: The p53 family in epidermal structure maintenance and wound healing

Figure 4: The p53 family in epidermis (structure maintenance and wound healing). A) Schematic depicting localization of p53 family member expression in normal skin. Epidermal structures are depicted summarizing the distribution of p53 family members in skin layers. p63 is found in the outer root sheath of the hair follicle and in basal keratinocyte stem cells and can thus function as a marker for stem cell presence. p53 and p73 are expressed in various cell types of the skin, while a delicate balance of p63 isoforms is crucial for skin development and maintenance. TAp63α is required for initiation of epithelial stratification, but ΔNp63α becomes the predominant isoform for maturing skin, promoting terminal differentiation of keratinocytes [De Laurenzi et al., 2000; Kamiya et al., 2004; Koster et al., 2004; Pellegrini et al., 2001]. B) Graph depicting relative levels of p63 isoform mRNA expressed during epidermal wound healing emphasizing the balance between TAp63 and ΔNp63 isoforms. Following wounding, ΔNp63 isoforms decrease within minutes or hours while TAp63 isoform levels increase (particularly the more transcriptionally active TAp63γ) during rapid migration and proliferation of new cells. Within 3 days of wounding, ΔNp63 expression levels increase and both TAp63 and ΔNp63 levels remain increased from day 7 to day 14. Subsequently, TAp63 expression levels decrease and ΔNp63 resumes its role in promoting terminal differentiation for maintenance of mature skin. By day 21 all isoforms return to their pre-wound levels. The graph was generated based on data obtained from [Bamberger et al., 2005; Noszczyk and Majewski, 2001].

The p63 protein is highly expressed in keratinocyte stem cells, but reduced in transient amplifying keratinocytes [Pellegrini et al., 2001]. A switch between expression of TAp63 and ΔNp63 isoforms occurs in epidermal formation during embryogenesis. The TAp63 isoforms are the first to be expressed and initiate epithelial stratification, but inhibit terminal differentiation, requiring these isoforms to be counterbalanced with ΔNp63 isoforms to allow skin to mature [Koster et al., 2004]. In normal adult skin, p63 (predominantly transcriptionally inactive ΔNp63α) is expressed in the nuclei of basal keratinocytes. However, total p63 expression decreases as cells leave the basal layer, withdraw from the cell cycle, undergo terminal differentiation, and enlarge to form mature, stratified skin. Immunohistochemical staining of total p63 protein expression can identify keratinocyte stem cells both in hair follicles and the basal keratinocyte layer [Pellegrini et al., 2001]. Analysis of total p63 expression by immunohistochemistry has also become a useful tool for marking keratinocytes with increased proliferative potential, such as in wound healing and neoplasticity [Parsa et al., 1999].

Skin wound healing involves epidermal regeneration and migration via hyperproliferation of keratinocytes. Serial biopsies of human skin healing taken at time intervals between 2 and 21 days after an injury [Noszczyk and Majewski, 2001] indicate that, initially, ΔNp63 mRNA levels are dramatically decreased in the epidermal tongue invading under the scab. TAp63γ mRNA (the TAp63 isoforms inhibit terminal differentiation) was detected in normal mouse skin 30 minutes through 14 days after wounding while TAp63α mRNA levels were absent immediately following wounding and increased steadily from 3 to 14 days after wounding [Bamberger et al., 2005]. Given that TAp63γ is the most transcriptionally active isoform of p63, it is not surprising that

this isoform is the first to be induced following wounding and initially predominates over ΔNp63γ concurrent with cellular proliferation, migration, and initial reepithelialization. However, five days after the injury, ΔNp63α mRNA is increased in basal keratinocytes, associated with normal keratinocyte differentiation, and by day 21, all p63 isoforms returned to pre-wound levels, with ΔNp63α predominating to aid in terminal differentiation and stratification. p63 immunostaining is also pronounced in psoriasis, both in the epidermal rete ridges of mature lesions and in acanthotic regions of early lesions, further suggesting p63's importance in epithelial proliferation [Shen et al., 2005].

1.5.5. The p53 family in cancer

As discussed above, the p53 protein has been well established as a tumor suppressor and is frequently mutated or inactivated in cancer [Hofseth et al., 2004]. However, the p73 and p63 genes are rarely mutated in cancer [Irwin and Kaelin, 2001]. Instead, the expression levels of p73 and p63 isoforms are frequently altered in epithelial cancers, as summarized in Table 2, suggesting either a causative role in cancer etiology or a selective pressure during tumorigenesis. Imbalances between ΔN and TA isoforms of the p53 family members in a variety of tumor types fosters debate about their function as tumor suppressors or as oncogenes [Moll and Slade, 2004]. The ΔN isoforms of p73 and p63 are frequently overexpressed in cancer and are inhibitors of the TA isoforms of all three p53 family members, leading to the loss of p53 family-dependent cell cycle arrest and/or apoptotic response [Frasca et al., 2003; Rocco and Ellisen, 2006; Rocco, Leong et al., 2006; Slade et al., 2004]. Mutant p53 can bind to and inactivate p73 [Gaiddon et al., 2001; Strano et al., 2000] and certain cancers exhibit a decrease or loss of p73 and p63 expression including mammary adenocarcinomas, squamous cell carcinomas, select

lymphoid malignancies, and bladder cancer [Ahomadegbe et al., 2000; Araki et al., 2002; Cai et al., 2000; Faridoni-Laurens et al., 2001; Ferru et al., 2005; Kamiya et al., 2004; Koga et al., 2003; Nenutil et al., 2003; Puig et al., 2003]. Deregulation of isoform expression is often associated with increased tumor invasiveness, treatment resistance, and poor patient prognosis [Koga et al., 2003; Masuda et al., 2003; Muller et al., 2005; Puig et al., 2003]. Extensive cross-talk among the many isoforms of the p53 family members [Murray-Zmijewski et al., 2006] has made it difficult to address the tissue-specific tumor suppressive or oncogenic roles each individual isoform might play and the stages of tumor progression that are driven by deregulation of p53 family member expression.

Table 2: Alterations of p63 and p73 isoform expression levels in human cancer - page 1

Cancer type	Expression level	Mechanism	Effect invasiveness of tumor?	Reference
Bladder	p73 down	LOH	more aggressive tumor growth, resistance to cisplatin	Matsumoto, 2004
Bladder	p63 down		higher tumor-node-metastasis	Koga, 2003
Bladder	p73 down		increased invasiveness	Puig, 2003
Bladder	p63 down		increased invasiveness	Urist, 2002
Bladder	p63 down	methylation		Park, 2000
Bladder	p73 up 22/23 tumors			Yokomizo, 1999
Bladder	p73 up 18/45 tumors			Chi, 1999
Breast	p73 down	LOH		Ahomadegbe, 2000
Breast	p73 up			Zaika, 1999
Breast	p73 up		poor prognosis	Dominguez, 2001
Breast	TAp63 up			Nylander, 2002
Breast	p73 up			Scwartz, 1999
Breast	p73 equal in N vs. T			Shishikura, 1999
Breast	ΔNp73 and TAp73 up		poor prognosis with ΔNp73 up	Dominguez, 2006
Breast	p73 up		advanced cases, but loss of p73 associated with distant metastasis	Yamamoto, 2002
Breast	p63 up		metaplastic disease associated with higher p63 than non-metaplastic	Koker, 2004
Breast	p63 up			Grenier, 2007
Breast	p63 down		reduced in carcinoma in situ and not expressed in invasive carcinoma	Wang, 2002
Cervical	p73 up		radiosensitivity	Liu, 2004
Cervical	ΔNp63 up			Lin, 2006
Cervical	p73α down, other p73 isoforms up		invasive islands of cells lose p73	Nenutil, 2003
Colorectal	p73 up			Sunahara 1998
Colorectal	ΔNp73 up			Dominguez, 2006
Colorectal	p63 up		poor differentiation	Carneiro, 2006
CNS	p73 up			Loiseau, 1999
CNS	p73 up			Nozaki, 2001
CNS	p73 down	methylation		Chu, 2006
Esophogeal	p73 up	Loss of imprinting		Cai, 2000
Esophogeal	p73 down		increased invasiveness	Masuda, 2003
Esophogeal	p63 up			Hall, 2001
Esophogeal	p63 and p73 up	ΔNp63α and TAp73β predominating		Cui, 2005
Esophogeal	ΔNp63 up			Hu, 2002; Geddert, 2003
Esophogeal	ΔNp63 up in majority		ΔNp63 loss = worse prognosis	Morita, 2005
Gastric	p73 down	LOH		Yokozaki, 1999
Gastric	p73 up	Loss of imprinting		Kang, 2000
Gastric	p73 down	methylation	specific to EBV-induced cancer	Ushiku, 2007
Gastric	p63 up			Tannapfel, 2001

Table 2: Alterations of p63 and p73 isoform expression levels in human cancer - page 2

Cancer type	Expression level	Mechanism	Effect invasiveness of tumor?	Reference
Hepatocellular	p73 equal in N vs. T			Mihara, 1999
Hepatocellular	p73 up			Herath, 2000
Hepatocellular	ΔNp73 and TAp73 up	isoform ratio crucial	ΔNp73 up = worse patient survival, TAp73 aided apoptosis	Muller, 2005
Hepatocellular	p73 up			Stiewe, 2004
Hepatocellular	TAp73 up			Qin, 2005
Hepatocellular	p73 up			Pan, 2002
Hepatocellular	p73 up			Zemel, 2002
Hepatocellular	p73 up			Tannapfel, 1999
Hepatocellular	p63 down			Ramalho, 2006
HNSCC	p73 down			Faridoni-Laurens, 2001
HNSCC	p63 up	gain of locus		Yamaguchi, 2000
HNSCC	p63 and p73 up			Weber, 2002
HNSCC	ΔNp63 up			Sniezek, 2004
Lung	p73 up			Mai, 1998
Lung	p73 up			Tokuchi, 1999
Lung	ΔNp73 and ΔNp63 up			Uramoto, 2006
Lung	differential p63 expression		adenocarcinoma = p63 down, SCC = p63 up	Camilo, 2006
Lung	p63 up			Massion, 2003
Lung	p63 up			Au, 2004
Melanoma	DeltaTAp73 up	splice alternative from first promoter		Tuve, 2004
Neuroblastoma	p73 up			Kovalev, 1998
Neuroblastoma	p73 up			Matos, 2001
Neuroblastoma	ΔNp73 up		reduced survival, poor outcome	Casciano, 2002
Neuroblastoma	ΔNp73 up			Douc-Rasy, 2002
Neuroblastoma	p73 up			Yang, 2000
Neuroblastoma	p73 up			Liu, 2000
Neuroblastoma	p73 down		poor prognosis	Ichimaya, 1999
Osteosarcoma	p63, p73 down			Park, 2004
Ovarian	p73 up			Imyanitov, 1999
Ovarian	p73 up			Ng, 2000
Ovarian	p73 up			Zwahlen, 2000
Ovarian	p73 up			Chen, 2000
Ovarian	p73 up		poor prognosis	Niyazi, 2003
Ovarian	DeltaTAp73 up			Concin, 2004
Ovarian	p63 down		increasing invasiveness	Poli-Neto, 2006
Prostate	ΔNp73 up			Guan, 2005
Prostate	p73 up			Takahashi, 1998
Prostate	p73 equal in N vs. T			Yokomizo, 1999
Prostate	p73 isoform disequilibrium			Arvanitis, 2004
Prostate	p63 down		prostate adenocarcinoma	Parsons, 2001
Renal	p73 equal in N vs. T			Mai, 1998
Thymic	p73 down			Ferru, 2005
Thymic	p63 up			Dotto, 2007

In one mouse model, mice heterozygous for p73 or p63 alone or in combination with each other or p53 (i.e. $p53^{+/-}/p63^{+/-}$, $p53^{+/-}/p73^{+/-}$, or $p63^{+/-}/p73^{+/-}$) developed multiple carcinomas, supporting tumor suppressive roles for p73 and p63 [Flores et al., 2005]. Heterozygosity of p73 and p63 in mice was associated with adenocarcinomas and SCC, while p53 heterozygous mice developed lymphomas and sarcomas, implying that p73 and p63 have tissue specificity for epithelial solid tumors compared to p53. This suggests tissue specificity for each p53 family member in tumor suppression. Since tumors arising in p63-heterozygous mice did not exhibit associated loss of p73 (thus tumor suppressive function of p73 should still have been intact), it was suggested that p73-dependent tumor suppression and apoptosis may require the presence of p63 [Rocco and Ellisen, 2006]. In a separately derived mouse model, however, p63 heterozygous mice did not develop any tumors, continuing the debate about the involvement of each p53 family member in tumor suppression [Keyes et al., 2006]. These carcinogenesis studies focused on mice with total knock-out of individual or combinations of p53 family members. Isoform specific roles in cancer have not been addressed for many tumor types, including SCC.

Recent studies have shown that reactivating p53 alone is sufficient to cause tumor regression in mice [Ventura et al., 2007; Xue et al., 2007]. Therefore, therapeutic strategies are being developed to make use of the p53 protein's apoptotic response to kill tumor cells [Levesque and Eastman, 2007]. Reintroducing active p53 through adenovirus-mediated transfer, reactivation of mutant p53 via small molecules, or disruption of the interaction of p53 inhibitors such as Mdm2 are methods being sought for therapeutics. However, the acute DNA damage response of the p53 protein may be contributing to the tissue damage and radiation sickness characteristic of many currently

used anti-cancer therapeutics. Thus strategies are also being sought to block p53-mediated toxicology [Gudkov and Komarova, 2005]. More research is needed to understand how to activate p53 for apoptotic response only in a tumor cell specific manner.

1.6 The p53 family as positive and negative regulators of DNA damage response to IR and UVB

All three p53 family members are induced in response to multiple DNA damaging agents [Moll and Slade, 2004] and can induce both cell cycle arrest and apoptosis [Dohn et al., 2001; Horn and Vousden, 2007; Jost et al., 1997]. The p53 and p73 proteins each have defined roles in cellular response to IR and p73 can induce apoptosis following DNA damage even in the absence of p53 [Chao et al., 2000; Liu, Chan et al., 2006; Liu, Leung et al., 2004; Oniscu et al., 2004]. However, the role of p63 in response to IR has not been well studied, though p63 is induced following IR treatment [Ongkeko et al., 2006]. Post-translational modifications often regulate activation of the p53 family members for mediation of cell fate. For example, in response to DNA damage by IR, which causes double strand DNA breaks, the ATM protein (mutated in ataxia-telangiectasia) and the related ATR protein (involved in cell cycle checkpoint arrest following DNA damage) can regulate p53 activation by mediating phosphorylation of p53 at serine-15, leading to the activation of downstream target genes. When ATM is mutated, the p53 response to IR is abrogated. However, in response to DNA damage with UV, which causes single stand DNA breaks (thymine dimer adducts), cells with mutated ATM can still induce p53 Serine-15 phosphorylation through ATR. Thus DNA damage sensor-mediated post-translational modification of p53 is one method by which cellular

response to different types of DNA damage (ie single vs. double strand) is carried out (reviewed in [Lakin et al., 1999].

The p73 protein is induced in an E2F1-dependent manner [Pediconi et al., 2003] and acetylation of p73 by both p300 and c-Abl helps differentiate p73-mediated cell cycle arrest response from the apoptotic response [Agami et al., 1999; Costanzo et al., 2002; Wang and Ki, 2001; Yuan et al., 1999]. Cell cycle checkpoint kinases, chk1 and chk2, that inhibit progression of cells through G1 and G2 phases of the cell cycle and delay progression through S phase, both mediate p53 and p73 induction [Urist et al., 2004]. The increase of p73 expression following DNA damage is cell cycle-dependent in that the p73 protein is increased only after progression through G1/S phase [Irwin et al., 2000; Wang and Ki, 2001] and TAp73 isoforms accumulate during S phase [Fulco et al., 2003]. However, increased expression of p73 is not specifically required for p73 to mediate cellular response to radiation [Liu, Chan et al., 2006]. The p73 protein is induced following treatment with UV [Lin et al., 2004] but its role in mediating UV response has not been well studied. However, the p63 and p53 proteins both are known to have a role in response to UVB. The ΔNp63α isoform was phosphorylated and downregulated in response to UVB in keratinocytes [Liefer et al., 2000; Westfall et al., 2005] and also exhibited a reduced affinity to p53 target gene binding sites due to phosphorylation [Papoutsaki et al., 2005]. Meanwhile, the TAp63γ isoform was upregulated in response to UV treatment [Katoh et al., 2000]. Thus the p53 family member isoforms mediate different cell responses to the various types of DNA damage and further work is needed to understand which isoforms are important, individually or synergistically, for mediating these responses.

1.7 Known mechanisms of cooperation and competition between p53 family members

Cooperative and competitive functional interactions between p53 family members are only beginning to be elucidated. There are multiple ways in which the p53 family members exert influence over one another. Examples are through direct protein-protein interactions between p53 family isoforms, through dominant negative inhibition of ΔN isoforms against TA isoforms, through transcriptional inter-regulation such that the transactivating isoforms govern the expression levels of other isoforms, through direct competition or cooperation at specific promoters of downstream target genes, and through interaction with common regulatory proteins such as Aspp1 and Aspp2 or Mdm2. Each of these mechanisms of inter-regulation between family members will be discussed in detail in the following section. Figure 5 illustrates the activating (A) or inactivating or aberrant (B) p53 family direct or indirect interactions.

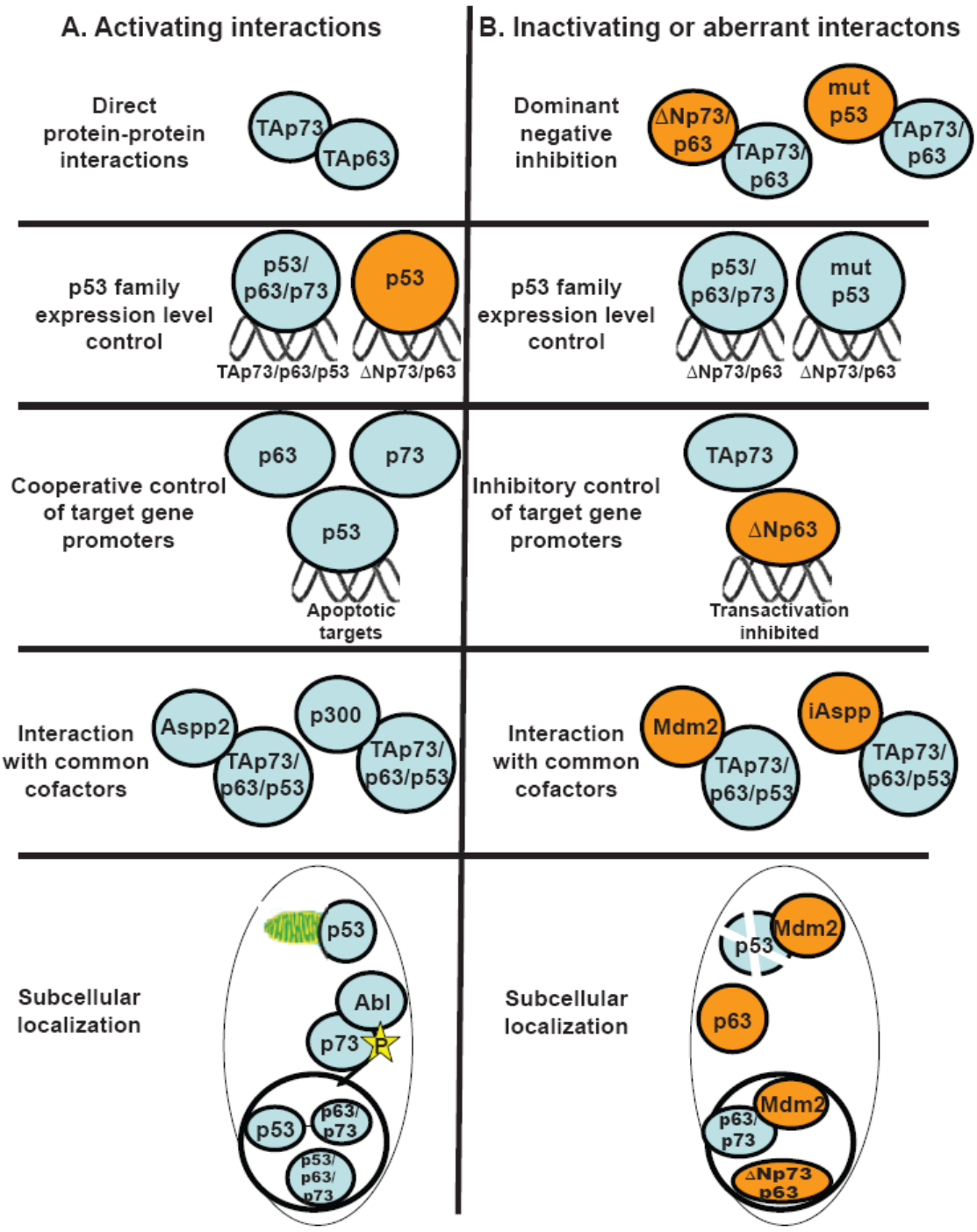

Figure 5: p53 family interactions (direct and indirect)

Figure 5: Model of known p53 family member interactions (direct and indirect). A) Schematic showing examples of activating interactions between p53 family members (direct and indirect). Activating interactions can be through direct protein-protein interactions of TA:TA (or in some cases TA:ΔN if the ΔN isoforms have a secondary transactivation domain) p63:p63, p73:p73, or p63:p73 isoforms, through p53 family members transactivating expression of TA isoforms or inhibiting transactivation of inhibitory ΔN isoforms, through cooperative upregulation of target gene expression such as the obligatory cooperation between p63 and p73 for p53-mediated apoptosis in some tissues, through interaction with activating cofactors, and through localization to the nucleus (or in the case of p53, to the mitochondria) to mediate cellular functions. Orange represents inhibition and blue represents activation. Consensus p53 DNA binding sites are shown as double helices . In the subcellular localization box, the yellow and green organelle represents mitochondria, the yellow starred "P" is a phosphorylation of p73 mediated by c-Abl to translocate p73 to the nucleus, the larger oval represents the cell membrane containing the cytoplasm, and the smaller oval represents the nucleus. B) Schematic showing examples of inactivating or aberrant interactions between p53 family members (direct and indirect). Inactivating interactions can be through dominant negative inhibition by direct protein-protein interactions of ΔN isoforms or mutant p53 to inhibit the TA isoforms, through p53 family members (including mutant p53) aberrantly upregulating expression of ΔN isoforms, through competition for consensus promoter binding sites such that the ΔN isoforms predominantly bind and inhibit TA isoforms from transactivating target genes, through interaction with inhibitory cofactors, and through alterations in subcellular localization. All of the published interactions are cited and discussed in detail in the Introduction section 1.7.

1.7.1. Direct p53 family member protein-protein interaction

The p53 protein transactivates its downstream targets by forming tetramers that interact with DNA [Stenger et al., 1992; Stenger et al., 1994; Sturzbecher et al., 1992]. The newly discovered p53 isoform, p53β, directly interacts with full length wild type p53 and enhances its transcriptional activity at the Bax promoter but has no effect on the transactivation of p21 [Murray-Zmijewski et al., 2006]. A thorough analysis of p53 isoform interactions has yet to be undertaken. The various isoforms of p63 interact with each other (e.g. heterotetramers of TA and ΔN isoforms were identified) [Yang et al., 1998]. Also the α and β isoforms of p73 were shown by yeast two hybrid screens to interact [Kaghad et al., 1997]. Further, cooperation between splice variants of p73 enhanced p73 transcriptional activity [Alarcon-Vargas et al., 2000; Ueda et al., 1999; Ueda et al., 2001].

Since p63 and p73 share approximately 40% homology with p53 in their oligomerization domains the question was raised as to whether these family members could interact with one another or with p53. Indeed, the isoforms of p63 and p73 are capable of directly associating with each other both *in vitro* and *in vivo*, but do not interact with wild type p53 [Davison et al., 1999]. Mutant p53 proteins that have altered DNA binding domain conformations, found in human tumors, interact with both p63 and p73, inhibiting their transcriptional activity [Bensaad et al., 2003; Gaiddon et al., 2001; Strano et al., 2002; Strano et al., 2000]. This inhibition by mutant p53 is thought to be one mechanism through which cell survival through inactivation of p53 family members is achieved since p63 and p73 are rarely mutated in cancer. Both the oligomerization domain and the DNA binding domains of p63 and p73 are required for interaction with

p53, but it is the DNA binding domain of p53 that mediates interaction with the other family members [Strano et al., 2000]. In fact, the only requirement for interaction of p53 with p63 and p73 is that p53 exhibit a conformational change such that the DNA binding domain is in its denatured state [Bensaad et al., 2003]. Analysis of direct protein-protein interactions between p53 family members to mediate cellular outcomes still has a long way to go. Clearly, even minor alterations in expression levels of the p53 family member isoforms, as are frequently observed in human cancer, would change the opportunity for the isoforms to interact, perhaps abolishing normal p53-family mediated cell cycle arrest and apoptosis and aberrantly promoting cell survival.

1.7.2. Dominant negative inhibition

The question was recently posed as to how many p53 mutant molecules were needed to render a p53 tetramer transcriptionally inactive. Interestingly, at least three mutants were required showing that wild type p53 is a very tenacious transactivator. However, only one ΔNp53 isoform (construct lacking the first 90 aa of p53) was required within a tetramer to completely abolish TAp53 transcriptional activity and this inhibition did not require p53 to be bound to DNA [Chan et al., 2004]. Similarly, the ΔN isoforms of p63 and p73 have been shown to be potent inhibitors of the transcriptionally active forms of the p53 family [Fillippovich et al., 2001; Lee et al., 2006; Senoo et al., 2001; Slade et al., 2004]. ΔNp63α was capable of abolishing TAp63γ transactivational activity [Yang et al., 1998] and recently it was demonstrated that in human head and neck squamous cell carcinoma (HNSCC), ΔNp63α was also a potent inhibitor of TAp73β-dependent apoptosis through both direct protein-protein interaction and through competition for binding at the promoters of apoptotic target genes [Rocco, Leong et al.,

2006]. The majority of TAp73β molecules found in HNSCC cells were in complex with ΔNp63α, which is the predominant p63 isoform found in normal epidermal tissues [Parsa et al., 1999] and is overexpressed in up to 80% of HNSCC tumors [Hu et al., 2002; Sniezek et al., 2004]. Since ΔN isoforms of both p63 and p73 are expressed in normal tissues, more studies are needed to address the regulatory roles of the ΔN isoform inhibition of p53 family member transcriptional activity. Understanding the p53 family member cooperative and competitive protein-protein interactions in normal cells may lead to mechanisms to restore that balance in cancer cells with altered expression levels of p53 family members.

1.7.3. Intra-family transcriptional control of p53 family member expression levels

Another level of inter-family regulation consists of feedback loops where family members directly transactivate other isoforms or mediate their stability. While wild type p53 is a transcriptional repressor of ΔNp63, in cancer cells ΔNp73α and mutant p53 are capable of transcriptionally upregulating ΔNp63, leading to cell survival due to repression of apoptosis [Lanza et al., 2006]. This aberrant upregulation of ΔNp63 was further enhanced upon cellular treatment with doxorubicin, showing a mechanism of cancer cell anti-chemotherapeutic survival mediated by misregulation of p53 family expression levels. The most transcriptionally active form of p63, TAp63γ activated expression of ΔNp63α. Upon loss of p53 expression, TAp63γ was stabilized, in turn driving increased expression of ΔNp63α [Li et al., 2005].

When p73 isoforms were ectopically expressed, the ΔNp73α isoform was the most stable with a half life of 4 Hrs. while TAp73α had a half life of 2.25 Hrs. and the

other isoforms were even less stable. Cotransfection of ΔNp73α and TAp73α lead to stabilization of TAp73α but in a non-transcriptionally active state due to inhibitory hetero-oligomer formation [Slade et al., 2004]. Localization of the inhibitory hetero-oligomers (e.g. DNA bound vs. unbound) was not evaluated. ΔNp73 was transactivated by TAp73 α, β, and γ and by p53 due to a p53 responsive element found within the ΔNp73 promoter [Grob et al., 2001; Kartasheva et al., 2002; Nakagawa et al., 2002; Vossio et al., 2002]. Increased expression of ΔNp73 inhibited p53 and p73-mediated transactivation of downstream target genes (including ΔNp73 itself) through competition for binding to the promoter and through formation of TAp73:ΔNp73 heterotetramers, forming an autoregulatory feedback loop. Another p53 responsive element was found in the promoter of TAp73 and it was shown that p53 and p73 could also upregulate TAp73 [Chen et al., 2001]. TAp73α stabilized the p53 protein and increased expression of p53 target genes, but the DNA binding activity of TAp73α was not required [Miro-Mur et al., 2003]. Furthermore, it was recently discovered that p53 has a p53-binding site in its own promoter and is transcriptionally regulated by both itself and by p73 [Wang and El-Deiry, 2006]. Thus the p53 family exerts multiple levels of control to govern the expression levels of the individual isoforms and the loss or aberrant expression of any of the isoforms in cancer could also affect the other family members, disrupting their activities.

1.7.4. Competition for or coordination of target promoters

Because of the significant degree of homology in their DNA binding domains, the three p53 family members interact with promoters of common, as well as divergent, downstream target genes as discussed in section 1.3.2. Some p53 family target genes have been summarized in Table 1, but the genes *p21*, *Gadd45*, *Bax*, *Noxa*, and *Puma* will

be discussed here in greater detail because they will be used as experimental targets to indicate cell cycle arrest response (*p21*), DNA damage repair response (*Gadd45*), or apoptotic response (*Bax*, *Noxa*, and *Puma*) of p53 family members in this study.

1.7.4.1. p21$^{waf1/CAP20/cip1/Sdi1}$

The cyclin-dependent kinase inhibitor, p21, is induced by both p53- dependent and independent mechanisms to cause cell cycle arrest following cellular stress, and is a part of a family of cyclin-dependent kinase (Cdk) inhibitors including p27 and p57, all involved in cell cycle checkpoint control, inhibiting all kinases involved in G1 to S progression of the cell cycle. The *p21* gene was first defined as a p53 downstream target [el-Deiry et al., 1993] and was later shown to be transactivated by both p73 and p63 [Dietz et al., 2002; Dohn et al., 2001; Zhu et al., 1998]. The p63 and p73 proteins also transcriptionally regulate p57 [Beretta et al., 2005; Blint et al., 2002; Vaccarello et al., 2006]. Mice lacking p21 are more prone to tumorigenesis, specifically spontaneously induced sarcomas and lymphomas and chemically induced malignant skin tumors [Topley et al., 1999]. Mice lacking p21 were completely defective in p53-mediated cell cycle arrest, and tumor formation was accompanied by aneuploidy and chromosomal abnormalities that were not present in mice expressing p21 [Barboza et al., 2006]. The p21 protein is a known negative regulator of apoptosis and is thought to be a first responder to DNA damage to stop the cell cycle and keep cells intact while damage is repaired [Gartel and Tyner, 2002]. The p21 promoter contains two separate p53 family DNA binding sites, both with strong homology to the p53 consensus DNA binding sequence, one at 2300 bp upstream of the start site (the stronger of the two in terms of

DNA binding affinity of the p53 family members) and the other at 1300 bp upstream of the start site [Resnick-Silverman et al., 1998].

1.7.4.2. Gadd45

After the p21 and related proteins arrest the cell cycle, proteins like Gadd45 (Growth arrest and DNA-damage-inducible gene) begin the process of DNA repair. Gadd45 is involved in regulating the nucleotide excision repair (NER) pathway following DNA damage, particularly by UVB or other single-stranded DNA-damaging agents [Smith et al., 1996; Zhan et al., 1996]. The main role of Gadd45 is to effect chromatin remodeling, allowing other DNA repair proteins to interact with the DNA [Smith et al., 2000]. Gadd45 is also involved in cell cycle arrest and can directly interact with p21 and proliferating cell nuclear antigen (PCNA, a sliding DNA clamp interacting with DNA polymerases to repair DNA damage), but must be released from p21 in order to carry out its NER pathway DNA repair function [Maeda et al., 2005]. In fact, following low-dose (<40 J/m^2) treatment with UV, cells target p21 for degradation in order to promote DNA repair [Bendjennat et al., 2003]. The *Gadd45* gene can be upregulated by all three p53 family members in response to DNA damage [Dohn et al., 2001; Hollander et al., 1993; Kastan et al., 1992; Vayssade et al., 2005]. The predominant p53 binding site is located in the third intron of the *Gadd45* gene [Hollander et al., 1993] and is strongly homologous to the p53 consensus DNA binding sequence.

1.7.4.3. Bax, Noxa, and Puma

Multiple common transcriptional targets of the p53 family are responsible for mediating apoptosis in cells (See Table 1). The Bax, Noxa, and Puma proteins are all pro-apoptotic Bcl-2 family members and *Bax* was discovered first as a p53 target [Miyashita

et al., 1994; Selvakumaran et al., 1994] followed by *Noxa* [Oda et al., 2000] and *Puma* [Nakano and Vousden, 2001]. The apoptotic target genes often contain p53 binding sites that are less homologous to the p53 consensus sequence than the cell cycle arrest or DNA damage repair-mediating target genes and are therefore less readily induced by p53 [Qian et al., 2002]. Bax exists as an inactive monomer in the cytoplasm of unstressed cells but, following DNA damage, dimerizes and translocates to the mitochondria to stimulate cytochrome c release and caspase activation, leading to the mitochondrial permeability transition and apoptosis [Hsu et al., 1997; Wolter et al., 1997]. Noxa translocates to the mitochondria and aids in activation of Caspase 9 [Oda et al., 2000], and Puma (existing as α and β isoforms) associates with Bcl-2, induces cytochrome c release, and activates procaspase 3 and 9 [Nakano and Vousden, 2001]. Family member cooperation with p53 for induction of apoptotic targets during development and in response to DNA damage is required, at least in some tissues, such as mouse embryonic fibroblasts (MEFs), and in the developing mouse central nervous system (CNS) [Flores et al., 2002]. However, this obligate cooperation for p53-dependent apoptotic response may be tissue specific, given that p63 and p73 were not similarly required in T cells [Senoo et al., 2004]. p53 itself was required for radiation- or etoposide-induced apoptosis in thymocytes [Clarke et al., 1993]. The p63 protein was shown to be localized at Bax and Noxa promoters even in the absence of p53 [Flores et al., 2002] and could mediate apoptosis through activation of Bax in the developing neuron without cooperation from p53, while p53 required p63 expression to mediate apoptosis [Jacobs et al., 2005]. The p73 protein was found to mediate apoptosis through induction of Puma and Bax and through impacting the translocation of Bax to the mitochondria (indirectly, as p73 remained localized in the

nucleus) [Melino et al., 2004]. The p63- and p73-mediated transactivation of apoptotic targets, including Puma, was enhanced by interaction with the cofactors Aspp1 or Aspp2 as will be discussed subsequently [Bergamaschi et al., 2004]. In SCC, the presence of ΔNp63α was shown to inhibit TAp73β-mediated transactivation of Noxa and Puma in a p53-independent manner through both competitive interaction at the promoters of Noxa and Puma and through direct inhibitory ΔNp63α:TAp73β hetero-oligomerization [Rocco, Leong et al., 2006]. Thus, the p53 family induction of pro-apoptotic targets and mediation of apoptosis exhibit multiple layers of cooperative or competitive interactions including direct protein-protein interactions between p53 family isoforms, dominant negative inhibition of ΔN isoforms against TA isoforms, competition or cooperation at specific promoters of downstream target genes, and interaction with common regulatory proteins.

1.7.5. Subcellular localization

The localization of p53 is crucial for its function in the cell. Clearly, as guardian of the genome, p53 must be localized in the nucleus to survey cellular damage and help mediate cell fate including cell cycle arrest, DNA repair, and apoptosis. Another role for p53 in mediating apoptosis has also been shown to require p53 localization to the cytoplasm, particularly direct localization to the mitochondria where it aids mitochondrial permeabilization [Erster et al., 2004; Mihara et al., 2003; Mihara and Moll, 2003]. Both p63 and p73 are mainly localized in the nuclei of cells and no role has yet been found for p63 or p73 at the mitochondria [Dobbelstein et al., 2005]. Aberrant cytoplasmic localization of p63 was found to be associated with poor prognosis in patients with lung cancer indicating that the predominant role of p63 for normal cellular function is nuclear

[Narahashi et al., 2006]. Endogenous p63 upregulation in lung cancer cells [Narahashi et al., 2006] and adenovirus-mediated forced p73 overexpression in neuroblastoma cell lines [Goldschneider et al., 2003] lead to nuclear accumulation of p53, suggesting that one method by which p63 and p73 aid p53 function is by maintaining p53 localization in the nucleus. In the case of TAp73, enhanced nuclear localization of p53 in an active conformation lead to upregulation of p21 and induced growth arrest in neuroblastoma cell lines where p53 had previously been inactive [Goldschneider et al., 2003].

The p73 protein is found in both nuclear and cytoplasmic compartments and its localization is mediated by interaction with other regulatory proteins. For example, the cytoplasmic tyrosine kinase Abl was shown to phosphorylate p73, activating it for apoptotic response to DNA damage as p73 translocated to the nucleus [Agami et al., 1999]. In fact, the presence of functional c-abl was required for the p73-mediated apoptotic response to IR-induced DNA damage in this study. Another interesting example of regulatory control by interacting proteins is the differentially regulated subcellular localization of p53 and p73 in response to increased expression levels of Mdm2. While p53 is exported from the nucleus and degraded by the proteosome following ubiquitination by Mdm2 (discussed in greater detail in the following section), nuclear p73 is stabilized by interaction with Mdm2. Mdm2 co-transfected with either TAp73α or β colocalized to form nuclear aggregates, reducing the transactivational activity of the TAp73 isoforms [Gu et al., 2001]. It would be interesting to see whether colocalization of p73 with Mdm2 competitively keeps Mdm2 from interacting with p53, thereby allowing p53 activity to be enhanced. This question was not addressed in this study.

1.7.6. Interaction with common cofactors

The p53 family of proteins are regulated both positively and negatively through interaction with multiple proteins. A list of some common upstream regulators of p53 family members is provided in Table 3 but specific attention is paid to negative p53 family regulation by Mdm2, transcriptional co-activation of p53 family members by p300, and co-activation of the p53 family for apoptotic control by Aspp1 and Aspp2.

1.7.6.1 Mdm2

The Mdm2 protein variably regulates stability and is a transcriptional target of all three p53 family members, establishing a feedback loop to specifically control p53 expression. Mdm2 was first shown to be a negative regulator of p53 because it inhibited transactivation of p53 target genes through interaction with p53 [Momand et al., 1992]. In fact, the Mdm2-p53 mechanistic feedback loop was first demonstrated through the p53 protein's ability to transactivate the *Mdm2* gene while the Mdm2 protein blocked this transactivation potential [Wu et al., 1993]. It was not until later that Mdm2 became also known as an E3 ubiquitin ligase targeting p53 for degradation by the proteosome [Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997]. Mdm2 interacts directly with DNA-bound p53 as has been demonstrated both *in vitro* using a DNA affinity immunoblotting assay (DAI) [Knights et al., 2003] and *in vivo* using chromatin immunoprecipitation (ChIP) [Jin et al., 2002]. A delicate balance is maintained by the cell to keep both p53 and Mdm2 in check. If unregulated by Mdm2, p53 can cause aberrant cell death [Moll and Petrenko, 2003] and inhibition of cell proliferation in tissues that are normally resistant to apoptosis [Ringshausen et al., 2006]. However, one

frequently employed mechanism of inactivating p53 in human cancer is by upregulation of Mdm2 which renders wild type p53 ineffectual as a tumor suppressor [Iwakuma and Lozano, 2003]. Small molecules that will interrupt aberrant p53-Mdm2 interactions have long been sought for use as anti-cancer agents, but further study is needed to ensure that the regulatory role of Mdm2 to keep p53 from killing normal cells is kept in check. Both p73 and p63 contain Mdm2 interaction domains and are capable of binding Mdm2 [Kadakia et al., 2001; Zeng et al., 1999]. Surprisingly, neither p63 nor p73 were targeted for proteosomal degradation following interaction with Mdm2, but instead Mdm2 inhibited p63-dependent transcription by transporting p63 out of the nucleus [Kadakia et al., 2001] and inhibited p73-dependent transcription by disrupting its interaction with cofactors [Zeng et al., 1999]. Thus far, little is known about whether interaction between Mdm2 and p53 family members is involved in cancer progression. ΔNp73 and Mdm2 together antagonized p53 more strongly than either antagonist individually [Kartasheva et al., 2002].

It bears mentioning that, besides Mdm2, the degradation of p53 family members is controlled by other E3-ubiquitin ligases including Cop1, Pirh2, and Itch. Cop1 (constitutively photomorphogenic 1) and Pirh2 were linked to degradation of p53 in mammalian cells in a manner independent of Mdm2 [Dornan, Wertz et al., 2004; Leng et al., 2003]. p53 transactivates both the Cop1 and Pirh2 genes, thereby forming two more p53-regulatory feedback loops. Cop1 was found upregulated in breast and ovarian adenocarcinomas [Dornan, Bheddah et al., 2004] and Pirh2 was found overexpressed in lung cancer [Duan et al., 2004]. The p63 and p73 proteins are not targeted for ubiquitylation and degradation by any of these three proteins that form regulatory

feedback loops with p53. Instead, the E3-ubiquitin ligase Itch/AIP4 promotes the degradation of both p63 and p73, but not p53 [Oberst et al., 2005; Rossi et al., 2005; Rossi et al., 2006]. Only the TAp73δ isoform was unaffected by Itch, but all other isoforms of p63 and p73 were ubiquitylated and degraded by this protein. The TAp73δ isoform of p73 (as well as the α, β, and γ isoforms) was cleaved by Calpain 1, suggesting that this may be the mechanism through which this isoform is regulated instead of through interaction with Itch [Munarriz et al., 2005]. Divergent regulation of degradation between the three p53 family members and their isoforms adds to the differential expression in various tissues and at various times in development and tumorigenesis.

1.7.6.2. p300

A link was first shown to exist between p300, an acetyltransferase alternatively known as cAMP-response element-binding protein (CREBP) binding protein, and p53 because viral proteins could interact with both p300 and p53, driving transformation of cells [Yaciuk et al., 1991]. However, it was later shown that p53 and p300 interacted directly without the presence of viral proteins and that this interaction activated p53 for DNA binding and transactivation of target genes [Lill et al., 1997]. The p53 family members are all three activated for transcription of downstream targets by p300 [Lill et al., 1997; MacPartlin et al., 2005; Zeng et al., 2000]. The p300 protein can acetylate the p53 family members, thereby activating them, and also acts to bring the p53 family members to their downstream target promoters. The acetylation activity of p300 is not required for stabilization of p73 at the promoters of downstream target genes and enhancement of p73 transcriptional activity [Zeng et al., 2001]. However, acetylation activity is required for activation of p63 [MacPartlin et al., 2005]. Disruption of p53

family-p300 interactions is one mechanism by which progression toward cancer can be mediated. Cancer cells with upregulated nuclear accumulation of Mdm2 have been shown to have lower levels of p300 [Alshatwi et al., 2006]. Further, the Mdm2 protein, when in complex with DNA-bound p53, kept p300 from interacting with p53, thereby abrogating p53 transactivation function [Knights et al., 2003; Kobet et al., 2000].

1.7.6.3. ASPP family of proteins

The Aspp family of proteins (Ankyrin repeats, SH3 domain, and Proline-rich region Protein or apoptosis stimulating protein of p53) consists of Aspp1, Aspp2, and inhibitory Aspp (iAspp), proteins that specifically regulate the p53 family members for apoptosis but not cell cycle function [Bergamaschi et al., 2004; Braithwaite et al., 2006; Samuels-Lev et al., 2001]. The iAspp protein is the most evolutionarily conserved Aspp family member and has been conserved from *C. elegans* to humans. Because of its function as an inhibitor of p53-mediated apoptosis and the fact that its expression confers cellular resistance to UVB and cisplatin treatment, iAspp is considered an oncoprotein [Bergamaschi, Samuels et al., 2003]. Aspp1 and Aspp2 stimulated the transactivation activity of p53, TAp63γ, and, modestly TAp73α on apoptotic targets Bax, Pig3, and Puma, but not Mdm2 or p21 [Bergamaschi et al., 2004; Samuels-Lev et al., 2001]. Human tumors expressing wild-type p53 have frequently downregulated Aspp1 and Aspp2 [Samuels-Lev et al., 2001; Sullivan and Lu, 2007], while iAspp has been found to be upregulated in cancer [Bergamaschi, Samuels et al., 2003]. Further, p53 mutants that have mutated residues important for contacting Aspp2 [Gorina and Pavletich, 1996] have been found in human cancer with relatively high frequency [Braithwaite et al., 2006]. Recently, a minimal peptide derived from p53 (37 evolutionarily conserved amino acids

from the DNA binding domain of p53) was shown to inhibit iAspp interaction with p73 regardless of p53 expression status and therefore activate p73 for apoptosis [Bell et al., 2007]. This inhibition of iAspp lead to inhibition of tumor growth *in vivo*, placing the Aspp family of proteins as a new potential target for cancer therapeutics to increase p53 family activity.

Table 3: Upstream regulators and cofactors of p53 family members

Protein	Function	Bind to p53 family members?			Reference
		p53	p73	p63	
p300/CBP	Acetylation, activation	+	+	+	Lill, 1997; Zeng, 2000; MacPartlin, 2005;
14-3-3 sigma	cell cycle	+	-	-	Yang, 2003
Aspp2/53BP2	cell cycle/ apoptosis	+	+	+	Bergasmachi, 2004
Mdm2	regulation, ubiquitylation	+	+	+	Michael, 2002
MdmX	regulation	+	+	-	Wang, 2001
Abl	signalling	+	+	-	Yuan, 1996; White, 1999
Chk 1,2	signalling	+	+	-	Dasika, 1999 Gonzalez, 2003
MAPK (p38)	signalling		involved in signalling to all three		Hildesheim, 2004; Sanchez-Prieto, 2002, Wu, 2004
PCAF	signalling	+	+	-	Liu, 1999; Zhao, 2003
PML	transcription	+	+	+	Bernassola, 2004; Bernasolla, 2005; Pearson, 2001
Sp1	transcription	+	+	+	Innocente, 2005; Koutsodontis, 2005; Romano, 2006
Sumo1	sumoylation	+	+	+	Rodriguez, 1999; Ghioni, 2005; Minty, 2000
Nedd4	neddylation	+	+	+	Miyazaki, 2003; Bakkers, 2005
Itch	degredation	-	+	+	Rossi, 2005; Rossi, 2006
Cop 1	Autoregulation	+	-	-	Dornan, 2004
Pirh2	Autoregulation	+	-	-	Leng, 2003
Pin 1	DNA damage response	+	+	-	Mantovani, 2004; Wiseman, 2005
ZEB	transcriptional repressor	+	+	-	Fontemaggi, 2001
E2F1	regulation	+	+	-	Waltermann, 2003; O'Connor, 1995
p14/p19Arf	cellular senescence	+	+	+	Guo, 2007; Kamijo, 1998
Pias-1	sumoylation	+	+	-	Munarriz, 2004; Okubo, 2004
c-Myc	inhibition	+	+	-	Watanabe, 2002; Sala, 1996
Daxx	mediator of fas signalling	+	+	+	Kim, 2003; Gostissa, 2004

A select list of regulators and cofactors of the p53 family members. Included are proteins that have been reported to interact with multiple p53 family members except for 14-3-3 sigma, Cop 1, and Pirh2, which have only been shown thus far to interact with p53. 14-3-3 sigma was included because it is also a transcriptional target of multiple family members (see Table 1) and Cop 1 and Pirh2 were included for completeness of autoregulatory cofactors of p53.

1.8. Objectives of the current study and approach

The purpose of this thesis project was to further define cooperative p53 family member inter-regulation in tumor suppression and cellular response to DNA damage in a clonal model of epidermal carcinogenesis endogenously expressing wild type p53 and multiple isoforms of both p73 and p63. The germane unanswered questions in the p53 family field that are addressed in this study are as follows:

1.8.1 Unanswered questions

1.8.1.1. In light of the expression of the multiple p53 family isoforms, does p73 have a distinct role in tumor suppression?

1.8.1.2. If p73 has a distinct role in tumor suppression can one p73 isoform mediate tumor suppression or is more than one isoform required?

1.8.1.3. Is there a functional interplay between p73 and the other p53 family members to coordinate downstream functions in keratinocytes, specifically in tumor suppression and response to DNA damage with IR and UVB?

1.8.2 Approach

The aims of this study were accomplished through:

1) Globally evaluating changes in endogenous expression levels of p53 and isoforms of p63 and p73 proteins during squamous cell carcinogenesis and following cellular treatment with IR and UVB in a mouse model of epidermal carcinogenesis.

2) Silencing expression levels of all p73 isoforms in initiated keratinocytes using siRNA and evaluating tumorigenesis and cellular response to DNA damaging agents.

3) Reconstituting initiated keratinocytes that had been selected for silencing of p73 expression with individual TAp73α or ΔNp73α isoforms and evaluating tumorigenesis and cellular response to DNA damaging agents.

4) Globally evaluating DNA binding capabilities of endogenous p53 and p63/p73 isoforms using a DNA affinity immunoblotting (DAI) assay. DNA binding capabilities of p53 family members were evaluated for changes during the process of carcinogenesis *in vitro*, in SCC tumors *in vivo*, and when expression of p73 was perturbed in initiated cells via either silencing p73 expression or reconstituting cells with individual TAp73α or ΔNp73α isoforms. DNA binding capabilities of the p53 family members were also evaluated following treatment with DNA damaging agents.

5) Evaluating p53 family inhibitory interactions with the Mdm2 protein in the presence and absence of p73 isoform expression.

1.8.3 Clonal epidermal cell model of carcinogenesis for evaluating changes in p53 family protein expression and function during tumor progression

The clonal keratinocyte model of carcinogenesis was developed to study cellular transformation in epithelial cells (the origin of most human cancers) rather than in fibroblasts (the model system in which most previous transformation studies had been conducted). In addition, epithelial cell transformation assays offered the means to study the role of differentiation and apoptosis in cancer etiology, prevention, and treatment with cells that had clear *in vivo* counterparts. Studies of epithelial cells often relied on easy to grow genomically unstable cell lines of human tumor origin, calling into question biological relevance to normal cellular behavior and protein activities. To address the need for relevant *in vitro/in vivo* epithelial transformation models, the Kulesz-Martin

laboratory optimized clonal growth conditions and established epidermal keratinocyte clones from neonatal mice. A schematic of the clonal epidermal model of carcinogenesis is provided in Figure 6. The cell lines derived from non-transformed clone 291 and sibling clones behaved "normally," growing in culture for up to 18 passages, undergoing terminal differentiation upon treatment with medium containing high calcium, and producing normal appearing epidermis upon transplantation as a skin graft. The epidermis produced by 291 cells *in vivo* was not "initiated" as it failed to produce tumors, even after tumor promoter tetradecanoyl-phorbol-13-acetate (TPA) treatment [Kulesz-Martin et al., 1985; Kulesz-Martin et al., 1988]. After initiation *in vitro* with dimethylbenz[α]anthracene (DMBA) and testing at intervals by inoculation into athymic nude mice, independent sequential lineages were produced of stages of carcinogenesis. The lineages include the initiated (precursor to SCC) clone (03C), and SCC-producing derivatives (03R) that rapidly establish poorly differentiated primary tumors in skin that, within several months, invade and metastasize to lung and lymph node. Other lineages include initiated 09C and papilloma-producing 09R, with a low risk of malignant conversion to SCC, and initiated 05C and moderately differentiating SCC-producing 05R. The carcinogenesis model has been used for direct measurements of DNA lesions induced by UVB and IR [Beehler et al., 1992; Maccubbin et al., 1995; Przybyszewski et al., 1998] as well as for studies of endogenous wild type p53 isoforms in cells treated with IR, UVB and other DNA damaging agents [Knights et al., 2003; Kulesz-Martin et al., 1994; Kulesz-Martin et al., 1995; Wu, Huang et al., 1997; Wu et al., 1994].

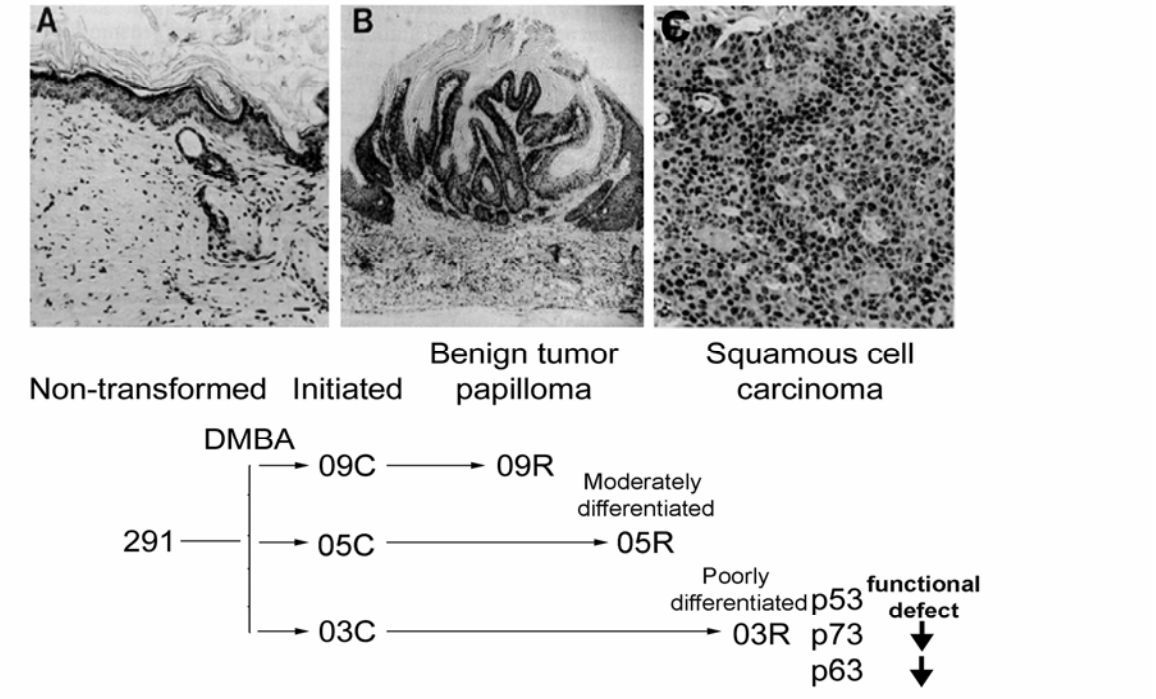

Figure 6: Mouse clonal epidermal cell model. Schematic of distinct lineages within the clonal model of epidermal carcinogenesis and histology (H&E staining) of skin graft sites and tumors produced by transplantation of cells. A) Reepithelialization of graft site 12 days after transplantation of cultured non-transformed 291 cells; secondary structures (sebaceous glands, hair follicles) are absent, as expected for a transplant site. B) Papilloma derived from transplantation of cultured 09R cells; the epithelial cells of the tumor converged with the surrounding host epidermis similarly to an endogenous tumor. C) Poorly differentiated SCC derived from transplantation of cultured 03R cells. The distinct lineages of initiation and malignant conversion include the 09R cells (corresponds with B) that produce benign papilloma, 05R cells that produce moderately differentiated SCC, and 03R cells (corresponds with C) that produce poorly differentiated, metastatic SCC [Kulesz-Martin et al., 1988].

1.8.4. DNA affinity immunoblotting assay for determination of p53 family member promoter occupation

The DNA affinity immunoblotting (DAI) assay (schematic provided in Figure 7) was developed as a sensitive, specific technique useful for evaluating DNA binding capabilities of endogenous proteins such as p53 and its relatives. It is more sensitive than traditional DNA binding assays such as EMSA and has been used to evaluate DNA binding of proteins and their associated co-factors both in cells and in tumor tissue [Knights et al., 2003; Liu et al., 2001; Liu, Lagowski et al., 2004]. An alternative DNA binding assay, ChIP, can be useful for determining the DNA binding of individual proteins in the context of chromatin and has been used to establish the localization of p53 family members to target promoters. While ChIP measures the protein occupancy of endogenous promoters in the cell, results can be limited if high affinity (immunoprecipitation quality) antibodies are not available (as is the case for isoform-specific p63 and p73 antibodies) or when one wants to evaluate the DNA binding capability of more than one protein or isoform at a time, as was required for this study. Thus, in this study, the DAI assay was used to globally evaluate endogenous p53 family member isoform DNA binding capabilities throughout the process of carcinogenesis and in response to DNA damage in wild type cells and cells that had perturbed p73 expression.

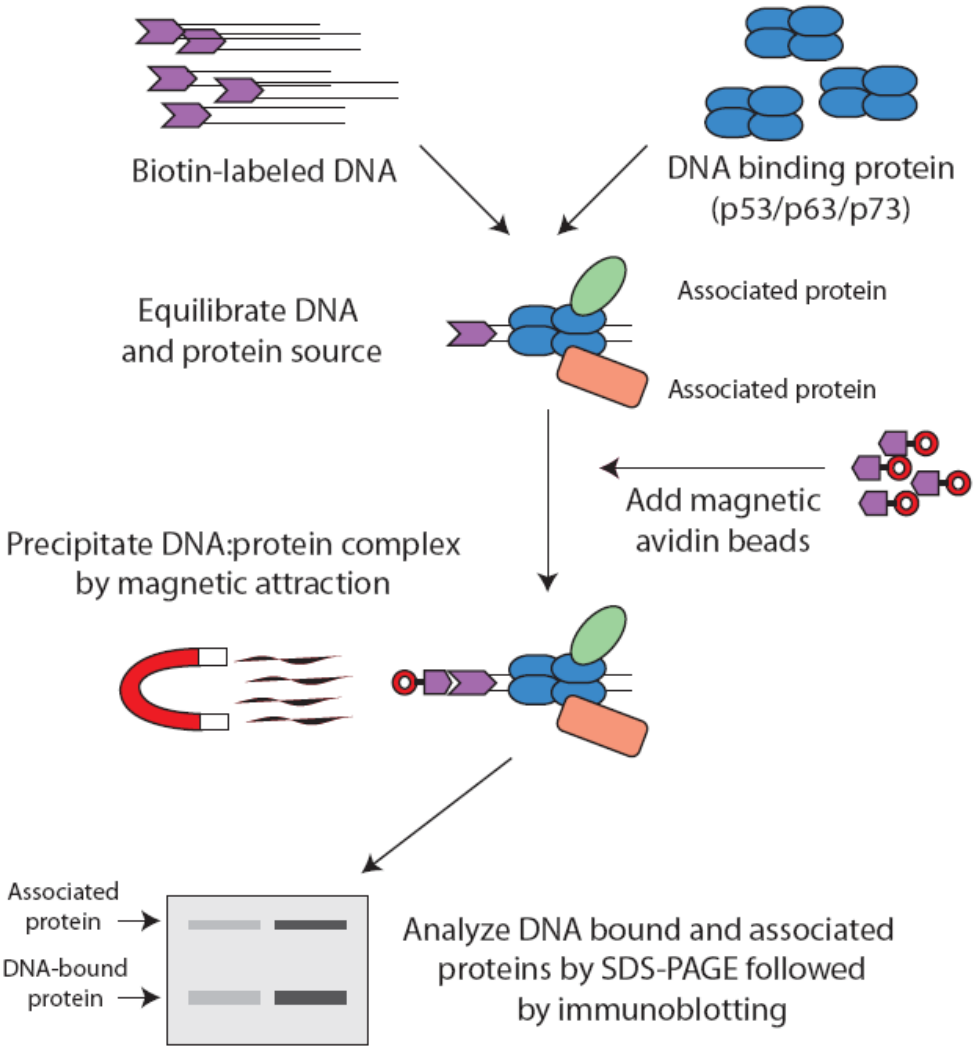

Figure 7: DNA affinity immunoblotting procedure schematic. DNA affinity immunoblotting (DAI) is a DNA binding assay that, because it is not volume restricted, is at least 3-fold more sensitive than EMSA that allows for the characterization of the DNA binding properties of endogenous cellular proteins. Lysates prepared from cultured cells or tissues are incubated with biotin-labeled DNA probes containing the binding site of interest. The DAI assay provides specificity at two levels, the DNA template and the antibody specific for the target DNA binding protein. The DAI assay also allows for the detection of proteins that associate with the DNA-bound protein of interest. The DAI assay was developed by Dr. Yuangang Liu [Liu et al., 2001] and the schematic was provided by Dr. Chad Knights from his Ph.D. thesis.

1.8.5 Overview of results

We found that p73 expression was spontaneously lost concomitant with malignant conversion in keratinocytes in the epidermal model of carcinogenesis. Further, silencing of p73 via siRNA in initiated $p53^{+/+}$ keratinocytes lead to conversion to squamous cell carcinoma (SCC) *in vivo* which was reversible upon reconstitution of TAp73α but not ΔNp73α. We investigated the cellular response to IR in the presence and absence of p73, showing that loss of p73 at malignant conversion was associated with resistance to IR *in vitro*. The loss of radiation sensitivity and malignant conversion was characterized by reduced steady state DNA binding levels of transcriptionally active p63 isoforms to the p21 promoter, failure to induce specific p53 family transcriptional targets, and failure to arrest in G1. Reconstitution of TAp73α, but not ΔNp73α, increased steady state DNA binding capabilities of TAp63β, TAp63γ, and ΔNp63γ, and increased steady state levels of p53 family target mRNA, but did not restore cellular sensitivity to IR. In the absence of TAp73α, we observed preferential DNA binding of the inhibitory ΔNp63α isoform both *in vitro* and *in vivo* in SCC suggesting that in the absence of TAp73α, a balance is tipped toward DNA binding of the inhibitory isoforms. We studied the role of the p53 family in keratinocyte response to UVB. Tumorigenic cells lacking p73 that were resistant to IR remained sensitive to UVB, accompanied by DNA binding of the TAp63γ isoform. This suggests that keratinocyte response to UVB is not dependent upon p73 and that a hierarchy of p53 family member responses to DNA damage may exist in which p73 is required for p53/p73 mediated G1/S response to DNA damage whereas p63 can mediate an apoptotic response to UVB in the absence of p73. Finally, we found that the p53 family inhibitor, Mdm2, was in complex with DNA-bound p53 family members in

malignant cells, but reconstitution of cells with TAp73α correlated with removal of Mdm2 from the complex, making them more like primary keratinocytes or initiated cells. Taking these data together, we suggest that p73 may be acting as a molecular shield to keep p53 family member inhibitors, such as ΔNp63α and Mdm2, away from DNA-bound p53 family members, allowing the TA p53 family isoforms access to their target promoters for transactivation of downstream genes. We conclude that the p73 protein is an important mediator of tumor suppression and DNA damage response in keratinocytes expressing wild type p53 and multiple isoforms of p73 and p63.

2. Materials and Methods:

For many of the subsequent methods sections, reference is made to sequence-specific features of p63 and p73 including apparent molecular weight, TA versus ΔN isoforms, specific exons differentiating C terminal splice variants, and target sequences against which siRNA is directed to silence p73. For reference, annotated, color coded nucleotide sequences of both p63 and p73 have been provided as Figures 8A and 8B.

Figure 8A: Annotated sequence of p63 page 1

GenBank AF075436
Mouse TAp63 mRNA sequence (full length)
2043 bp BASE COUNT: 536 a 622 c 471 g 414 t

When Translated:
TAp63 α = 680 aa
TAp63 β lacks exon 13 = 555 aa
TAp63 γ alternate exon 11, lacks exons 12-14 = 483 aa

Key:
Start atg
5′ UTR + Exons 1-4
Exon 5
Exon 6
Exon 7
Exon 8
Exon 9
Exon 10
Exon 11
Exon 12
Exon 13
Exon 14

Instead of grey highlighted sequence, ΔNp63 uses a start site within the third intron and contains the sequence:
atgttgtacctg gaaaacaatg cccagactca atttagtgag

gamma alternate exon 11 contains the sequence:
tctcctttca gcctgcttca ggaatgagct tgtggagccc cggggagaag ctccgacaca gtctgacgtc ttctttagac attccaaccc cccaaaccac tccgtgtacc catag

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   1 atgaattttg aaacttcacg gtgtgccacc ctacagtact gccccgaccc ttacatccag
  61 cgtttcatag aaaccccagc tcatttctcg tggaaagaaa gttattacag atctgccatg
 121 tcgcagagca cccagacaag cgagttcctc agcccagagg tcttccagca tatctgggat
 181 tttctggaac agcctatatg ctcagtacag cccatcgagt tgaactttgt ggatgaacct
 241 tccgaaaatg gtgcaacaaa caagattgag attagcatgg attgtatccg catgcaagac
 301 tcagacctca gtgaccccat gtggccacag tacacgaacc tggggctcct gaacagcatg
 361 gaccagcaga ttcagaacgg ctcctcgtcc accagcccct acaacacaga ccacgcacag
 421 aatagcgtga cggcgccctc gccctatgca cagcccagct ccacctttga tgccctctct
 481 ccatcccctg ccattccctc caacacagat tacccgggcc cacacagctt cgatgtgtcc
 541 ttccagcagt caagcactgc caagtcagcc acctggacgt attccaccga actgaagaag
 601 ctgtactgcc agattgcgaa gacatgcccc atccagatca aggtgatgac cccaccccca
 661 cagggcgctg ttatccgtgc catgcctgtc tacaagaaag ctgagcatgt caccgaggtt
 721 gtgaaacgat gccctaacca tgagctgagc cgtgagttca atgaggaca gattgcccct
 781 cccagtcatc tgattcgagt agaagggaac agccatgccc agtatgtaga agatcctatc
 841 acgggaaggc agagcgtgct ggtcccttat gagccaccac aggttggcac tgaattcaca
 901 acagtcctgt acaatttcat gtgtaacagc agctgcgtcg gaggaatgaa cagacgtcca
 961 attttaatca tcgttactct ggaaaccaga gatgggcaag tcctgggccg acggtgcttt
1021 gaggcccgga tctgtgcttg cccaggaaga gaccggaagg cagatgaaga cagcatcaga
1081 aagcagcaag tatcggacag cgcaaagaac ggcgatggta cgaagcgccc tttccgtcag
1141 aatacacacg gaatccagat gacttccatc aagaaacgga gatccccaga tgatgagctg
1201 ctgtacctac cagtgagagg tcgtgagacg tacgagatgt tgctgaagat caaagagtca
1261 ctggagctca tgcagtacct ccctcagcac acgatcgaaa cgtacaggca gcagcagcag
1321 cagcagcacc agcacctact tcagaaacag acctcgatgc agtctcagtc ttcatatggc
1381 aacagttccc cacctctgaa caaaatgaac agcatgaaca agctgccttc cgtgagccag
1441 cttatcaacc cacagcagcg caatgccctc actcccacca ccatgcctga gggcatggga
1501 gccaacattc ctatgatggg cactcacatg ccaatggctg gagacatgaa tggactcagc
1561 cctacccaag ctctccctcc tccactctcc atgccctcca cctcccactg caccccacca
1621 ccgccctacc ccacagactg cagcattgtc agtttcttag caaggttggg ctgctcatca
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Figure 8A: Annotated sequence of p63 page 2

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1681 tgcctggact atttcacgac ccaggggctg accaccatct atcagattga gcattactcc
1741 atggatgatt tggcaagtct gaagatccct gaacagttcc gacatgccat ctggaagggc
1801 atcctggacc acaggcagct gcacgacttc tcctcacctc ctcatctcct gaggacccca
1861 agtggtgcct ctaccgtcag tgtgggctcc agtgagaccc gtggtgaacg tgtgatcgat
1921 gccgtgcgct ttaccctccg ccagaccatc tcttttccac cccgtgacga gtggaatgat
1981 ttcaactttg acatggattc tcgtcgcaac aagcagcagc gtatcaaaga ggaaggagaa
2041 tga
```

Figure 8B: Annotated sequence of p73 page 1

GenBank NM_011642
Mouse TAp73 mRNA sequence (full length)
2452 bp BASE COUNT: 515 a 832 c 694 g 411 t

When Translated:
TAp73 α = 636aa
TAp73 β lacks exon 13 = 499aa
TAp73 γ lacks exon 11 = 475aa
TAp73 δ lacks exons 11-13 = 404aa
TAp73 ε lacks exons 11 and 13 = 555aa
TAp73 ξ lacks exons 11 and 12 = 540aa

Key:
Start atg
5′ UTR + exons 1-4
Exon 5
Exon 6
Exon 7
Exon 8
Exon 9
Exon 10
Exon 11
Exon 12
Exon 13
Exon 14

Instead of grey highlighted sequence, ΔNp73 uses a start site within the third intron and contains the sequence:
atgctttacg tcggtgaccc catgagacac ctcgccac

Sequence used for siRNA KD of all p73 isoforms

qPCR primers used for detection of p73 α and β mRNA

```
   1 tggtcccgct tcgaccaaga ctccggctac cagcttgcgg gccccgcgga ggaggagacc
  61 ccgctggggc tagctgggcg acgcgcgcca agcggcggcg ggaaggaggc gggaggagcg
 121 gggcccgaga ccccgactcg ggcagagcca gctggggagg cggggcgcgc gtgggagcca
 181 ggggcccggg tggccggccc tcctccgcca cggctgagtg cccgcgctgc cttcccgccg
 241 gtccgccaag aaaggcgcta agcctgcggc agtcccctcg ccgccgcctc cctgctccgc
 301 acccttataa cccgccgtcc cgcatccagg cgaggaggca acgctgcagc ccagccctcg
 361 ccgacgccga cgcccggccc ggagcagaat gagcggcagc gttggggaga tggcccagac
 421 ctcttcttcc tcctcctcca ccttcgagca cctgtggagt tctctagagc cagacagcac
 481 ctactttgac ctccccccagc ccagccaagg gactagcgag gcatcaggca gcgaggagtc
 541 caacatggat gtcttccacc tgcaaggcat ggcccagttc aatttgctca gcagtgccat
 601 ggaccagatg ggcagccgtg cggccccggc gagcccctac accccggagc acgccgccag
 661 cgcgcccacc cactcgccct acgcgcagcc cagctccacc ttcgacacca tgtctccggc
 721 gcctgtcatc ccttccaata ccgactaccc cggcccccac cacttcgagg tcaccttcca
 781 gcagtcgagc actgccaagt cggccacctg gacatactcc ccactcttga agaagttgta
 841 ctgtcagatt gctaagacat gccccatcca gatcaaagtg tccacaccac cacccccggg
 901 cacggccatc cgggccatgc ctgtctacaa gaaggcagag catgtgaccg acattgttaa
 961 gcgctgcccc aaccacgagc ttggaaggga cttcaatgaa ggacagtctg ccccggctag
1021 ccacctcatc cgtgtagaag gcaacaacct cgcccagtac gtggatgacc ctgtcaccgg
1081 aaggcagagt gtggttgtgc cgtatgaacc cccacaggtg ggaacagaat ttaccaccat
1141 cctgtacaac ttcatgtgta acagcagctg tgtgggggggc atgaatcgga ggcccatcct
1201 tgtcatcatc accctggaga cccgggatgg acaggtcctg ggccgccggt ctttcgaggg
1261 tcgcatctgt gcctgtcctg gccgtgaccg caaagctgat gaagaccatt accgggagca
1321 acaggctctg aatgaaagta ccaccaaaaa tggagctgcc agcaaacgtg cattcaagca
1381 gagcccccct gccatccctg ccctgggtac caacgtgaag aagagacgcc acggggacga
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Figure 8B: Annotated sequence of p73 page 2

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1441 ggacatgttc tacatgcacg tgcgaggccg ggagaacttt gagatcttga tgaaagtcaa
1501 ggagagccta gaactgatgg agcttgtgcc ccagcctttg gttgactcct atcgacagca
1561 gcagcagcag cagctcctac agaggccgag tcacctgcag cctccatcct atgggcccgt
1621 gctctcccca atgaacaagg tacacggtgg tgtcaacaaa ctgccctccg tcaaccagct
1681 ggtgggccag cctcccccgc acagctcagc agctgggccc aacctggggc ccatgggctc
1741 cgggatgctc aacagccacg gccacagcat gccggccaat ggtgagatga atggaggcca
1801 cagctcccag accatggttt cgggatccca ctgcaccccg ccacccccct atcatgcaga
1861 ccccagcctc gtcagttttt tgacagggtt ggggtgtcca aactgcatcg agtgcttcac
1921 ttcccaaggg ttgcagagca tctaccacct gcagaacctt accatcgagg accttggggc
1981 tctgaaggtc cctgaccagt accgtatgac catctggagg ggcctacagg acctgaagca
2041 gagccatgac tgcggccagc aactgctacg ctccagcagc aacgcggcca ccatctccat
2101 cggcggctct ggcgagctgc agcggcagcg ggtcatggaa gccgtgcatt tccgtgtgcg
2161 ccacaccatc acaatcccca accgtggagg cgcaggtgcg gtgacaggtc ccgacgagtg
2221 ggcggacttt ggctttgacc tgcctgactg caagtcccgt aagcagccca tcaaagagga
2281 gttcacagag acagagagcc actgaggaac gtaccttctt ctcctgtcct tcctctgtga
2341 gaaactgctc ttggaagtgg gacctgttgg ctgtgcccac agaaaccagc aaggaccttc
2401 tgccggatgc cattcctgaa gggaagtcgc tcatgaacta actccctctt gg
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Figure 8: Annotated sequences of p63 and p73 used for studies. A) Annotated sequence of mouse p63 (GenBank AF075436) describing features pertinent to these studies, i.e. sequences coding each exon and alternative sequences encoding ΔNp63 and the p63γ alternative exon 11. B) Annotated sequence of mouse p73 (GenBank NM_011642) describing pertinent features as for p63 but also including the sequence used for siRNA silencing of p73 isoforms via siRNA and qPCR primers used for detection of p73 mRNA.

2.1. Cell culture and treatment (IR and UVB)

As described in [Kulesz-Martin et al., 1988], the non-transformed murine epidermal cell line 291 as well as the p53-null epidermal cell line NK1, obtained from A. Balmain (UCSF Comprehensive Cancer Center, San Francisco, CA), were grown in "low calcium medium" composed of Eagle's minimal essential medium (EMEM) with L-Glutamine, non-essential amino acids, and Eagle's salts without $CaCl_2$ (Invitrogen). This medium base is supplemented with 5% (v/v) fetal calf serum (Atlanta Biologicals) treated with Chelex-100 resin (Bio-Rad) to reduce Ca^{2+} concentration, 10% (v/v) mouse dermal fibroblast conditioned medium, 10 ng/ml of epidermal growth factor (EGF) (Upstate Biotechnology), 0.8% (v/v) antibiotic/antimycotic solution (Invitrogen) and 0.04 mM Ca^{2+}. Primary keratinocyte cell lysate was provided by Dr. Kathryn King in Dr. Wendy Weinberg's laboratory, FDA, Bethesda, MD. Initiated 03C and malignant 03R derivatives of the 291 parental strain were maintained in EMEM lacking EGF or conditioned medium but containing 5% native fetal calf serum and 1.4 mM Ca^{2+}. The 03C si-p73, 03C si-Control, and 03C si-p73 + Vector, + ΔNp73α, or + TAp73α cells were maintained under the same conditions used for 03C cells except that the medium was supplemented with 2.5 μg/ml puromycin to maintain integration of the siRNA into the genome and/or 400 μg/ml G418 to maintain integration of p73 isoforms. Additional cell clones generated for this study but not used to obtain data are listed in Table 5a. Cells in log phase growth at ~ 50-70% confluence were treated with IR using a ^{137}Cs source irradiator or UVB with lightbulbs from National Biological Corporation (Twinsburg, OH). IR and UVB doses used for this study were selected based on dose response curves (a range of doses from 4 Gy to 30 Gy IR and from 70 to 270 J/m^2 UVB were tested) for

cell cycle arrest versus apoptotic response of cells in the epidermal carcinogenesis model as well as publications citing optimal conditions. Doses as low as 0.5 Gy impact cell cycle progression while the apoptotic index only increases after 4 Gy (16% cell death 48H post treatment with 4 Gy IR vs. 48% cell death 48H post treatment with 32 Gy IR) [Bishay et al., 2001]. The UVB dose of 135 J/m^2 was selected as a mid-range dose to allow both cell cycle arrest and apoptotic cellular response in our cell lines. This dose correlated with a study showing that MCF7 human breast cells treated with 125 J/m^2 UVB had a 37% death rate while nearly 100% of cells treated with 300 J/m^2 UVB died [Koch-Paiz et al., 2004].

2.2. Cell and tissue protein lysate preparation

For whole cell extraction, cells were lysed at 4^{o}C for 1 Hr. in whole cell extraction buffer (20 mM HEPES [pH 7.5], 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, adjusted to pH 7.5 and supplemented with 1 mM Na_3VO_4, 50 mM NaF, 1 mM DTT, 0.5 mM PMSF, 1 μM leupeptin, 1 μM pepstatin, and 0.1 μM aprotinin), centrifuged at 13,000 rpm for 10 min., and the supernatants stored at -80^{o}C. For preparation of lysate from tumor tissue, tumors were snap frozen in liquid nitrogen and pulverized in liquid nitrogen using a mortar and pestle. Pulverized tumor was transferred into a 2 mL snap freezing tube until all liquid nitrogen had evaporated. Tissue was washed once in 600 μl wash buffer (100 mM KCl, 20 mM HEPES, 10 mM EDTA, 20% glycerol, 1 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin) and centrifuged at 2000 rpm for 10 min at 4^{o}C. Wash buffer was removed and the pellet was resuspended in whole cell extraction buffer for lysis at 4^{o}C for 1 Hr., centrifuged at 13,000 rpm for 10 min., and the supernatants stored at -80^{o}C.

2.3. Detection of p53 family member protein expression by immunoblotting

Protein concentrations were determined by spectrophotometry readings at 595 nm visible light comparing to bovine serum albumin (BSA) standards (New England Biolabs) using Bradford protein dye reagent (Bio-Rad). Whole cell lysate aliquots of 40 μg were incubated for 5 min. at 95^{o}C in sample buffer (100 mM Tris HCl [pH6.8], 100mM DTT, 20% glycerol, 2% SDS, and bromophenol blue) and separated by 8% SDS-PAGE in electrophoresis buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). Following electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher and Schull) in 4^{o}C transfer buffer (25 mM Tris, 192 mM glycine, and 20% (v/v) methanol) at 100 volts for 1 Hr. Membranes were blocked in either TBS-t (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.05% Tween-20) containing 5% nonfat milk powder (for p63), or TBS-t containing 5% BSA (Sigma) (for p53/p73). Proteins were reacted with specific antibodies to p53 (pAb122 hybridoma cells purchased from ATCC, also called pAb421 Ab-1 from Calbiochem), pan p63 (4A4 monoclonal from Santa Cruz, detects all isoforms), p63α (H-129 from Santa Cruz), ΔNp63 (p40 Ab-1 from Oncogene), p73 (monoclonal 5B1288 from Imgenex, detects all isoforms), Mdm2 (2A10 from Calbiochem) or actin (C-2, Santa Cruz) as loading control. See Table 4 for complete information about antibodies and protocols (all antibodies listed were raised against human epitopes). Following reaction with specific primary antibody, membranes were incubated for 1 Hr. in TBS-t + milk with a 1:2000 dilution of secondary IgG-HRP conjugate antibody and washed three times (20 Min. each) with TBS-t milk. Membranes were incubated for 5 min. in Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) and proteins visualized by exposure to Kodak XO-MAT film. Transfection

of Myc-tagged p63 isoform plasmids (TAp63α, TAp63γ, TA*p63α, TA*p63β, TA*p63γ, ΔNp63α, ΔNp63γ provided by Dr. Frank McKeon, Harvard Medical School, Boston, MA) and Flag-tagged p73 plasmids of the various isoforms (TAp73α, TAp73β, ΔNp73α, ΔNp73β, Ex2delp73α, Ex2delp73β generously provided by Dr. Ute Moll, University of Stony Brook, New York, NY) were used as positive controls for size comparison to determine endogenous p63 and p73 isoform expression within the SCC model.

Table 4: Antibodies and conditions used in this study

Antibody	Source	Human Epitope	Clonicity	Species recognized	Blocking buffer	Time/temp	Dilution	Dilution buffer	Time/temp
p53 pAb122	Hybridoma, ATCC	371-380	m	m, h	5% milk in TBS-t	overnight/ 4 C	1ml:2ml	Tbs-t	1 hr./ RT
p53 pAb421 (ab-1)	Calbiochem	371-380	m	m, h	5% milk in TBS-t	overnight/ 4 C	1:500	Tbs-t	1 hr./ RT
pan p63 (4A4)	Santa Cruz	1-205	m	m, h	5% milk in TBS-t	overnight/ 4 C	1:1000	Tbs-t	1 hr./ RT
p63α (H-129)	Santa Cruz	513-641	p	m	5% BSA in TBS-t	1 hr./ RT	1:500	Tbs-t	overnight/ 4 C
ΔNp63 (p40 Ab-1)	Calbiochem	N-terminus	p	m	5% BSA in TBS-t	overnight/ 4 C	1:500	Tbs-t	1 hr./ RT
p73 (Clone 5B429)	Imgenex	N-terminus	m	m, h	5% BSA in TBS-t	overnight/ 4 C	1:500	Tbs-t	1 hr./ RT
Mdm-2 (Ab-2, 2A10)	Calbiochem	294-339	m	m	5% milk in TBS-t	overnight/ 4 C	1:500	Tbs-t	1 hr./ RT
Actin (C-2)	Santa Cruz	C-terminus	m	m, h	5% milk in TBS-t	overnight/ 4 C	1:1000	Tbs-t	1 hr./ RT
Goat anti-mouse IgG-HRP	Pierce		m				1:2000	5% milk in TBS-t	1 hr./ RT
Goat anti-rabbit IgG-HRP	Jackson Immunoresearch		m				1:2000	5% milk in TBS-t	1 hr./ RT

Antibody information for optimal detection of proteins by immunoblotting. The human epitope against which the antibodies were raised has been provided, but all antibodies used are capable of cross reacting with mouse proteins.

2.4. Silencing of p73 via siRNA

To silence all isoforms of p73 in the initiated 03C cells, the Dharmacon siDesign Center (www.dharmacon.com/DesignCenter/DesignCenterPage.aspx) was utilized to obtain likely sequences for targeting p73 by siRNA and the si-p73 and si-Control sequences were blasted against the mouse genome to determine specificity (www.ncbi.nlm.nih.gov/BLAST). Three siRNA sequences against p73 were tested for specificity by transient transfection before choosing one for cloning. Oligos were synthesized by the OHSU oligonucleotide and sequencing core to contain the chosen 19 nucleotide siRNA p73 sequence (5'GGGACTTCAATGAAGGACA3') or siRNA control sequence (5' GTTCACCGTTCAAAGCCAG3') and subcloned into the pSilencer vector (Ambion) modified to contain a puromycin selectable marker (termed pSilencerpuro) as instructed by Ambion using *ApaI* and *EcoRI* cloning sites. A complete list of plasmids generated for this study, some of which were not used to obtain data, is given in Table 5b. The pSilencerpuro vectors (2.5 μg DNA purified using Qiagen HiSpeed plasmid purification kit per 100 mm plate) were transfected into the 03C cells at 50% confluence using the lipofectamine 2000 reagent (Invitrogen) at 20μl per 100 mm plate. DNA:lipid complexes were incubated for 30 min. in 1 mL medium at room temperature before being brought to 3 mL volume and incubated with the cells for 4 Hrs. at 37^{o}C. The volume per 100 mm plate was brought to 10 mL for overnight incubation. The following day, the medium was replaced with medium containing puromycin (Sigma) at 2.5 μg/ml final concentration and refreshed every 3 days. Colonies were selected within 2 weeks of adding the drug. Silencing of p73 was confirmed by immunoblotting and by qPCR.

Clones were designated 03C si-p73 or 03C si-Con followed by a clone identification number.

2.5 Reconstitution of p73 isoforms to si-p73 cells

To reconstitute TAp73α or ΔNp73α in the 03C si-p73 cells, cells were transfected as above (2.4.) with pcDNA3 plasmids expressing Flag-tagged p73 isoforms and clonally selected after growth in medium containing G418 (Sigma 400 μg/ml media) and purimycine (2.5 μg/ml final concentration). The siRNA sequence used to silence mouse p73 isoforms was (5'GGGACTTCAATGAAGGACA3'), immediately proceeded in the mouse sequence by an AA dinucleotide (GenBank accession NM_011642 nucleotides 985-986 followed by the siRNA target, nucleotides 987-1005). This same siRNA target can be found in the human p73 sequence (GenBank accession NM_005427 nucleotides 712-730) with the exception of a cytosine substitution at position 11 in the human sequence (compared to a thymine in the mouse). However, a GA dinucleotide immediate proceeds this target in the human p73 sequence (nucleotides 710-711), making the siRNA effective only against mouse p73 isoforms according to rules for siRNA design, Ambion technical bulletin #506, www.ambion.com. Constitutive expression of the p73 isoforms was confirmed by immunoblot and clones were designated using the clone identification number from the 03C si-p73 parental cells, an indication of which isoform was expressed, and a new clone identification number.

Table 5a: Cell clones generated for this study

Cell clone name	Parent cell	Experimental use	Constructed by	Cell bank location
03C siControl 4	03C	Control cells that maintain "03C-like" qualities for testing against 03C si-p73 clones	Jodi Johnson	13-2
03C siControl 5	03C	Control cells that maintain "03C-like" qualities for testing against 03C si-p73 clones	Jodi Johnson	13-2
03C siControl 1, 3, 6 clones	03C	Control cells that maintain "03C-like" qualities for testing against 03C si-p73 clones	Jodi Johnson	13-2
03C si-p73 2-3	03C	Silencing of p73 (all isoforms) in 03C cells	Jodi Johnson	13-2 and 19-2
03C si-p73 2-4	03C	Silencing of p73 (all isoforms) in 03C cells	Jodi Johnson	13-2 and 19-2
03C si-p73 2-1 thru 2-12 clones (12 clones total)	03C	Silencing of p73 (all isoforms) in 03C cells - MAJORITY REGAINED p73 EXPRESSION	Jodi Johnson	13-2 and 19-2
03C si-p73 + PCDNA3 vector only clone 7	03C si-p73 2-3	Control of 03C with silenced p73 to test against clones where have added back single p73 isoforms	Jodi Johnson	19-5
03C si-p73 + PCDNA3 vector only clone 8	03C si-p73 2-4	Control of 03C with silenced p73 to test against clones where have added back single p73 isoforms	Jodi Johnson	19-5
03C si-p73 + ΔNp73α clone 3	03C si-p73 2-4	03C with silenced p73 stably expressing ΔNp73α (human)	Jodi Johnson	19-5
03C si-p73 + TAp73α clone 1	03C si-p73 2-4	03C with silenced p73 stably expressing TAp73α (human)	Jodi Johnson	19-5
03C si-p73 + TAp73α clone 4	03C si-p73 2-4	03C with silenced p73 stably expressing TAp73α (human)	Jodi Johnson	19-5
03C si-p73 + TAp73α clone 10	03C si-p73 2-3	03C with silenced p73 stably expressing TAp73α (human) (has lost expression of ΔNp63α isoform)	Jodi Johnson	19-5

Clones 2-3 and 2-4 maintained stable silencing of p73 (termed 03C si-p73-1 and 03C si-p73-2 respectively throughout the described studies). Clones 2-1, 2-2, 2-5, 2-6, and 2-7 retained silenced p73 up to passage 14 post transfection, but by passage 25 had regained expression of p73. Clones 2-8 thru 2-12 had regained p73 expression at passage 14 post transfection. Clones 2-1, 2-3, 2-4, 2-6, and 2-9 expressed increased levels of Mdm2 (comparable to the 03 cells), while the remaining clones expressed levels of Mdm2 comparable to the untransfected 03C or 03C si-Control cells. Only the 2-3 and 2-4 clones were used to obtain data for this study but the other clones are available for future studies.

Table 5b: Plasmids constructed for this study

Plasmid name	Parent vector	Experimental use	Constructed by/Source	Glycerol stock number
pSilencer 1.0-U6			Ambion	417
pSilencerpuro	pSilencer 1.0-U6	Cloning target siRNA for selection in cells with puromycin	Sally El-Hizawi	432
pSilencerpuro p63 1-5	pSilencerpuro	siRNA against p63 clone 1-5 for stable selection in cells with puromycin	Jodi Johnson	565
pSilencerpuro p73 1-5	pSilencerpuro	siRNA against p73 clone 1-5 for stable selection in cells with puromycin	Jodi Johnson	566
pSilencerpuro p73 2-5	pSilencerpuro	siRNA against p73 clone 2-5 for stable selection in cells with puromycin	Jodi Johnson	567
pSilencerpuro Noxa 1-5	pSilencerpuro	siRNA against Noxa clone 1-5 for stable selection in cells with puromycin	Jodi Johnson	568
pSilencerpuro Noxa 2-4	pSilencerpuro	siRNA against Noxa clone 2-4 for stable selection in cells with puromycin	Jodi Johnson	569
GST p73α-myc	pGEX-2T	expression of human p73α	Freda Miller Lab	496
pcDNA6/TR	pcDNA6	expression of tetracyclin repressor	Invitrogen	501
pcDNA4-GFP	pcDNA4	to test tet repressor cells for inducibility of gene expression (GFP)	Stephen Lanker lab	538
pTREX DEST 30		for expressing gene of interest in tet-repressable system	Invitrogen	502
pTREX DEST 31		for expressing gene of interest in tet-repressable system - contains his tag	Invitrogen	503
pENTRp53T21E	pENTR/D Topo	Entry vector for Gateway system for initial cloning of PCR amplified p53T21E (from 452)	Jodi Johnson	504
pENTRp73α	pENTR/D Topo	Entry vector for Gateway system for initial cloning of PCR amplified p73a (from 496)	Jodi Johnson	505
pDEST30p53T21E	pTREX DEST 30	Subcloned from 504 so is now ready for use in tet-repressable system	Jodi Johnson	515
pDEST31p53T21E	pTREX DEST 31	Subcloned from 504 (his tag) so is now ready for use in tet-repressable system	Jodi Johnson	516
pDest30p73α	pTREX DEST 30	Subcloned from 505 so is now ready for use in tet-repressable system	Jodi Johnson	517
pDEST31p73α	pTREX DEST 31	Subcloned from 505 (his tag) so is now ready for use in tet-repressable system	Jodi Johnson	518
pENTRwtp53	pENTR/D Topo	Entry vector for Gateway system for initial cloning of PCR amplified wild type p53 (from 422)	Jodi Johnson	556
pDEST30wtp53	pTREX DEST 30	Subcloned from 556 so is now ready for use in tet-repressable system	Jodi Johnson	557
pDEST31wtp53	pTREX DEST 31	Subcloned from 556 (his tag) so is now ready for use in tet-repressable system	Jodi Johnson	558
GSTBBP		Generating peptide to make BBP (Aspp2) antibody	Charles Lopez lab	423
pcDNA3TAp63α	pcDNA3	Expression of myc-tagged p63 isoform (mouse)	Frank McKeon lab	506
pcDNA3TAp63γ	pcDNA3	Expression of myc-tagged p63 isoform (mouse)	Frank McKeon lab	507
pcDNA3TA*p63α	pcDNA3	Expression of myc-tagged p63 isoform (mouse)	Frank McKeon lab	508
pcDNA3TA*p63β	pcDNA3	Expression of myc-tagged p63 isoform (mouse)	Frank McKeon lab	509
pcDNA3TA*p63γ	pcDNA3	Expression of myc-tagged p63 isoform (mouse)	Frank McKeon lab	510
pcDNA3ΔNp63α	pcDNA3	Expression of myc-tagged p63 isoform (mouse)	Frank McKeon lab	511
pcDNA3ΔNp63γ	pcDNA3	Expression of myc-tagged p63 isoform (mouse)	Frank McKeon lab	512
pcDNA3TAp73α	pcDNA3	Expression of Flag-tagged p73 isoform (human)	Ute Moll lab	589
pcDNA3TAp73β	pcDNA3	Expression of Flag-tagged p73 isoform (human)	Ute Moll lab	590
pcDNA3ΔNp73α	pcDNA3	Expression of Flag-tagged p73 isoform (human)	Ute Moll lab	591
pcDNA3ΔNp73β	pcDNA3	Expression of Flag-tagged p73 isoform (human)	Ute Moll lab	592
pcDNA3Δex2p73α	pcDNA3	Expression of Flag-tagged p73 isoform (human)	Ute Moll lab	593
pcDNA3Δex2p73β	pcDNA3	Expression of Flag-tagged p73 isoform (human)	Ute Moll lab	594

Vectors used for the current studies are described in the main text but the remaining vectors listed here are available for future studies.

2.6. Cell morphology studies

Cells were plated at a density of 2.5 million cells per 150 mm culture plate (Fisher Scientific) and photographed daily to observe growth characteristics. A Leica DMIRE2 microscope (Leica Microsystems) was used at 200X magnification and cells were photographed using a Magnafire Mega-Pixel digital camera (Media Cybernetics) and Magnafire imaging software. After 96 Hrs., cells were trypsinized in 0.25% Trypsin (Invitrogen) and re-counted to determine the growth rate.

2.7. Colony formation (viability) assays

Cells were trypsinized 24 Hrs. following either mock treatment or treatment with 4 Gy IR and plated to obtain at least 100 colony forming cells per control 60mm plate in triplicate. Eleven days after plating, the cells were fixed with 100% methanol for 2 Hrs., stained with 10% (v/v) Giemsa (Sigma Diagnostics), and washed three times with deionized water. Colonies were photographed with a digital camera and counted using Imagepro Plus software (Media Cybernetics). Standard deviation and graphical representation of this data was performed with Microsoft Excel.

2.8. Flow cytometry for cell cycle analysis

Triplicate plates of cells were grown to 70% confluence and either mock treated or treated with 4 Gy IR or 135 J/m^2 UVB. After 24 Hrs., cells were trypsinized and resuspended in cold calcium-free medium. After centrifugation and counting, 2 million cells from each of the individual triplicate plates were transferred to siliconized borosilicate glass tubes (Fisher Scientific). Cells were washed twice in Phosphate Buffered Saline (PBS Invitrogen) and resuspended in 1 ml PBS before fixation in ice-cold 100% ethanol with vortexing. Cells were stored at 4^oC until staining with propidium

iodide (PI Molecular Probes). For PI staining, cells were washed twice in cold PBS to remove ethanol and resuspended in PI solution (PBS, 0.1% Triton X-100, 200μg/ml PI, 100 μg/ml RnaseA (Amersham)) with incubation at 37^{o}C for at least 30 min. before analysis by the OHSU Cancer Center FACS core. At least 10,000 events were counted and the Modfit cell cycle analysis program was used for data analysis.

2.9. Caspase-3 apoptosis assays

Cells were either mock treated or treated with IR (8 Gy or 30 Gy), or UVB (270 J/ m^2) and harvested by scraping into the medium 15Hr after treatment. Samples were centrifuged in 50 mL falcon tubes at 1000 rpm for 5 min. and the cell pellet was resuspended in 1 mL cold PBS and transferred to a 1.5 mL eppendorf tube. The cells were centrifuged for 8 min. at 800 rpm at 4^{o}C, PBS was removed, and the cells were resuspended in 50 μl cytosolic extraction buffer (50 mM HEPES [pH 7.5], 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$). Cells were lysed by freeze/thawing through emersion of the tubes in a dry ice/ethanol bath followed by a 37^{o}C water bath for five cycles. Tubes were centrifuged at 13,000 rpm at 4^{o}C for 12 min. and the protein concentration was measured by spectrophotometry as above (2.3.). The caspase-3 colorimetric substrate (Ac-DEVD-pNA EMD Biosciences) was diluted to a final concentration of 2 mM (1.3 mg/mL) in reaction buffer (100 mM HEPES [pH7.5], 10% glycerol, 0.1% CHAPS, 0.1 mg/mL BSA) and stored in aliquots at -80^{o}C. The assay was performed in triplicate for each sample in a 96 well plate using 40 μg of protein lysate and 200 μM Ac-DEVD-pNA diluted to 250 μl final volume in reaction buffer. Absorbance at 405 nm was measured every Hr. for 12 Hrs. using a Spectramax Plus plate reader and SoftMAX Pro software (Molecular

Devices Corporation) and the average slope of the triplicate reactions was calculated to determine caspase-3 activity.

2.10. *In vivo* mouse tumorigenesis studies – newborn injections and xenografts

Newborn Balb/C mice (3 days old) were injected in the subcutaneous space between the shoulder blades with 2.5 million cells (03C cells, 03C si-Con cells, 03R cells, 03C si-p73 cells or 03C si-p73 cells + Vector, + ΔNp73α, or + TAp73α) in 50 μl of 1.4 mM Ca^{2+} serum-free EMEM using a 22g needle. For skin grafting experiments, cells were trypsinized and centrifuged at 1000 rpm for 4 min. at 4^{o}C and resuspended in 1.4 mM Ca^{2+} serum-free EMEM so that the slurry was at a concentration of 5 million cells per 30 μl serum-free EMEM. Cells were placed on ice until ready to be transplanted. Mice were anaesthetized using oxygen:isoflorane (provided by the OHSU department of animal care surgery core) and two wound sites were produced on the mouse's back using a 6 mm biopsy punch. The cells were pipetted into the wound sites (5 million cells per graft site), Duoderm (ConvaTec) was used to dress the wounds, and the wound area was bandaged with tape. Viability of cells was confirmed by re-plating cells from the slurry at the end of the grafting procedure. Mice were monitored weekly for tumor development and tumors were measured using calipers. When a tumor reached 1 cm in length or width, the animal was euthanized and the tumor was fixed in formalin for analysis by hematoxylin and eosin (H&E) staining as well as snap frozen in liquid nitrogen for subsequent preparation of protein lysate as described above (2.2.).

2.11. DNA affinity immunoblotting (DAI)

2.11.1 Preparation of probes

Specific biotinylated probe DNA sequences with p53 consensus sites in blue of the mouse distal p21 promoter (2300bp upstream of the transcriptional start site,: ggactagctt tctggccttc aggaacatgt cttgacatgt), the mouse Mdm2 (located in the first intron tgtggggctg gtcaagttgg gacacgtccg gcgtcggctg tcggaggagc taagtcctga catgtctcca gc), mouse Noxa (located in the promoter aggcttgccccggcaagttg), mouse Puma (located in the first intron ctgcaagccccgacttgtcc), mouse Bax (located 500bp upstream of the transcriptional start site tgacaagcatatcccaggcaagctttgaacttgcgg), or a negative control sequence lacking p53 binding sites (Neg PCR amplified from a region 3400 bp upstream of the p21 transcriptional start site) were generated by PCR amplification from 291 cell genomic DNA template to best represent the native p53 binding sites found in each promoter. Each PCR reaction included 1.25 ng of each primer (sequence shown in Table 6), 1 µl genomic DNA template, 1 µl 10 mM dNTPs (Fermentas), 5 µl 10X pfu reaction buffer (200 mM Tris-HCl [pH 8.8], 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)SO_4$, 1% Triton X-100, 1 mg/ml BSA), 1.25 units pfu DNA polymerase (Stratagene), and RNAse free water (Ambion). PCR reactions were performed in a Mastercycler Gradient thermocycler (Eppendorf) using 30 cycles of 95°C for 30sec, 58°C for 30sec, and 72°C for 30 sec with a 3 min. extension time at 72°C. The products were electrophoresed in a 1.5% agarose gel and purified according to the manufacturer's instructions using QIAquick gel purification kit (Qiagen). To ensure plentiful pure probe, a further round of PCR amplification was performed using the PCR products from the first reaction as

template followed by gel purification. Sequences were verified at the OHSU Cancer Institute sequencing core facility.

Table 6: Primers used for DAI probe amplification

Probe	Length (bp)	5' biotinylated PCR primer	3' PCR primer
Neg	164	5'TCTAAAGTCCCTGCCTTCC3'	5'CGAGATAGTTCAGTGAATGCC3'
p21	158	5'GCATCAGTCCTCCCATCC3'	5'GCCCCACCTCTTCAATTCC3'
Mdm2	150	5'ACGGGTGGGACTGGGCTGGG3'	5'ACGGCTGCGGAAACGGGG3'
Noxa	199	5'TGCCCCAGCAATGGATAC3'	5'GAACCGAGCGAAGTGGAG3'
Puma	199	5'CTCTGGCTGCCGGGAAAC3'	5'CTCTATGCCGCGTCTCAG3'
Bax	144	5'CAATACTCCCGTCCTGCC3'	5'CCCCGCCCCCATTATTTC3'

2.11.2. DAI analysis of p53 family member DNA binding capabilities in cells and in tumor tissue

The DAI assay protocol has been described [Liu et al., 2001; Liu, Lagowski et al., 2004]. Figure 9 exhibits the reliability and sensitivity of the DAI assay as well as correlation with ChIP. Aliquots of 400 μg lysate were prepared from mock treated cells, cells harvested 3 Hrs. or 24 Hrs. following treatment with 4 Gy IR, cells harvested 5 Hrs. or 24 Hrs. following treatment with 135 J/m^2 UVB, or tissue harvested from mouse SCC tumors. The lysate was diluted in 1X DNA binding buffer (DBB) [20 mM Tris (pH 7.2), 3% glycerol, 1 mM EDTA, and 0.06% Triton X-100 supplemented with 50 mM NaF, 1 mM Na_3VO_4, 5 mM DTT, and 10 μg salmon sperm DNA) and incubated for 30 min. with rocking at 4°C in a microcentrifuge tube with 2 nm of the specific biotinylated probe DNA sequences described above (2.11.1.). The DNA:protein complexes were precipitated with 0.1 mg of magnetic streptavidin beads (Promega) with rocking for 1.5 Hrs. and washed 3 times with 1X DBB +NaCl (same as DBB above, but with 150 mM NaCl added). The DNA-bound lysate was loaded on 8% SDS-PAGE gels for analysis by immunoblotting with antibodies as described above (2.3.).

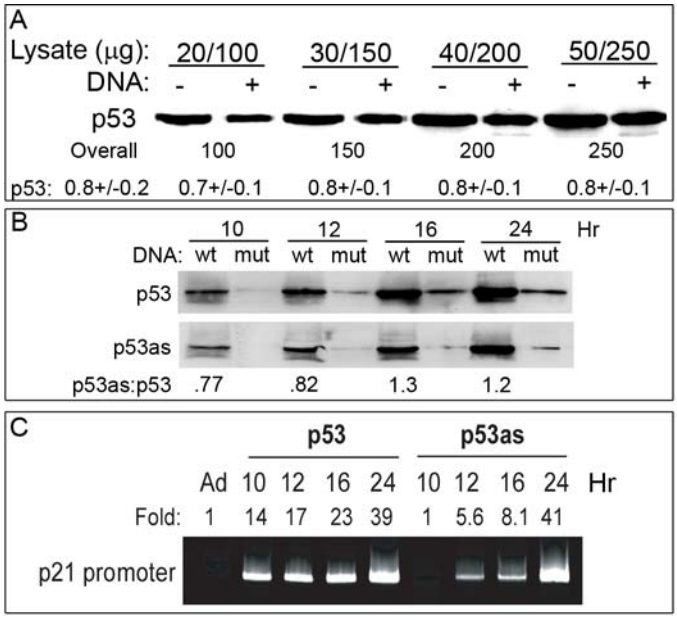

Figure 9: DAI as quantitative method – reliability, sensitivity, specificity, and correlation with ChIP. A) Reliability (linearity) of DAI for analysis of DNA binding activities of p53 in MCF7 cells. The signal intensities of total (-) p53 protein (20-50 μg loaded) vs. DNA-bound (+) protein (100-250 μg loaded) as indicated were quantified and expressed as a functional index as summarized directly below the immunoblot. The mean functional index is shown from three DAI assays with cell lysates from three independent harvests of MCF7 cultures (one experiment is shown). B) DAI assay comparing DNA-bound p53 to DNA bound p53as (alternatively spliced). We reported that p53as is delayed in ability to locate its DNA binding sequences compared to p53 due to the lack of the basic C terminus [Liu, Lagowski et al., 2004]. Cell lysates from indicated time points were subjected to DAI with biotinylated p53 consensus DNA (wt) and mutant p53 consensus DNA (mut). Quantification of the ratio of p53as to p53 below the immunoblot shows that while p53 is bound to the consensus site 10 hours following IR treatment, p53as does not reach full DNA binding capacity until later time points. C) ChIP to determine p53 and p53as present at the p21 promoter *in vivo* showing the delay of p53as binding to the p21 promoter in the context of chromatin compared to p53. The bound p21 promoter DNA was amplified with primers labeled with infrared dye 800 and quantified using an infrared imaging system. The relative quantification was calculated with signal intensity of $p21^{Waf1}$ DNA from H1299 cells infected with control adenovirus (Ad) as a reference. These experiments and the figure were provided by Dr. Yuangang Liu, modified from [Liu et al., 2001; Liu, Lagowski et al., 2004].

2.12. Immunoprecipitation (IP)

To determine p53 family member interactions in the mouse model of SCC, aliquots of 400 μg lysate were prepared from mock treated or treated cells. The cell lysate was brought to equivalent volumes by addition of whole cell extraction buffer (as in 2.2.) and to a total of no more than 125 mM NaCl with RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.3% SDS) then pre-cleared with 50 μg protein A sepharose beads (Amersham Pharmacia) for 30 min. with rocking at 4^{o}C. The sample was centrifuged at 2000 rpm for 5 min., beads removed, and 1 μg of each antibody specific to the p53 family members as described in 2.3. was added for incubation with rocking at 4^{o}C overnight. Antibody was precipitated by addition of 50 μg protein A sepharose beads for 1.5 Hrs. The beads were centrifuged at 2000 rpm for 5 min. and were washed four times (first and fourth washes with whole cell extraction buffer and second and third washes with SNNTE buffer: 50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1% Nonidet P-40, 500 mM NaCl, and 5% sucrose). A final centrifugation at 13,000 rpm was performed and the precipitated antibodies resuspended in sample buffer as in 2.3. The resulting p53 family complexes were separated by 8% SDS-PAGE and detected by immunoblotting as in 2.3.

2.13. Chromatin immunoprecipitation (ChIP)

To detect p53 family members bound to the p21 promoter in the context of chromatin, each plate of 1 X 10^{7} mock treated or treated cells was cross-linked with 1% formaldehyde (Fisher) and incubated for 15 min. with rocking at room temperature. The reaction was stopped with 125 mM glycine (final volume) and the cells were washed twice with cold PBS. Cells were collected by scraping in 5 ml of hypotonic buffer (1%

NP-40, 50 mM NaCl, 10 mM Tris [pH 8.0]) and incubated at 4°C for 10 min. then centrifuged at 2000 rpm for 5 min. at 4°C and resuspended in 600 μl/plate of cold RIPA buffer (as in 2.12.). Cells were sonicated with a Branson 250 sonifier at "3" setting ten times for 10 sec. each to generate 200-1000 bp DNA fragments. Debris was pelleted by centrifugation for 10 min. at 13,000 rpm at 4°C, 30 μl supernatant was aliquoted to be used as "input" control for the PCR step, and the supernatant was pre-cleared for 30 min. with rocking at 4°C with 50 μg of protein G sepharose beads. Aliquots of 200 μg supernatant were diluted to 1.2 ml in RIPA buffer and incubated overnight at 4°C with 2 μg of each p53 family member antibody (as in 2.3.) or negative control IgG isotype control antibody in the presence of 50 μg of protein G blocked beads. The beads had been blocked overnight with rocking at 4°C in 200 μg/mL sonicated salmon sperm DNA (Invitrogen) and 260 μg/mL BSA. Immunocomplexes were washed nine times with RIPA buffer followed by one wash with TE buffer then eluted with 200 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$ + 10 μg RNAse A per sample) for 15 min. at room temperature and beads were removed. The DNA-protein cross-links were reversed by adding 200 mM NaCl and incubating at 65°C overnight. DNA was purified by extraction with equal volume (200 μl) phenol:chloroform:isoamyl alcohol followed by centrifugation at 14,000 rpm. The top layer was transferred to another 1.5 ml eppendorf tube and extracted with equal volume of chloroform and centrifuged as above. The top layer was transferred to a fresh tube and precipitated by adding one tenth volume 3M Na Acetate, 2 μl of glycogen (Fermentas), and 2.5 volumes of 100% ethanol followed by overnight incubation at -20°C and final wash with 70% ethanol. DNA was centrifuged at

14,000 rpm for 10 min. and resuspended in 40 μl of TE buffer. Triplicate qPCR reactions (20 μl) for each sample and primer set were performed in a 384 well plate (Applied Biosystems, Inc) in the presence of UDP-N-glycosidase (Invitrogen) and SYBR-Green I dye (Applied Biosystems Inc.). Primers (6 μM) were 5' AGCAGGCTGTGGCTCTGATT 3' and 5' CAAAATAGCCACCAGCCTCTTCT 3' for the p21 promoter (contains the same p53 binding site found 2300kb upstream of the p21 transcriptional start site that was PCR amplified to generate the p21 probe for the DAI assay in section 2.11.1) and 5' CCAGCTGGGCTCTGCAATT 3' and 5' GCTGAGAGGGTACTGAAGGGAAA 3' for the negative control (7878kb downstream of the p21 transcriptional start site so that sheared chromatin containing the p21 promoter will not also contain this negative control sequence) [Gomes et al., 2006]. Data was collected using a 7900HT thermocycler (Applied Biosystems, Inc.) and analyzed using the $\Delta\Delta C_T$ method (Applied Biosystems Incorporated user bulletin #2, December 11, 1997).

2.14. Preparation of RNA and cDNA

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen), treated with DNAse I (Invitrogen) to remove genomic contamination, and purified using the RNeasy mini RNA extraction kit according to the manufacturer's specifications (Qiagen). One μg of RNA was reverse transcribed with AMV reverse transcriptase (Roche) and random hexamers (Integrated DNA Technologies) at 42°C for 1 hr. A "negative (-) RT reaction", lacking reverse transcriptase, was performed to verify lack of DNA contamination. One μl of the resulting cDNA products were amplified with β-actin gene specific primers [β-actin1 (AGGCTGGCCTTTGACACCTACCAGG) and β-actin2

(TCTGTTGTGTTTCCTCCCTGTTGG)] using a Mastercycler gradient PCR machine (Eppendorf) to confirm the presence of cDNA (300 bp) and absence of genomic DNA (gDNA 481bp).

2.15. Quantitative reverse transcriptase polymerase chain reaction (qPCR) of p53 family target genes

Triplicate qPCR reactions (15 μl) for each sample and primer set were performed in a 384 well plate (Applied Biosystems, Inc) using gene-specific primers (6 μM each summarized in Table 7, validated for linearity and target specificity) in the presence of UDP-N-glycosidase (Invitrogen) and SYBR-Green I dye (Applied Biosystems Inc.). SYBR-Green I fluoresces upon binding to the minor groove of double-stranded DNA, allowing the quantification of the double-stranded amplicon in real time. Gene expression data was collected using a 7900HT thermocycler (Applied Biosystems, Inc.). Data was analyzed using the $\Delta\Delta C_T$ method (Applied Biosystems Incorporated user bulletin #2, December 11, 1997).

Table 7: qPCR primers for p53 family downstream targets

Primer name	5' Primer sequence	3' Primer sequence
Mp21	5' CCATGTCCAATCCTGGTGATG 3'	5' CGAAGAGACAACGGCACACTT 3'
Mnoxa	5' ACTGTGGTTCTGGCGCAGAT 3'	5' TGAGCACACTCGTCCTTCAAGT 3'
Mpuma	5' GCGGCGGAGACAAGAAGAG 3'	5' TCCAGGATCCCTGGGTAAGG 3'
Mbax	5' GGGAAGGCCTCCTCTCCTACT 3'	5' GAGGACTCCAGCCACAAAGATG 3'
Mgadd45	5' TGGTGACGAACCCACATTCA 3'	5' CACGGGCACCCACTGATC 3'
Mp73	5' GAGTCACCTGCAGCCTCCAT 3'	5' CACCACCGTGTACCTTGTTCAT 3'
M18s	5' CGGCTACCACATCCAAGGAA 3'	5' CCTGTATTGTTATTTTTCGTCACTAC CT 3'

2.16. Detection of p63 and p73 RNA isoform expression by RT-PCR

To detect the various p63 isoforms by RT-PCR, primer and PCR reactions specified by collaborator Dr. Dennis Roop (Baylor University, Waco, TX) were performed with modifications. The methods used to determine the p63 isoforms expressed in mouse keratinocytes are shown in Figure 10 including immunoblotting with isoform-specific antibodies, RT-PCR, and comparison with published results from [King et al., 2006]. Primers for p63 or p73 were selected at the specific exon/intron junctions (see figures 8A and 8B) of α, β and γ forms for the 3' primer, and TA vs. ΔN specific junctions for the 5' primers (summarized in tables 8 and 9). PCR reactions to detect the shorter N terminal or C terminal p63 and p73 fragments were 40 cycles of 92°C for 30 sec, 56°C for 1 min, and 72°C for 1 min with a 15 min extension time at 72°C. PCR reactions to detect the long fragments (i.e. TAα, TAβ, etc.) were 40 cycles of 92°C for 1 min, 56°C for 2 min, and 72°C for 3 min with a 15 min extension time at 72°C. Myc-tagged p63 plasmids or FLAG-tagged p73 plasmids of the various isoforms were used as positive controls. Because the relative level of p73 mRNA was nearly undetectable by RT-PCR we used qPCR for investigations of p73 mRNA (Table 7).

Table 8: p63 isoform RT-PCR primers:

Primer name	Primer sequence	
M63ΔNF	5' TTGTACCTGGAAAACAATG 3'	
M63TAF	5' TCGCAGAGCACCCAGACA3'	
63SEQ.2F	5' CCACCACAGGTTGGCACTG 3'	
63SEQ.3F	5' CCAGATGATGAGCTGCTGTACC 3'	
M63-2R	5' GCATCGTTTCACAACCTCG 3'	
M63-4R	5' TAGTCCAGGCATGATGAG 3'	
M63-βR	5' CAGACTTGCCAAATCCTGAC 3'	
M63-γR	5' GGCTCCACAAGCTCATTC 3'	
Primer Combination	**Product length (bp)**	**Specific for:**
M63TA.F + M63-2R	~600	TA N-terminus
M63ΔNF + M63-2R	~400	ΔN N-terminus
63SEQ.3F + M63-4R	~450	α C-terminus
63SEQ.3F + M63-βR	~450	β C-terminus
63SEQ.2F + M63-γR	~450	γ C-terminus
M63TAF + M63-4R	~1600	TAp63α
M63ΔNF + M63-4R	~1400	ΔNp63α
M63TAF + M63-βR	~1550	TAp63β
M63ΔNF + M63-βR	~1350	ΔNp63β
M63TAF + M63-γR	~1250	TAp63γ
M63ΔNF + M63-γR	~1050	ΔNp63γ

Table 9: p73 isoform RT-PCR primers:

Primer name	5' Primer sequence	3' Primer sequence
MTAp73	5' TCTCTAGAGCCAGACAGCAC 3'	5' GTGGAGCTGGGCTGCGCGTAC 3'
MΔNp73	5' TTTACGTCGGTGACCCCA 3'	5' GTGGAGCTGGGCTGCGCGTAC 3'
Mp73α	5' CTGAAGGTCCCTGACCAGTA 3'	5' CTCTGTGAACTCCTCTTTGA 3'
Mp73β	5' GACCGCAAAGCTGATGAAGA 3'	5' TGACTCGGCCTCTGTAGGAG 3'
Mp73γ	5' CGGGATGCTCAACAGCCAC 3'	5' TGCAGGTGGTAGATGCTCTG 3'

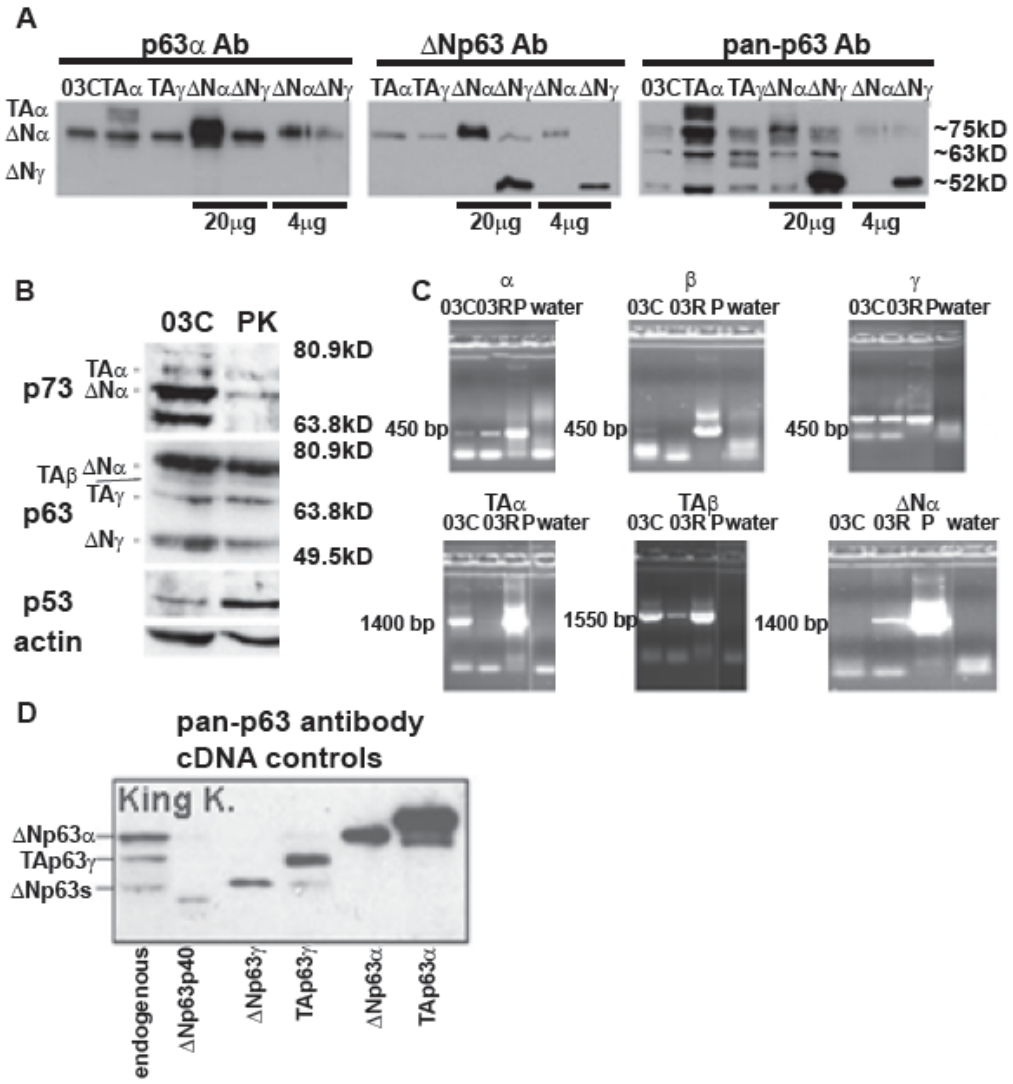

Figure 10: Determination of p63 isoform expression in clonal epidermal carcinogenesis model

Figure 10: Determination of p63 isoform expression. A) Immunoblot of 03C cells transfected with p63 isoforms. Cells were transfected with TAp63α, TAp63γ, ΔNp63α, or ΔNp63γ and blotted for ΔNp63 or p63α in addition to blotting with the pan-p63 4A4 antibody that detects all isoforms. Size comparison indicated that the cells expressed, in order of apparent molecular weight from greatest to least, ΔNp63α (~75kD), TAp63β (~73kD), TAp63γ (~63kD), an unknown p63 isoform (suspected to be ΔNp63β based on migration size, but may also be ΔNp63s), and ΔNp63γ (~52kD). B) Immunoblot of p53 family member protein expression levels in 03C cells and primary mouse keratinocytes (PK). All p63 isoforms expressed in the initiated 03C cells are also detected in the primary cells as well as p53 and at least 2 isoforms of p73. C) RT-PCR analysis of basal p63 mRNA expression in 03C and 03R cells (performed by the undergraduate summer research assistant that I mentored for three summers, Sarah Lawson, Gonzaga University). Plasmid DNA (P) was used as a positive control and water as a negative control. Sequencing at the OHSU core laboratory confirmed the presence of the short α, β, and γ as well as the long TAα, TAβ, and ΔNα sequences of p63 isoforms. Though TAp63α was detected at the RNA level in 03C cells, the TAp63α protein was not detected. D) Immunoblot figure taken from [King et al., 2006] showing p63 isoform expression in mouse primary keratinocytes compared to keratinocytes transfected with p63 isoform cDNA controls as indicated.

2.17. Microarray analysis

The global gene expression changes associated with loss of p73 protein expression in the 03C si-p73 cells was analyzed compared to the 03C si-Con cells and the 03R cells. Gross data are presented in the Appendix but individual gene changes are not discussed. RNA from biological replicates was isolated using TRIzol followed by purification with the RNeasy mini RNA extraction kit (Qiagen). 10 μg of RNA was submitted to the OHSU Cancer Institute Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resource for processing. Affymetrix mouse GeneChip® 430 2.0 (Affymetrix, Inc.) was used for hybridization, and data was analyzed using Microarray Suite (MAS) version 4.0 or 5.0 (Affymetrix) and Excel (Microsoft, Bellevue, WA). A novel unbiased microarray data analysis method, positional ranking and normalization (PRNP), was also used to rank gene expression changes between cell samples (analysis performed by Carl Pelz, OHSU). Probe sets with small or no shifts represented the invariant genes between samples that were used to normalize the data. When calculating fold changes, the samples were first normalized to an idealized reference sample (normally the average of the control samples) using these invariant probe sets. When ranking the most up/down regulated genes between groups of biological replicates, a consensus of all possible pair wise comparisons between the biological replicates was used to produce a robust ranking. Another method, distance weighted discrimination (DWD, modified from [Benito et al., 2004] by Pelz) was used to analyze discriminating overall genetic “signatures” or metagenes of compared cell lines. Microarray analyses comparing cells in the murine model of epidermal carcinogenesis have been reported in [Kulesz-Martin et al., 2005; Wang et al., 2002].

3. Results: p73 loss triggers conversion to squamous cell carcinoma reversible upon reconstitution with TAp73α

The manuscript prepared from the results discussed in Chapter 3 has been accepted for publication in *Cancer Research* in the August 15, 2007 publication (issue 67, volume 16 pp. 1-8). This manuscript is entitled "**p73 loss triggers conversion to squamous cell carcinoma reversible upon reconstitution with TAp73α"** by Jodi Johnson, James Lagowski, Alexandra Sundberg, Sarah Lawson, Yuangang Liu, and Molly Kulesz-Martin [Johnson et al., 2007].

3.1. Malignant conversion to SCC is accompanied by aberrant expression of p53 family member proteins:

The protein expression of the three p53 family members was examined in distinct stages of keratinocyte squamous cell carcinogenesis including genetically related non-transformed 291 cells, initiated 03C cells, and malignant 03R cells (Figure 11A). Spontaneous conversion from initiation to malignancy corresponded with nearly total loss of p73 protein expression, reduced p63 protein expression, and failure to induce p53 following DNA damage with 4 Gy IR (lanes 6-8). The precursor 03C cells (lanes 3-5) expressed at least three isoforms of p73 protein and at least five isoforms of p63 protein detectable by immunoblotting. The TAp73α isoform and the more predominantly expressed ΔNp73α isoform were confirmed by size comparison with known isoforms overexpressed via plasmid transfection. A third, lower molecular weight p73 isoform is expected to be TAp73β based on migration size but this could not be confirmed because multiple p73 isoforms migrate in the same location. In order from greatest to least apparent molecular weight, the predominantly expressed ΔNp63α isoform, the expected

TAp63β isoform, and the TAp63γ and ΔNp63γ isoforms were detected by immunoblotting with 4A4 pan-p63 antibody and confirmed by size comparison with known isoforms overexpressed via plasmid transfection. A fifth p63 isoform detected between the TAp63γ and ΔNp63γ isoforms is expected to be ΔNp63β. The 03C cells expressed the same isoforms of p63 and p73 (with increased expression of ΔNp73α) as their non-transformed parental cell line, 291 (lane 2), an independent p53 null mouse keratinocyte cell line, NK1 (lane 1), and cultured primary keratinocytes (Figure 10 B and D).

Because the 03R cells that are known to produce poorly differentiated SCC when transplanted into mice exhibited nearly total loss of p73 protein expression, we next examined the expression levels of the p53 family members in independently derived clonally selected initiated cells, 05C and 09C, and their tumor-forming derivatives, 05R and 09R (Figure 11B). The 05C cells (lane 2), like the 03C cells (lane 6), had upregulated p73 expression compared to the non-transformed 291 precursors (lane 1). While the 05R cells that form moderately differentiated SCC had reduced p73 expression (lane 3), the benign papilloma-forming 09R cells (lane 5) had upregulated p73 to levels comparable to the initiated 05C and 03C cells. The down-regulation of p73 was not as complete in the 05R cells as in the 03R cells (lane 7).

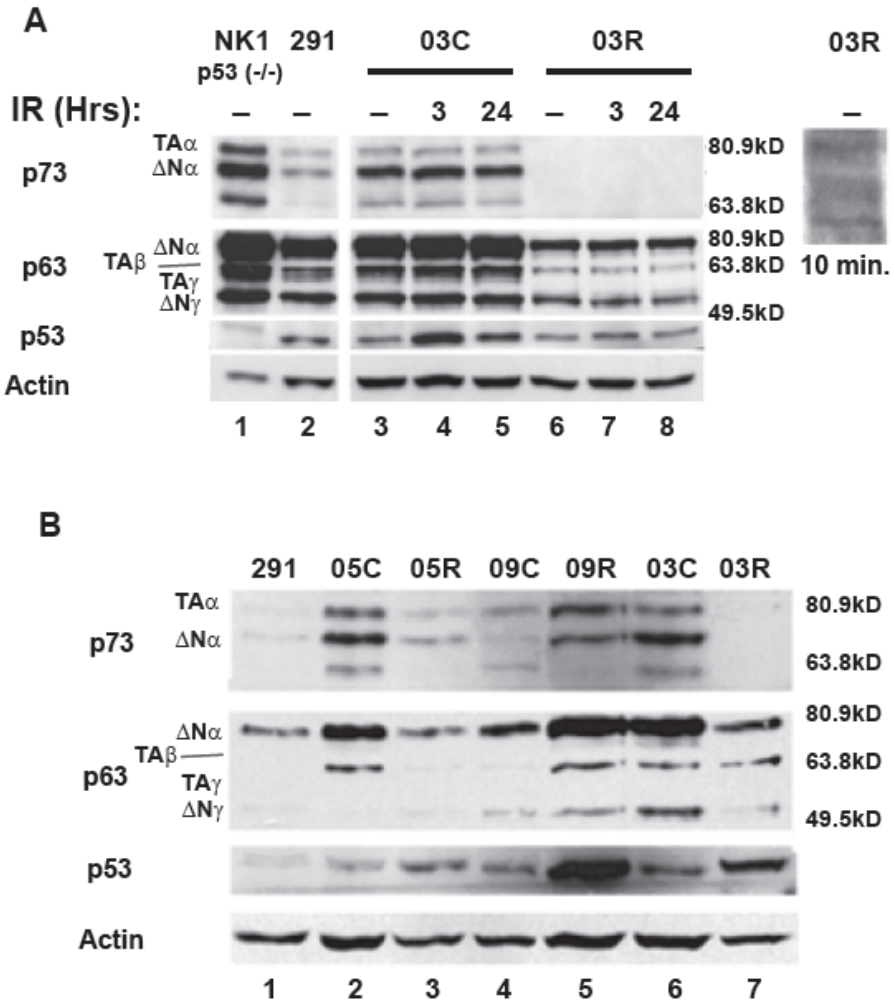

Figure 11: Malignant conversion to SCC is accompanied by aberrant expression of p53 family member proteins

Figure 11: Malignant conversion to SCC is accompanied by aberrant expression of p53 family member proteins. A) Immunoblot of p53 family member protein expression in non-transformed 291 cells, initiated 03C cells, and malignant 03R cells in lanes 2-8 as indicated. Cells were either mock treated (-) or harvested 3 or 24 Hrs. following treatment with 4 Gy IR. A 10 min. exposure of p73 in 03R cell lysate (compared to a 2 min. exposure for p73 in lanes 1-8) emphasizes the nearly complete loss of total p73 protein expression (upper right panel). An independent p53 null ($p53^{-/-}$) keratinocyte line NK1 showed the same p63 and p73 isoform expression pattern (Lane 1). B) Immunoblot of p53 family protein expression levels in non-transformed precursor 291 cells (lane 1), three independent DMBA-initiated cell lineages (05C, 09C, and 03C lanes 2, 4, and 6) and matched derivative tumorigenic cell lineages (05R, 09R, and 03R, lanes 3, 5, and 7). The p73 protein expression was increased in initiated cells compared to the non-transformed precursor and further increased in benign (papilloma) tumorigenic cells (09R), but reduced in SCC tumorigenic lineages (05R that produces moderately differentiated SCC and 03R that produces poorly differentiated SCC). For this immunoblot all cells were grown under identical culture conditions in low calcium medium as described in the Methods Section. Refer to Figure 6 for a schematic of related cell lineages.

3.2. Initiated cells with knocked down p73 exhibited characteristics of SCC cells *in vitro* and undergo malignant conversion to form SCC *in vivo*:

The p73 protein expression was down-regulated during malignant conversion to moderately or poorly differentiated SCC in the clonal model of epidermal carcinogenesis. Therefore, we examined whether targeted reduction in total p73 protein expression in initiated 03C cells could lead to similar alterations in cellular growth characteristics and to malignant conversion. To this end, 03C cell clones were selected for stable expression of siRNA directed against either p73 or a mismatched sequence that did not alter expression of any p53 family members. Data are shown for two independent 03C si-p73 knock-down clones (designated 03C si-p73-1 and -2) and one control 03C clone (designated 03C si-Con). The siRNA in the knock-down clones was specific to p73 (Figure 12A, lanes 3 and 4 upper panel), and did not alter the protein expression of the other p53 family members (Figure 12A, lower panels). Somewhat higher expression levels of the p53 protein in the 03C si-p73 clones than in the 03C cells are not strictly attributable to loss of p73 protein expression. Quantification of p53 levels in 03C versus 03R (lanes 1 and 2) and in 03C si-p73 clones (lanes 3 and 4) versus the vector control 03C Si-Con cells (lane 5) relative to actin loading control revealed less than 30% difference in total p53 expression. Quantitative real time PCR (qPCR) confirmed the reduction of p73 mRNA expression in the 03C si-p73 cells (data not shown).

In culture, the 03C si-p73 clones exhibited morphological characteristics similar to the 03R SCC cells, including pronounced multilayering of cells compared to the 03C cells (Figure 12B). To determine whether the loss of p73 expression lead to malignant conversion, we subcutaneously injected the 03C si-p73 cells, 03R cells as positive control

for cancerous growth, or 03C or 03C si-Con cells as negative controls into newborn Balb/C mice. The two independent clones of the 03C si-p73 cells formed poorly differentiated SCC (percent of mice with tumors graphed in Figure 13A) histologically indistinguishable from 03R-derived SCC (Figure 13B). The 03C si-p73-1 and si-p73-2 clones formed tumors at different rates and the si-p73-2 clone formed fewer tumors. These differences were not attributable to differences in siRNA efficiency against p73 or alterations in p53 family member expression levels (Figure 12A). Clonal selection of the 03C si-p73-1 versus si-p73-2 clones may have resulted in genetic variations to account for these phenotypic differences. Even so, the common feature of both clones was specific silencing of p73 and increased tumorigenicity relative to mice injected with 03C or 03C si-Con cells (open diamonds and squares respectively).

To determine the total gene expression changes associated with silencing of p73 and malignant conversion in the 03C cells, we harvested total RNA from biological replicates of the 03C si-Con, 03C si-p73-1, and 03R cells for comparison by microarray analysis. Gross data depicting the genetic similarities between 03C si-p73-1 cells and the 03R cells following analysis by Microarray Suite (MAS) and Excel are presented in the Appendix showing that the 03C si-p73-1 cells were nearly identical to the 03R cells and that both 03C si-p73-1 and 03R cells diverged from the 03C si-Con cells in gene expression profile. Further analysis was performed using the positional ranking and normalization (PRNP) and the distance weighted discrimination (DWD) methods ([Benito et al., 2004] modified by Pelz) to analyze discriminating overall genetic "signatures" or metagenes of compared cell lines. The metagene profile of the 03C si-p73 cells fell into the same quandrant as the 03R cells, confirming the genetic similarity of

03C si-p73 cells to the 03R cells as compared to the 03C si-Con cells (data not shown). Thus loss of p73 protein expression coordinated with changes in cellular morphology, global gene expression profile changes, and malignant conversion.

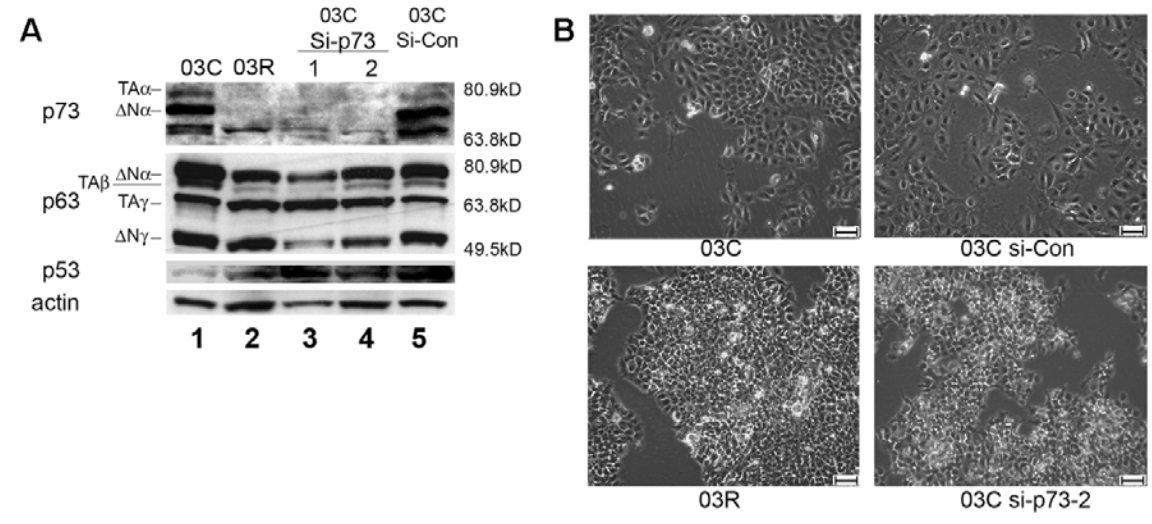

Figure 12: Disruption of p73 expression in initiated keratinocytes alters cells toward malignant phenotype *in vitro*. A) Immunoblot of p53 family members in 03C si-Con cells, 03C si-p73-1 cells, and 03C si-p73-2 cells compared to the 03C and 03R p53 family expression levels. B) Cell morphology of 03C, 03C si-Con, 03R, and 03C si-p73-2 cells grown for 72 Hrs. following plating at equal densities with inset bar representing 50 μm.

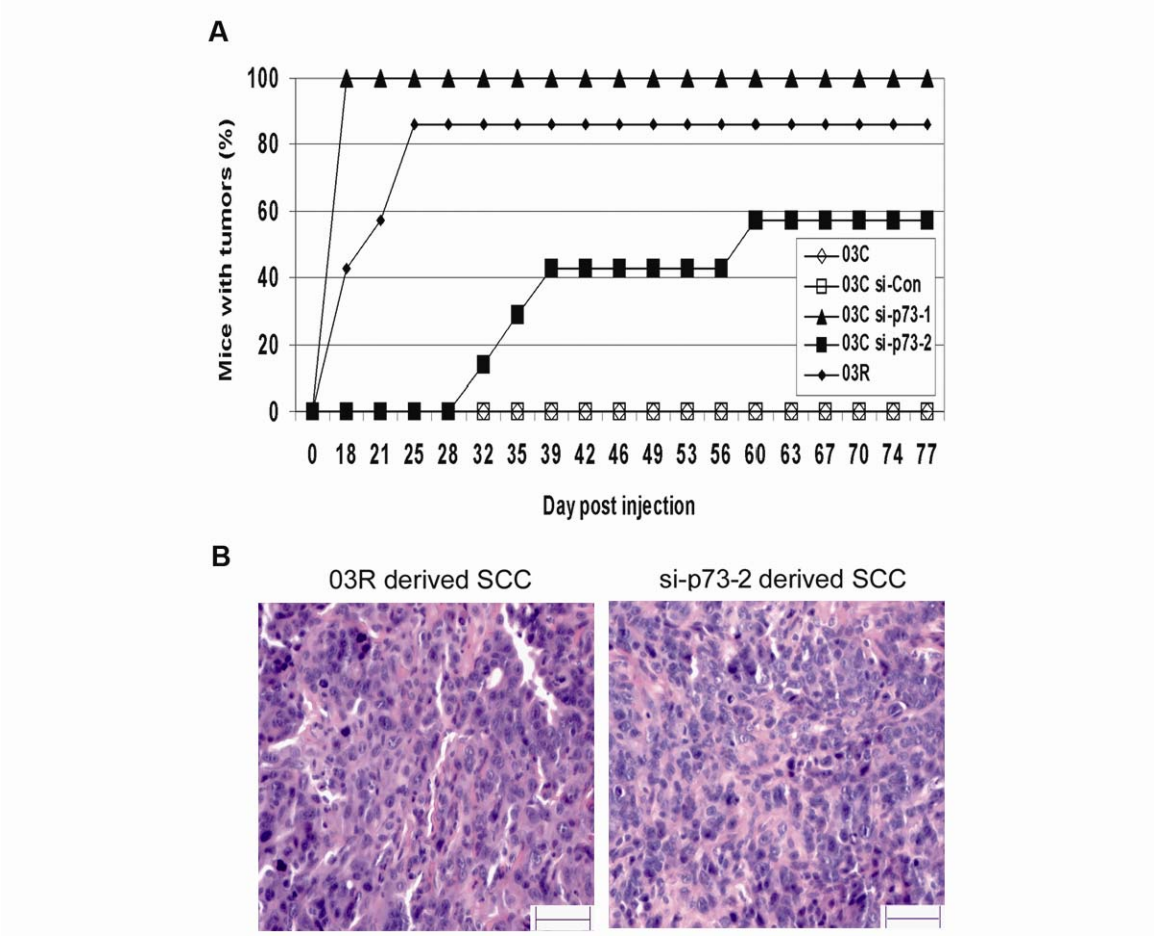

Figure 13: Initiated cells with knocked down p73 undergo malignant conversion to form SCC *in vivo*. A) Newborn mice injected with 03C cells (n=4), 03C si-Con cells (n=4), 03C si-p73-1 cells (n=6), 03C si-p73-2 cells (n=7), or 03R cells (n=7) were monitored for tumor growth with the endpoint being tumor measuring 1 cm in diameter. Mice injected with 03C si-p73 cells (▲, ■) or 03R cells (◆) formed tumors while mice injected with 03C (◇) or 03C si-Con (□) cells did not form tumors. The Kaplan-Meier method was used to calculate the number of days until tumor appearance in mice injected with each cell line compared to mice injected with 03C si-Con cells. Log-Rank statistics were used to test for overall differences among groups and a p-value less than 0.05 was considered statistically significant. 03R p=0.0131, 03C si-p73-1 p=0.0027, 03C si-p73-2 p=0.049. B) Histologically indistinguishable poorly differentiated SCC tumors harvested from mice injected with 03C si-p73 cells or 03R cells (H & E staining) with inset bar representing 50 μm.

3.3. Stable reconstitution of malignant si-p73 cells with TAp73α but not ΔNp73α restored cellular morphology comparable to initiated cells:

In both the 03R cells and the 03C si-p73 cells, loss of total p73 protein expression correlated with malignant conversion. To address isoform-specificity of p73 in malignant conversion, the 03C si-p73 cells were stably transfected with either pcDNA3 vector alone or with vectors containing FLAG-tagged human TAp73α or ΔNp73α cDNA. The TAp73β isoform, though it is the most transcriptionally active p73 isoform, was not included in the p73 recapitulation studies because it could not be confirmed that TAp73β was endogenously expressed in the keratinocytes. Even though the lowest molecular weight p73 isoform in the 03C cells is expected to be TAp73β based on migration size, the identity of this endogenously expressed isoform was not confirmed by size migration or RT-PCR analysis. Immunoblotting confirmed that physiologically relevant levels of TAp73α and ΔNp73α proteins were stably expressed comparable to those in the precursor 03C cells (Figure 14A). Further, the si-p73 cell clones expressing specific p73 isoforms (+ ΔNp73α or + TAp73α) or empty vector (+ Vector) expressed p63 and p53 protein at levels similar to the 03C cells. Interestingly, 03C si-p73 cells reconstituted with TAp73α reverted to morphological characteristics of initiated 03C cells in culture including a more adherent monolayer growth pattern compared to 03C si-p73 + Vector or + ΔNp73α cells (Figure 14B). The growth rate of the cells was examined for two vector-expressing clones, one ΔNp73α-expressing clone, and two TAp73α-expressing clones compared to the 03C cells. The TAp73α-expressing cells grew at a rate similar to the 03C cells while cells lacking p73 expression or expressing ΔNp73α proliferated more rapidly (Figure 14C).

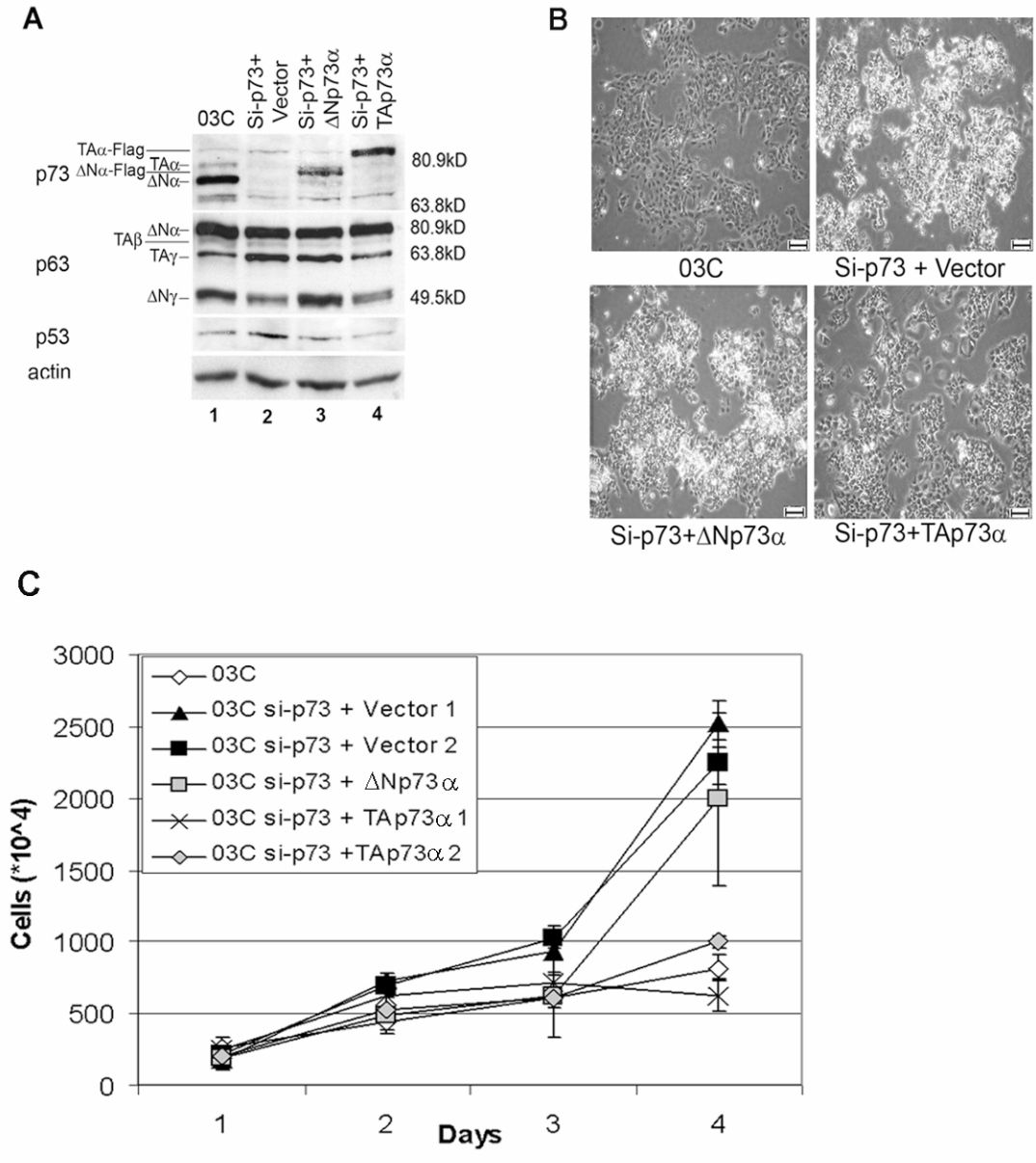

Figure 14: Stable reconstitution of TAp73α in SCC cells restores cells to premalignant phenotype

Figure 14: Stable reconstitution of TAp73α in SCC cells restores cells to pre-malignant phenotype. A) Immunoblot of p53 family protein expression levels in 03C si-p73 cells that were stably reconstituted with either pcDNA3 vector, Flag-tagged human ΔNp73α, or Flag-tagged human TAp73α. B) Cell morphology of 03C cells or 03C si-p73 + Vector, + ΔNp73α, or + TAp73α cells grown for 72 Hrs. after plating at equal densities with inset bar representing 50 μm. The 03C si-p73 + TAp73α cells remained in monolayers while the 03C si-p73 + Vector or + ΔNp73α cells formed multilayers like their si-p73 precursors shown in Figure 12B. C) Graph depicting the number of cells counted on each of four days following plating of $2.5x10^6$ cells in 150 mm tissue culture plates. The 03C si-p73 + TAp73α cells (X, ◇) divided at a rate similar to the initiated 03C cells (◇) while the 03C si-p73 + ΔNp73α cells (□) divided at a rate similar to 03C si-p73 + Vector cells (■, ▲) lacking p73.

3.4. Reconstitution of TAp73α protein expression in SCC cells suppressed tumorigenicity *in vivo*:

We next examined whether the cells reconstituted with TAp73α exhibited a less tumorigenic phenotype than cells expressing vector only or ΔNp73α. Newborn Balb/C mice injected with the 03R cells, the si-p73 + Vector cells or the si-p73 + ΔNp73α cells rapidly developed SCC (Figure 15). However, only one of 7 mice injected with 03C si-p73 + TAp73α cells developed a tumor. These results were confirmed in a replicate experiment in which three independent 03C si-p73 clones expressing TAp73α collectively developed tumors in only 20% of mice compared to 85-90% of mice injected with cells lacking p73 or expressing ΔNp73α (n=8 mice each group). We conclude that expression of TAp73α but not ΔNp73α restored tumor suppression in keratinocytes that had previously undergone malignant conversion induced by loss of p73. None of the tumors analyzed by immunoblotting had lost p53 or p63 protein isoform expression. However the small percentage of tumors that did develop from cells reconstituted with TAp73α had lost the ectopic expression of this isoform (an example of one such tumor is shown in Figure 21, discussed in Chapter 4).

To confirm these results in the malignant 03R cells as opposed to the malignant converted 03C si-p73 cells, attempts were made to introduce TAp73α and ΔNp73α isoforms into the 03R cells both through transient transfection and through selection using a tetracycline-inducible system (See Table 5b for plasmids constructed for these transfections). The tetracycline-inducible system was not successfully established in the 03R cells. Furthermore, transfection of the specific p73 isoforms into the 03R cells (either transiently or stably) was repeatedly unsuccessful. This may indicate that the 03R

cells have developed biological mechanisms through which to prevent the expression of p73. However, the more likely explanation is that the 03R cells are difficult to transfect and transfection efficiency was too low for the p73 isoforms to be detected.

We conclude that the loss of p73 expression, either spontaneously or through siRNA targeted against p73, is sufficient to trigger malignant conversion to poorly differentiated SCC and that restoration of TAp73α, but not ΔNp73α in malignant converted cells restored tumor suppression.

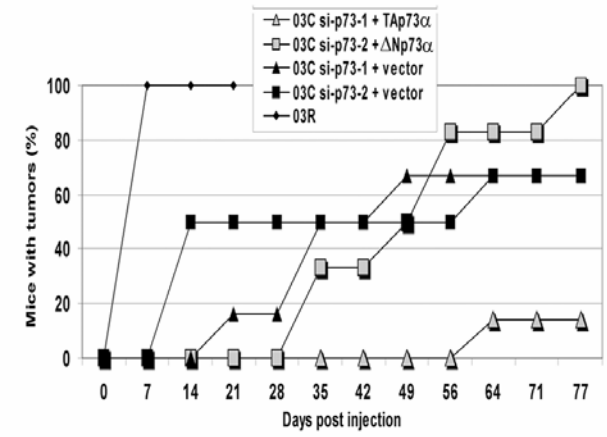

Figure 15: Reconstitution of TAp73α protein expression in SCC cells reduces tumorigenicity *in vivo*. Graph depicting the percentage of mice developing tumors following injection with cells + TAp73α (n=7,△), + ΔNp73α (n=6,□), + Vector (n=6 for both groups,▲,■), or known SCC-producing 03R cells (n=3,◆). Only 1/7 of the mice injected with 03C si-p73 + TAp73α cells developed a tumor (p=0.0351 using the Kaplan-Meier method and Log-Rank statistics to analyze differences in time to first appearance of tumor compared to mice injected with 03C si-p73 + Vector cells).

4. Results: The p53 family isoform cooperation; DNA binding and response to DNA damage

The manuscript prepared from the results discussed in Chapter 4 is being revised for submission after initial review. This manuscript is entitled "**p73 expression alters p63 and Mdm2 as components of DNA binding complexes in squamous cell carcinogenesis**" by Jodi Johnson, James Lagowski, Sarah Lawson, Yuangang Liu, and Molly Kulesz-Martin.

4.1. Cells lacking p73 expression exhibit IR resistance which is not corrected by reconstitution with TAp73α or ΔNp73α

We have reported previously that the malignant 03R cells exhibit resistance to IR *in vitro* characterized by increased cell viability, failure to undergo G1 arrest, and failure to induce p21 protein expression following DNA damage by treatment with 4 Gy IR as compared with initiated 03C cells [Knights et al., 2003]. The malignant cells also were defective in apoptosis following IR treatment, exhibiting no change in caspase-3 activity with treatments up to 30 Gy IR (discussed further in Figure 22). To test the role of p73 in helping mediate cellular responsiveness to IR, we examined the 03C si-p73 cells for IR sensitivity. The 03C si-p73 cells, like the 03R cells, exhibited IR resistance (survival when replated in colony forming assays) while the 03C si-Con cells retained IR sensitivity, forming fewer colonies (Figure 16A). Further, the 03C si-p73 cells failed to arrest in G1 compared to the 03C si-Con cells (Figure 16B). qPCR was used to test induction of endogenous *p21*, *Noxa*, *Puma*, *Bax*, and *Gadd45*, all known to be regulated by p53 family members following DNA damage (Table 1). While the 03C si-Con cells exhibited reproducible 2- to 3-fold induction of the tested p53 downstream targets, the

03R and 03C si-p73 cells did not induce any of these genes (Figure 16C upper graph). We conclude that p73 is involved in mediating keratinocyte sensitivity to DNA damage by IR and that loss of p73 renders the cells incapable of undergoing G1 arrest.

To determine whether reconstitution of 03C si-p73 cells with TA- or ΔNp73α isoforms would restore cellular sensitivity to IR, we tested the 03C si-p73 + Vector, + ΔNp73α, and + TAp73α for colony forming efficiency, cell cycle arrest, and induction of downstream target genes following treatment with 4 Gy IR. The reconstitution of these individual p73 isoforms was not sufficient to restore radiation responsiveness. The cells remained viable in colony forming assays comparable to the 03R cells and 03C si-p73 cells regardless of p73 expression status. They did not undergo G1 arrest following treatment with IR (data not shown). Reconstituting 03C si-p73 cells with TA- or ΔNp73α did not lead to induction of p53 target genes following treatment with IR relative to vector controls (Figure 16C lower graph; under two-fold mRNA induction is considered below the level of detection of the assay). The induction of p21 mRNA in the ΔNp73α-expressing cells was within 30% that of vector control and did not correlate with cell cycle arrest (data not shown). However, the 03C si-p73 + TAp73α cells expressed higher basal steady-state levels of p53 target mRNA (*p21*, *Noxa*, and *Gadd45*) compared to 03C si-p73 + Vector cells while 03C si-p73 + ΔNp73α cells did not induce, and appeared to marginally repress, basal expression of these targets (Figure 16D).

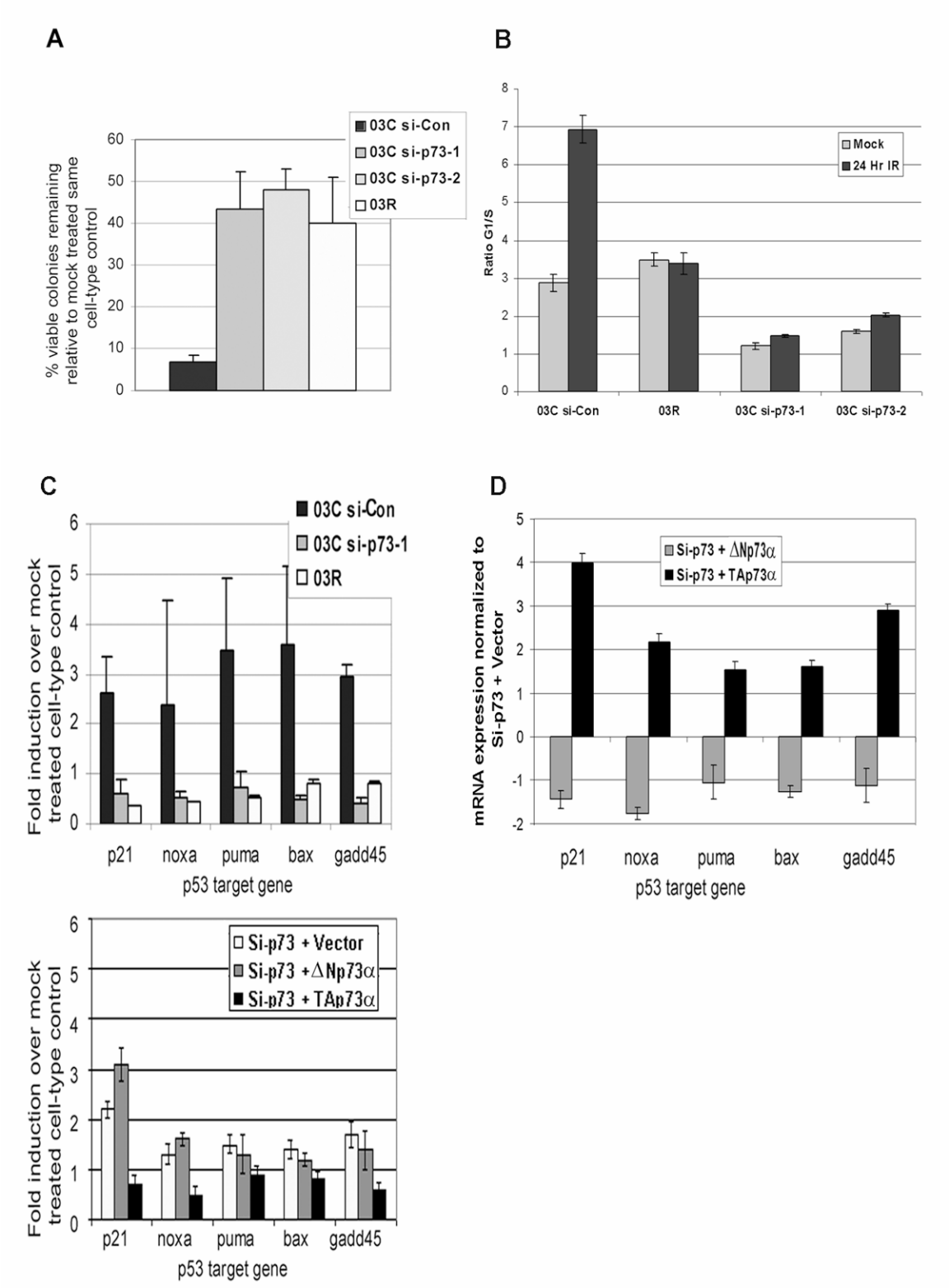

Figure 16: Cells lacking p73 expression exhibit IR resistance which is not corrected by reconstitution with TAp73α or ΔNp73α

Figure 16: Initiated cells lacking p73 exhibit resistance to IR which is not corrected by reconstitution with TAp73α or ΔNp73α. A) Viability assays to determine cellular sensitivity to IR. The graph represents the percent of viable colonies remaining after IR treatment compared to the mock treated cell type control (average of triplicate culture plates +/- SD). B) Flow cytometry to determine cellular G1/S arrest following treatment with IR. The graph represents the ratio of G1/S in cells as indicated. Ratios are calculated from the average (+/- SD) of triplicate analysis. C) Analysis by qPCR of p53 downstream target mRNA induction (*p21*, *Noxa*, *Puma*, *Bax*, and *Gadd45*) in 03C si-Con, 03C si-p73, and 03R cells (top graph) or 03C si-p73 + Vector, + ΔNp73α, or + TAp73α cells (bottom graph) 24 Hrs. following treatment with 4 Gy IR. The graphs represent fold induction over mock treated cell type control, and the error bars represent SD of triplicate reactions on two separate qPCR plates. D) Analysis by qPCR of p53 downstream target mRNA levels in cultured cells. The graph represents mRNA levels in 03C si-p73 + ΔNp73α or + TAp73α cells normalized to si-p73 + Vector cells with error bars representing SD of triplicate reactions on two separate qPCR plates.

4.2. Basal p53 family member isoform DNA binding capabilities were reduced in IR-resistant keratinocytes undergoing malignant conversion to SCC:

We were interested to know the potential mechanisms through which p73 could be mediating tumor suppression and cellular responsiveness to IR and why cells expressing TAp73α, though they did not regain IR sensitivity, formed fewer tumors than cells lacking p73 or expressing ΔNp73α. Since the p53 family members exhibit significant homology in their DNA binding domains (Figure 1), we questioned whether defective DNA binding capabilities of p53 family members may be leading to tumorigenesis and IR resistance. To begin, we globally compared the DNA binding capabilities of the p53 family member isoforms in the non-transformed 291, initiated 03C, and malignant 03R cells using the DNA Affinity Immunoblotting Assay (DAI) [Knights et al., 2003; Liu et al., 2001; Liu, Lagowski et al., 2004]. In the non-transformed (lane 3) and initiated cells (lane 6), p53, all expressed isoforms of p63, and both TAp73α and ΔNp73α were capable of binding to a DNA probe containing the distal p53 binding site of the p21 promoter (Figure 17A). However, only the dominant negative ΔNp63α isoform and none of the p73 or lower molecular weight p63 isoforms were capable of binding DNA in the malignant cells (lane 9).

The DNA binding capability of p53 increased in the 291 and 03C cells following treatment with IR compared with the mock treated cells (lanes 3 and 6), while p53 was neither induced nor capable of robustly binding the p21 probe in the malignant 03R cells (lane 9). TAp73α bound to the p21 probe in the 291 (lane 3) and 03C (lane 6) cells, predominating in the DNA-bound state over the more abundantly expressed ΔNp73α isoform (lanes 1 and 4), while the malignant cells had virtually no p73. This profile of

p53 family DNA binding capabilities was reproducible in four separate DAI experiments with biological replicates (one representative is shown). Thus the malignant 03R cells exhibited an overall defect in p53 family protein expression, most notably, the loss of p73 and specific losses of DNA binding capabilities of TAp63β, TAp63γ and ΔNp63γ isoforms disproportionate to their total protein expression. The percent of the total p63 isoform that is bound to the p21 probe is depicted graphically in Figure 17B using the formula [((isoform bound to p21 probe-scan background)-(isoform bound to negative control probe-scan background))/ (direct IB isoform * 5)]*100, which adjusts for 40μg of total lysate for direct immunoblotting (IB) relative to 200μg used for each DAI assay.

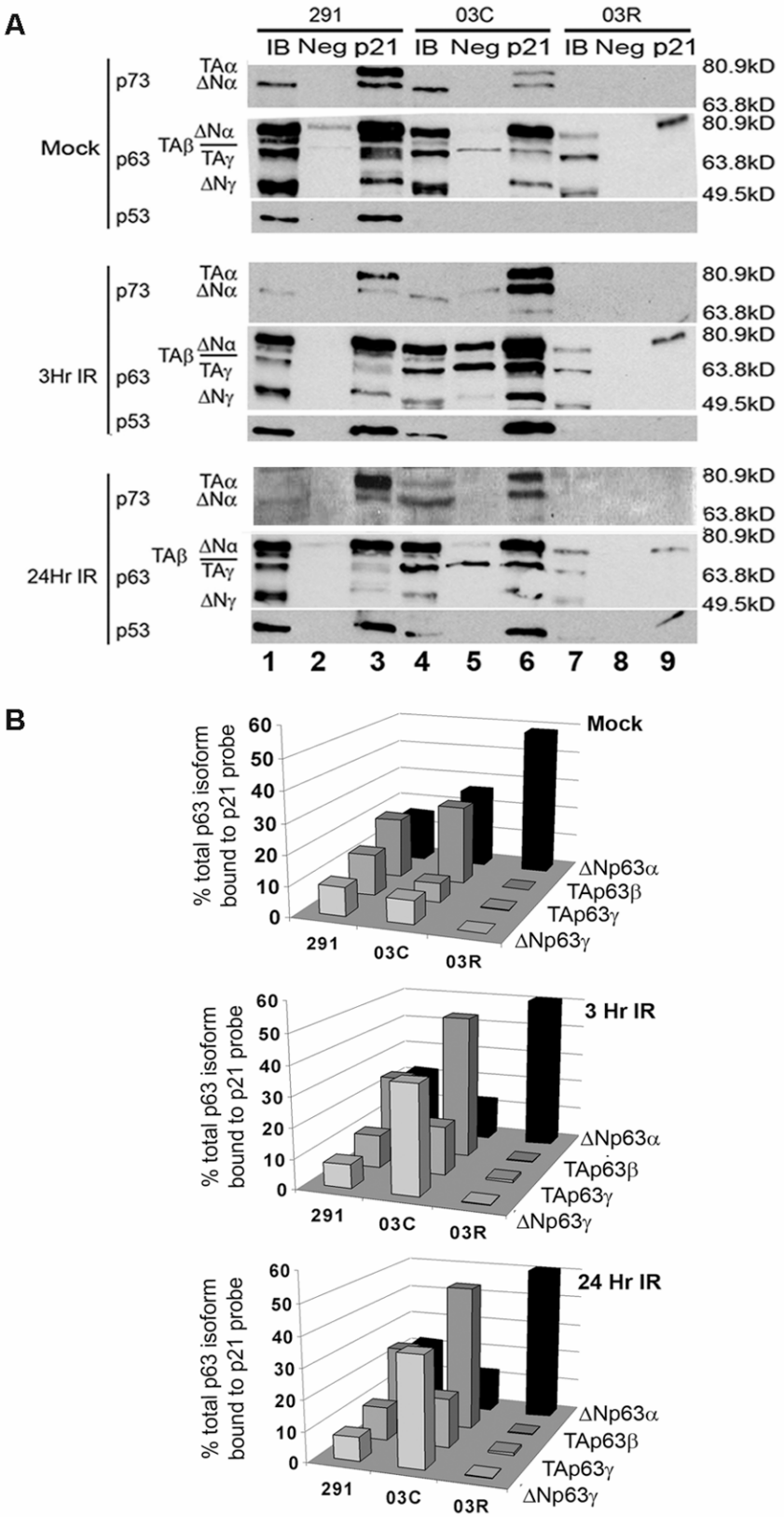

Figure 17: Malignant 03R cells exhibit reduced p53 family member DNA binding capability compared to initiated precursors

Figure 17: 03R cells exhibit reduced DNA binding of 73 and p63 isoforms when compared to 03C precursors. A) DAI analysis of p53 family member DNA binding in the SCC model cells that were either mock treated or treated with 4 Gy IR and harvested after 3 Hrs. or 24 Hrs. Lanes 1,4, and 7 (IB) are direct immunoblots of 40μg of whole cell lysate. Lanes 2, 5, and 8 (Neg) are DAI assays detecting p53 family member isoforms bound to the negative control probe. Lanes 3, 6, and 9 (p21) are DAI assays detecting p53 family member isoforms bound to the p21 probe. The malignant cells have reduced *in vitro* DNA binding of p53, p73, and the lower molecular weight p63 isoforms (TAp63β, TAp63γ, ΔNp63γ) to the p21 probe as compared to non-transformed and initiated cells. B) Graphical representation of the percent of each p63 isoform bound to the p21 probe based on the formula [((isoform bound to p21 probe-scan background)-(isoform bound to negative control probe-scan background))/(direct IB isoform * 5)]*100.

4.3. Silencing of p73 expression leads to reduced DNA binding of TAp63 isoforms and reconstitution with TAp73α aids TAp63 DNA binding:

We examined whether p53 family member isoforms might cooperate with each other for DNA binding efficiency. First we determined if the DNA binding capabilities of p63 and p73 isoforms were dependent on p53 expression by examining p53 null NK1 mouse keratinocytes for DNA binding capabilities of p63 and p73 isoforms. The p73 and p63 isoforms were capable of binding to the p21 probe in the absence of p53 (Figure 18A). In fact, the p73 protein DNA binding capability appeared enhanced in the absence of p53 although p63 DNA binding was not examined at the time of this experiment (Figure 18B).

We then tested whether loss of p73 protein expression altered the DNA binding capabilities of p63 isoforms. The DAI assay revealed that, similar to the DNA binding pattern of the p63 isoforms in the 03R cells, the 03C si-p73 cells had reduced steady state DNA binding of TAp63β, TAp63γ and ΔNp63γ to the p21 probe, as compared to 03C or 03C si-Con cells (Figure 19A). To determine if TAp73α or ΔNp73α might be mediating interaction of the lower molecular weight p63 isoforms with DNA, we tested the 03C si-p73 cells that had been reconstituted with the individual p73 isoforms. DAI analysis revealed that while the 03C si-p73 cells + Vector or + ΔNp73α displayed the defective p63 isoform DNA binding characteristics of 03R and 03C si-p73 cells (Figure 19B lanes 4-9 compared to 03C si-Con lanes 1-3), the cells expressing TAp73α exhibited restored DNA binding capabilities of TAp63β, TAp63γ, and ΔNp63γ to the p21 probe (lanes 10-12 and quantified in graph). This suggests that TAp73α specifically facilitates the DNA binding of TAp63β and TAp63γ and ΔNp63γ. Increasing autoradiography exposures of

the DNA binding of p63 in 03C si-p73 + Vector and 03C si-p73 + TAp73α cells (Figure 20) showed that the altered DNA binding capabilities of lower molecular weight p63 isoforms in the presence of TAp73α isoform expression was not simply an artifact of higher p63 protein expression. The lower molecular weight p63 isoforms were already detectable binding to DNA in the 03C si-p73 + TAp73α cells in a 30 second autoradiography exposure. However, only very faint p63 isoform bands were detectable in the 03C si-p73 + Vector cells even after one hour of exposure. The increased DNA binding capabilities of lower molecular weight p63 isoforms in 03C si-p73 + TAp73α cells corresponded with the increase in basal p53 downstream target mRNA levels observed in these cells (Figure 16D). However, the increased DNA binding capability of specific p63 isoforms was not sufficient to restore IR responsiveness in the cells reconstituted with TAp73α.

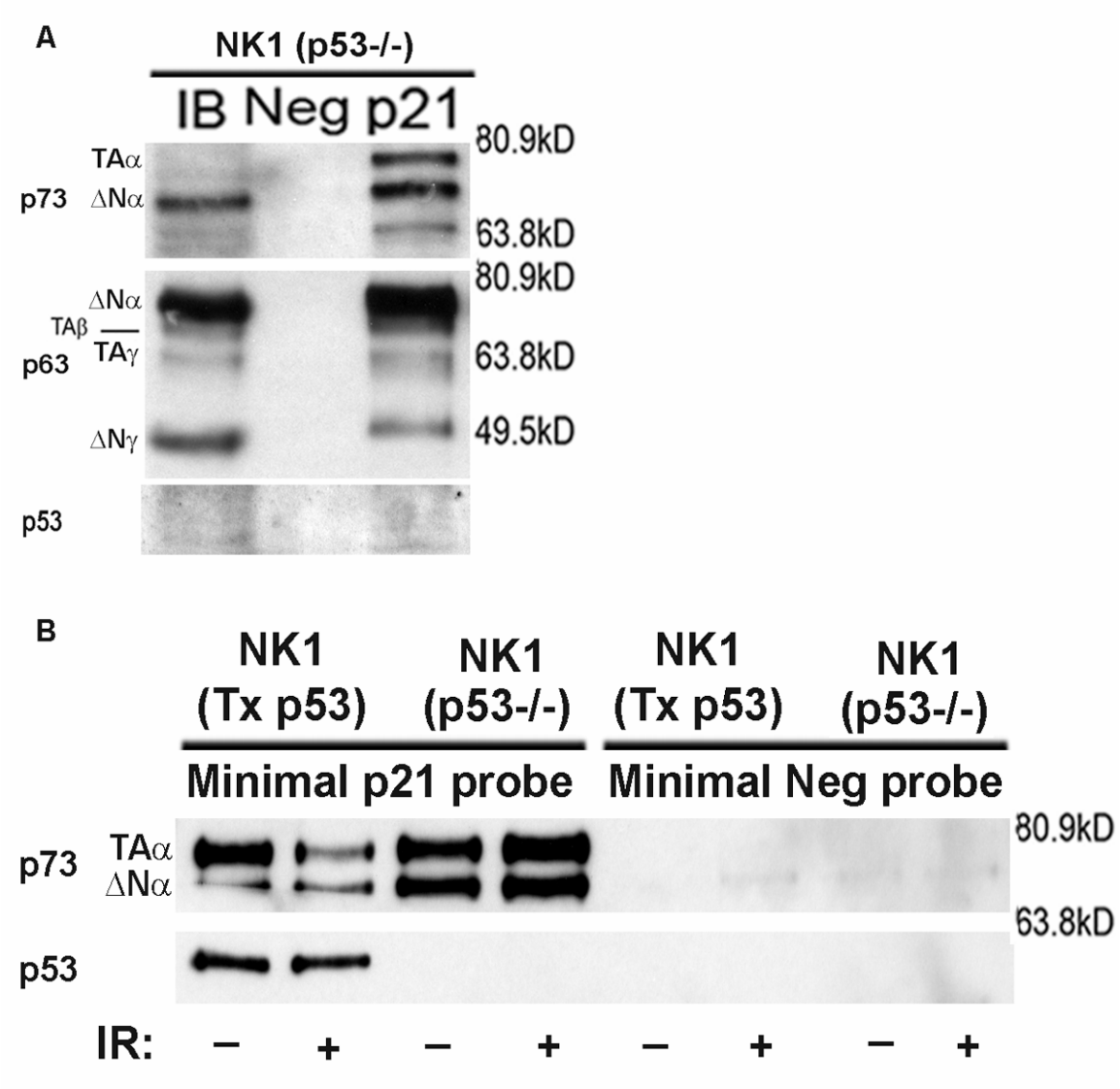

Figure 18: p53 is not required for p63/p73 DNA binding. A) DAI analysis of p73 and p63 DNA binding capabilities in p53 null NK1 cells. B) DAI analysis of p73 in NK1 cells either transfected with empty vector or wild type p53 and either mock treated or harvested 3 Hrs. following treatment with 4 Gy IR. Lysate was incubated with a minimal (33bp) p21 promoter probe with sequence CTGGCCATCAGGAACATGTCCCAACATGTTGAG or a minimal (24 bp) negative control probe with sequence TTGAGGTCAGGCAGTGCACTGCAC. The p63 protein DNA binding capability was not determined in this experiment.

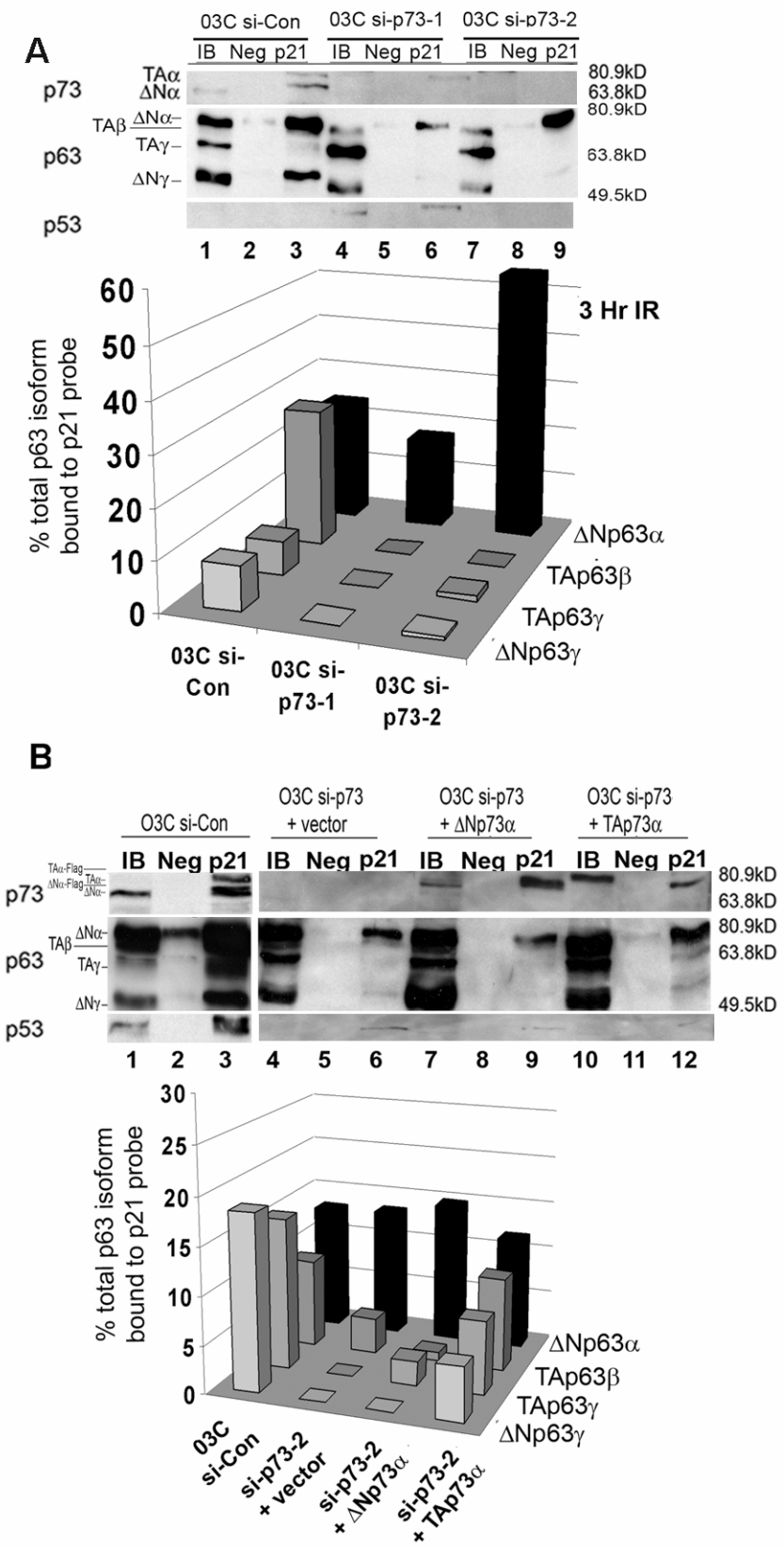

Figure 19: Silencing of p73 expression leads to reduced DNA binding of TAp63 isoforms but reconstitution with TAp73α aids TAp63 DNA binding

Figure 19: Silencing of p73 leads to reduced DNA binding of p63 isoforms which is reversed by reconstitution with TAp73α. A) DAI analysis of p63 isoform DNA binding capabilities in 03C si-Con vs. 03C si-p73 cells. The percent of each p63 isoform bound to the p21 probe is depicted graphically as in Figure 17. B) DAI analysis of p63 isoform DNA binding capabilities in 03C si-Con cells and si-p73-2 cells stably reconstituted with vector, ΔNp73α, or TAp73α. Like their precursor si-p73 cells, the cells + vector or + ΔNp73α exhibited defective TAp63β, TAp63γ, ΔNp63γ isoform DNA binding capabilities while introduction of TAp73α lead to increased p63 isoform DNA binding to the p21 probe as displayed graphically.

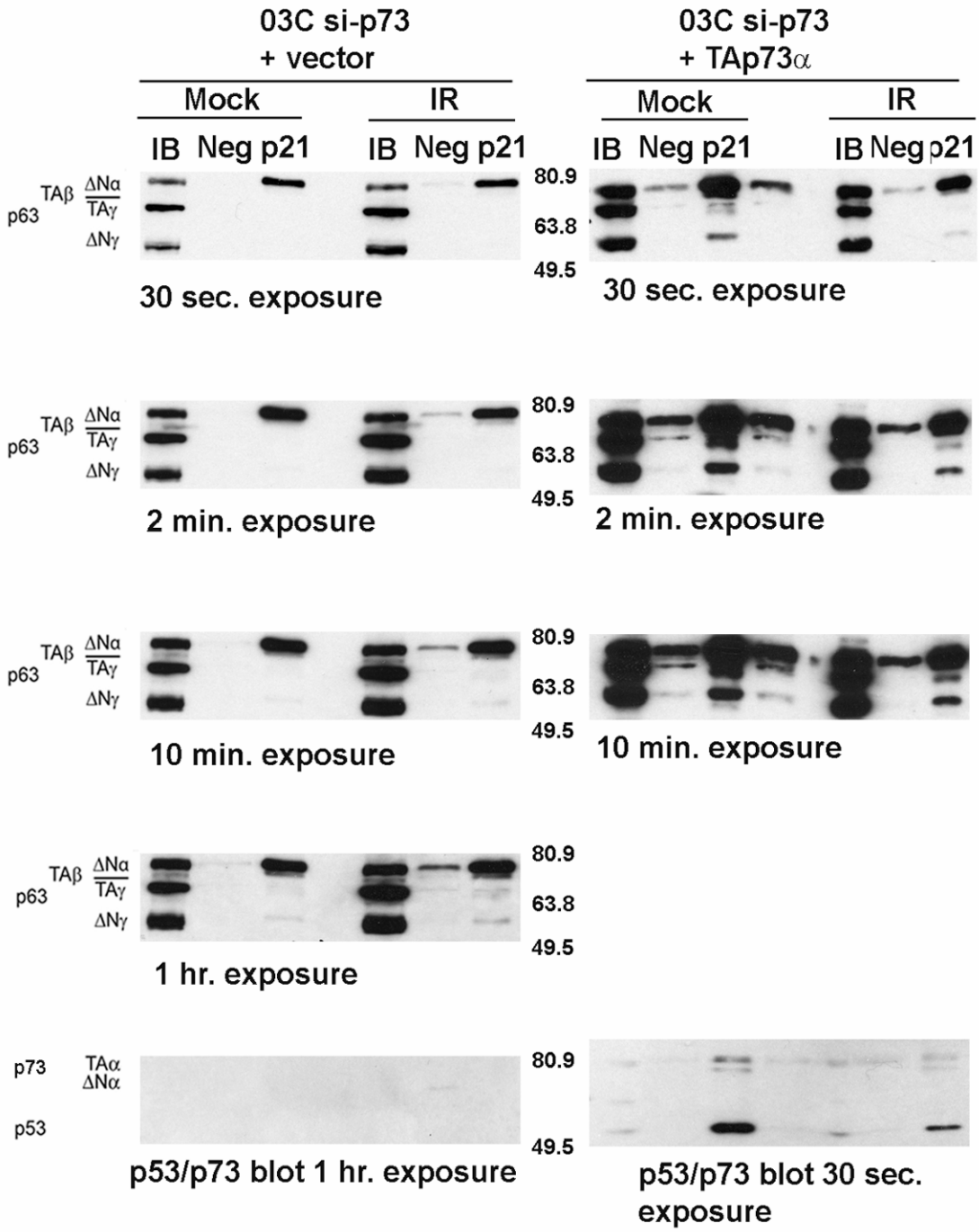

Figure 20: Increasing exposures of p63 DNA binding to show reduced DNA binding capability of lower molecular weight p63 isoforms in cells lacking p73 expression and improved DNA binding capabilty in cells expressing TAp73α

Figure 20: Increasing exposures of p63 to show reduced DNA binding capabilities of lower molecular weight p63 isoforms in cells lacking p73 expression and improved p63 isoform DNA binding capabilities in cells expressing TAp73α. DAI analysis was performed to determine DNA binding capabilities of p53 family members in lysate collected from mock or IR-treated 03C si-p73 + Vector cells or 03C si-p73 + TAp73α cells. The lower molecular weight p63 isoforms were not detected bound to the p21 probe in 03C si-p73 + Vector cells with autoradiography up to one hour. However, lower molecular weight p63 isoforms were visualized bound to the p21 probe after only 30 seconds of autoradiography. The lower panels show p53 and p73 DNA binding in the cells lacking p73 or expressing TAp73α. No p53 or p73 DNA binding was detected in the si-p73 + Vector cells even after one hour of exposure to the film whereas p53 and TAp73α were detected bound to the p21 promoter probe after a 30 second exposure.

4.4. SCC tumors exhibit diminished p53 family member DNA binding capabilities suggesting preferential ΔNp63α DNA binding in SCC:

DAI analysis was performed on lysate prepared from 03R- and 03C si-p73-derived SCC tumors to determine p53 family DNA binding capabilities (Figure 21). While p53 and multiple isoforms of p63 were expressed in the tumors, only the ΔNp63α isoform was capable of robustly binding to the p21 probe, not TAp63β, TAp63γ, ΔNp63γ, p73, or p53. The outcome did not change in tumors derived from cells expressing ΔNp73α or TAp73α and the tumors derived from cells expressing TAp73α had lost expression of this isoform. Thus defective p53 family DNA binding capability was a hallmark of SCC tumors *in vivo* as well as cultured malignant cells *in vitro*. We conclude that in SCC tumors *in vivo* the inhibitory ΔNp63α isoform predominantly binds DNA.

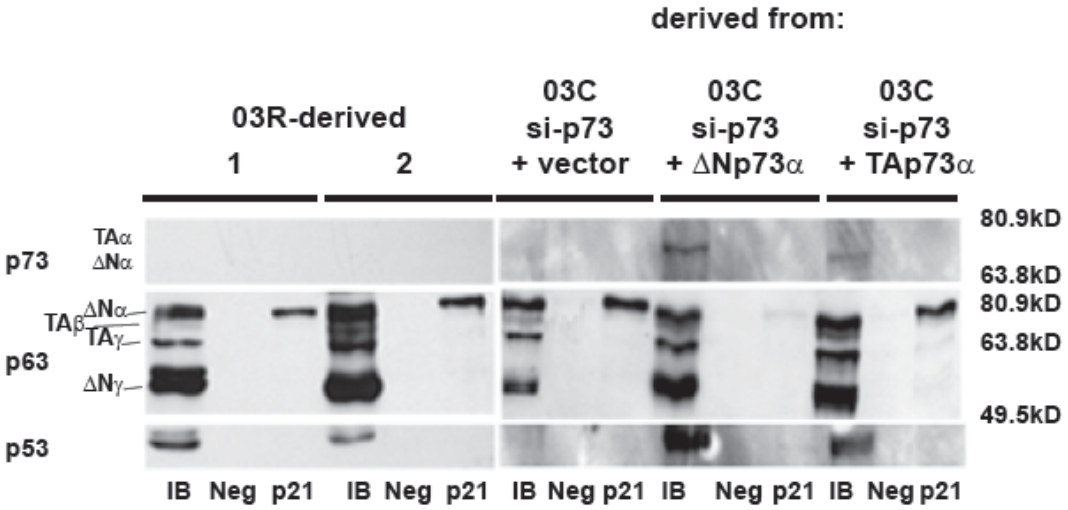

Figure 21: The ΔNp63α preferentially binds DNA in SCC tumors. DAI analysis of SCC tumors derived from mice injected with 03R, 03C si-p73 + Vector, 03C si-p73 + ΔNp73α or 03C si-p73 + TAp73α cells. Though p53 and multiple isoforms of p63 are expressed in the SCC tumors only the inhibitory ΔNp63α isoform is capable of robustly binding DNA. The SCC tumor derived from 03C si-p73 + TAp73α cells has lost expression of the TAp73α isoform.

4.5. The malignant 03R cells, though resistant to IR, remain sensitive to UVB:

When doing a dose-response study of the malignant 03R cells to determine the extent of their resistance to IR, we included a test for the keratinocyte response to UVB as another form of DNA damage (UVB causes single strand DNA breaks while IR causes double stranded breaks and the damage is sensed through different DNA damage pathways). We tested for apoptotic response using a colorimetric assay to detect activity of Caspase-3, an apoptotic enzyme. While the 03R cells did not induce Caspase-3 activity in response to doses up to 30 Gy IR (Figure 22A), we were surprised to observe that the 03R cells showed a 5-fold increase in Caspase-3 activity following treatment with 270 J/m^2 UVB compared to mock treated controls. The IR resistance of 03R cells corresponded with lack of induction of p53, p63, and p73 up to 30 Gy of IR (Figure 22B). However, the 03R cells consistently exhibited induction of p53 over mock treated cells at 5 hours following treatment with $135J/m^2$ UVB (Figure 22C lane 5). The p53 protein expression level had returned to steady state by 24 hours following UVB treatment in the 03R cells (lane 6), while in the initiated 03C cells, p53 induction persisted through 24 hours after treatment with UVB (Lanes 2 and 3).

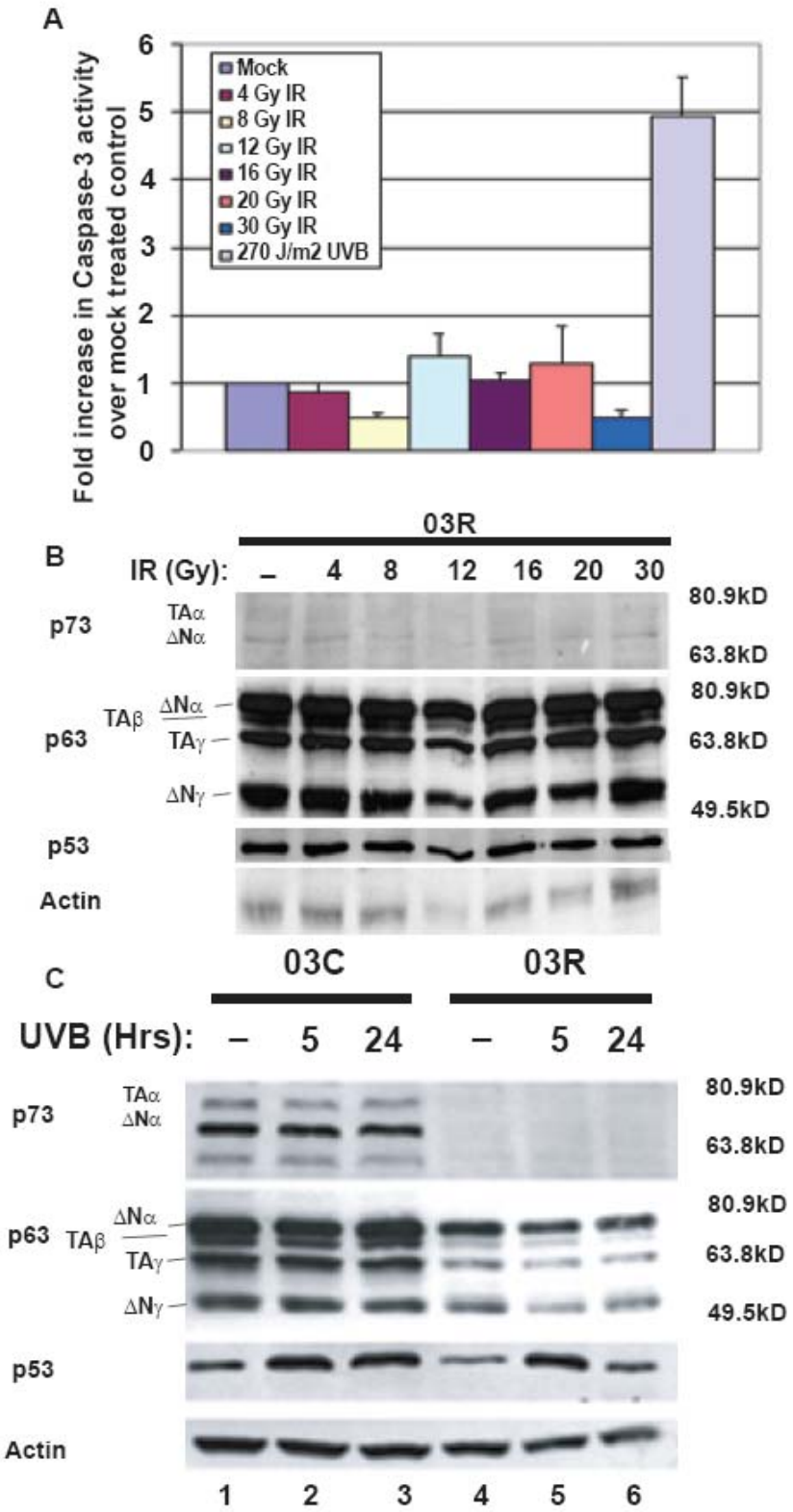

Figure 22: Malignant 03R cells, though resistant to IR, remain sensitive to UVB

Figure 22: Malignant 03R cells, though resistant to IR, remain sensitive to UVB. A) Colorimetric determination of Caspase-3 activity. The malignant 03R cells were tested for apoptotic Caspase-3 activity 24 Hrs. following treatment with varying doses of IR from 4 Gy to 30 Gy with no increase in Caspase-3 activity compared to mock treated controls. However, 03R cells treated with 270 J/m^2 UVB exhibited greater than 5-fold increase in Caspase-3 activity compared to the mock treated control. B) Immunoblot to detect p53 family member expression in 03R cells either mock treated or treated with IR at increasing doses as indicated. The p53 family member proteins were not induced in response to IR in the 03R cells. C) Immunoblot of p53 family member response to UVB in initiated 03C and malignant 03R cells. Cells were either mock treated (-) or harvested 5 or 24 Hrs. following treatment with 135 J/m^2 UVB. One representative (out of 5 biological replicates) is shown to indicate that total steady state level of p53 protein is induced in the 03C and 03R cells by 5 Hrs. (Lanes 2 and 5) following treatment with UVB and, while p53 remains induced in the 03C cells (Lane 3), levels have returned to the basal steady state level in the 03R cells by 24 Hrs. (Lane 6).

4.6 Keratinocytes lacking expression of p73 remain sensitive to UVB suggesting p73 is dispensable for some cellular responses to DNA damage:

We tested whether the initiated 03C cells with silenced p73 were also sensitive to treatment with UVB. Colony viability assays showed that, though resistant to treatment with 4 Gy IR, the 03C si-p73 and 03R cells remained sensitized to 135 J/m^2 UVB when compared to mock treated controls (Figure 23A). We performed flow cytometry to determine if the cells were able to undergo G1 arrest in response to UVB even though they had not arrested in G1 in response to treatment with IR. The 03C si-Con, 03C si-p73, and 03R cells all exhibited a decrease in the ratio of G1/S indicating that more cells were in S phase than in G1 following treatment with UVB (Figure 23B). The percentage of cells in each phase of the cell cycle is shown in Figure 23C demonstrating that cells accumulated in S phase following treatment with UVB regardless of p73 expression status. This suggested that the cellular response to UVB was more likely apoptotic than cell cycle arrest and DNA damage repair. We conclude that the p73 protein was dispensable for keratinocyte responsiveness to UVB even in cells that had become resistant to IR and undergone malignant conversion upon loss of p73 expression.

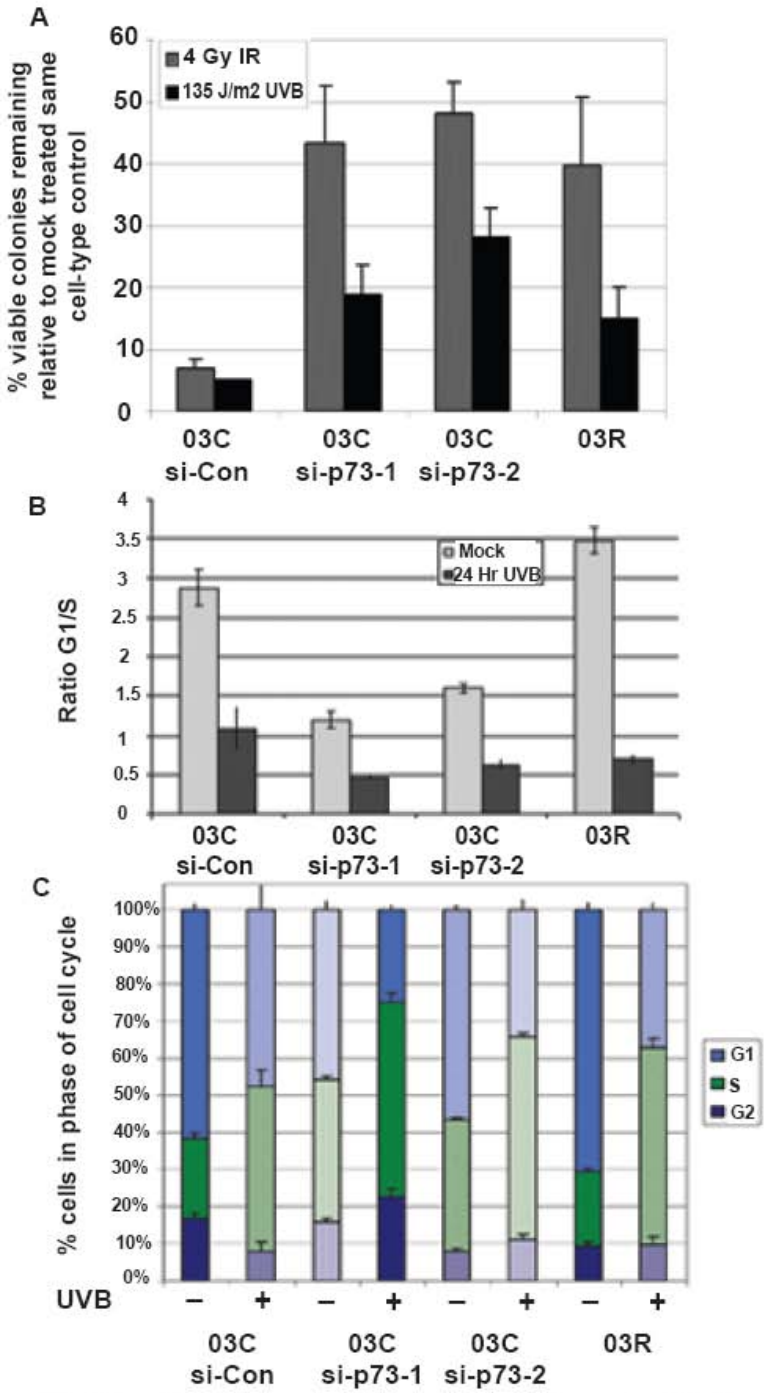

Figure 23: Keratinocytes lacking expression of p73, though resistant to IR, remain sensitive to UVB

Figure 23: Silencing of p73 in initiated keratinocytes does not render them resistant to UVB. A) Viability assays to determine cellular sensitivity to UVB compared to cellular sensitivity to IR. The graph represents the percent of viable colonies remaining after IR or UVB treatment compared to the mock treated cell type control (average of triplicate culture plates +/- SD). The 03C si-p73 cells and 03R cells exhibited greater sensitivity to UVB than to IR. B) Flow cytometry to determine cellular G1/S arrest following treatment with 135 J/m^2 UVB. The graph represents the ratio of G1/S in cells as indicated. Ratios are calculated from the average (+/- SD) of triplicate analysis. C) Alternative analysis of flow cytometry to determine % of cells in each phase of the cell cycle following mock treatment or 24 Hrs. after treatment with 135 J/m^2 UVB (+/- SD for triplicate analysis). UVB treatment corresponded with fewer cells in the G1 and G2 phases of the cell cycle compared to mock treatment, thus appearing as increases in cells in S phase.

4.7 The TAp63γ isoform may mediate keratinocyte UVB response:

We next examined the induction of p53 target genes in response to UVB treatment in the initiated 03C cells vs. the malignant 03R cells. While the initiated cells exhibited upregulation of *p21*, the 03R cells upregulated *p21* in addition to greater upregulation of apoptotic targets *Noxa* and *Puma* (Figure 24A). Similarly, the 03C si-Con cells showed induction of *p21* while the 03C si-p73 cells exhibited increased *p21*, *Noxa*, and *Puma* mRNA expression consistent with the idea that the cellular response to UVB was more likely apoptotic than cell cycle arrest and DNA damage repair (Figure 24B). We tested the DNA binding capabilities of p53 family members to both the p21 promoter probe and the Noxa promoter probe in the 03C and 03R cells before and after treatment with 135J/m^2 UVB using the DAI assay (Figure 24C). The DNA binding pattern of the p53 family members in the 03C cells following treatment with UVB was similar to 03C cells treated with IR (compare to Figure 17) in that TAp73α isoform predominated in the DNA-bound state and there were increases in p53 and p63 DNA binding to the p21 promoter following treatment. This was consistent with induction of *p21* mRNA observed in the 03C cells. In the 03R cells, where the ΔNp63α isoform had predominated in the DNA-bound state following treatment with IR, we observed increases in lower molecular weight TAp63 isoform DNA binding capabilities on the p21 probe and specific increase in TAp63γ DNA binding capability on the Noxa probe, consistent with preferential induction of *Noxa* mRNA (though *p21* was also induced in the 03R cells). This led us to conclude that the TAp63γ isoform was important for mediating the 03R response to UVB regardless of expression status of the p73 protein.

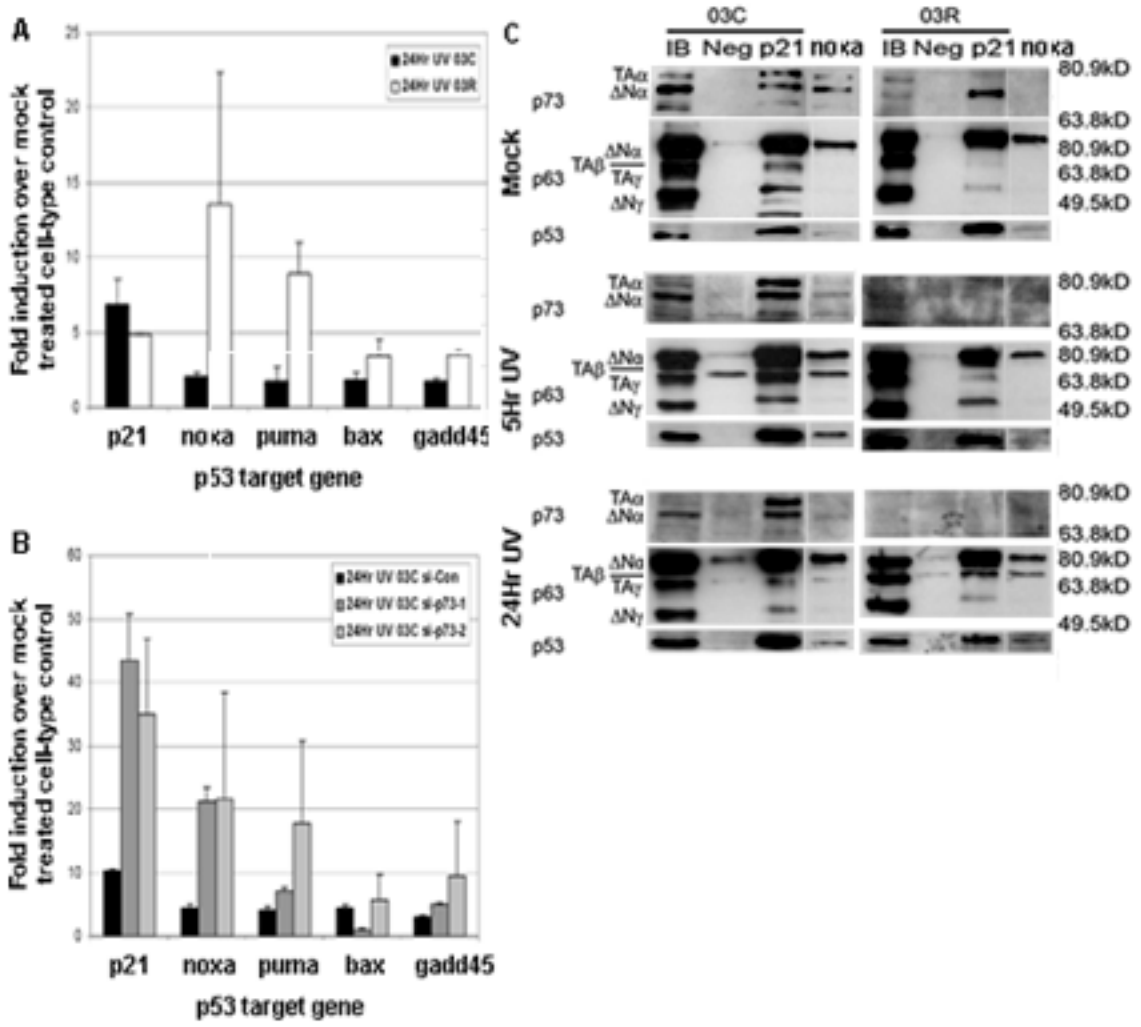

Figure 24: The TAp63 isoforms may coordinate keratinocyte response to UVB

Figure 24: The DNA binding of TAp63 isoforms correlated with keratinocyte response to UVB. A) Analysis by qPCR of p53 downstream target mRNA induction (*p21*, *Noxa*, *Puma*, *Bax*, and *Gadd45*) in 03C and 03R cells 24 Hrs. following treatment with 135 J/m^2 UVB. The 03R cells preferentially induce apoptotic targets following UVB treatment. B) Analysis by qPCR of p53 downstream target mRNA induction (*p21*, *Noxa*, *Puma*, *Bax*, and *Gadd45*) in 03C si-Con, 03C si-p73-1, and 03C si-p73-2 cells 24 Hrs. following treatment with 135 J/m^2 UVB. The graphs represent fold induction over mock treated cell type control, and the error bars represent triplicate reactions on two separate qPCR plates. C) DAI analysis of p53 family DNA binding capabilities to the p21 and Noxa probes in 03C and 03R cells. Cells were mock treated or harvested 5 or 24 Hrs. following treatment with UVB (135 J/ m^2). The Noxa lane was from the same gel as the IB, Neg, and p21 lanes, but an unrelated intermediate lane was removed to generate the figure. DNA binding of TAp63 isoforms in the 03C and 03R cells correlated with sensitivity to UVB and induction of downstream targets.

4.8 p73 as a molecular shield against the p53 family inhibitor Mdm2

Besides p53 family member proteins themselves, activities of the p53 family of transcription factors are impacted by many positive and negative regulatory proteins (Table 3). The Mdm2 protein is a known negative regulator of the transactivation efficiency of all three p53 family members. We observed increases in Mdm2 protein expression in the 03R cells as well as in 4/11 clones of 03C si-p73 cells compared to 0/5 clones of 03C si-Con cells (two representatives of 03C si-p73 clones overexpressing Mdm2 shown in Figure 25A), partially explaining why p53 activity was abrogated in these cells. Adding back TAp73α or ΔNp73α to the 03C si-p73 cells did not reduce the high Mdm2 expression levels in the cells. Mdm2 has been shown to be present in p53-DNA binding complexes, inhibiting p53-mediated transactivation of downstream targets [Jin et al., 2002; Knights et al., 2003]. We tested Mdm2 association with DNA-bound p53 complexes in primary keratinocytes, initiated 03C cells, and malignant 03R cells using the DAI assay. While Mdm2 (starred, Figure 25B) was not in complex with the DNA-bound p53 family members in primary keratinocytes or in the 03C cells, Mdm2 was in the complex in the malignant 03R cells as had been reported in [Knights et al., 2003]. To address whether p73 had any impact on the presence of the Mdm2 in complex with DNA-bound p53 family members on the p21 probe, we examined the 03C si-p73 cells + Vector, + ΔNp73α, or + TAp73α for Mdm2 by DAI. The 03C si-p73 cells + Vector or + ΔNp73α exhibited Mdm2 in the DNA-bound p53 family complex. However, the cells expressing TAp73α had lost Mdm2 from this complex (Figure 25C). The reduced Mdm2 in complex with DNA-bound p53 family members was concomitant with increased DNA binding of lower molecular weight p63 isoforms (Figure 25D). Thus the

TAp73α protein is both a positive regulator of DNA binding of p63 isoforms and a negative regulator of the inhibitor of p53 family transactivation, Mdm2.

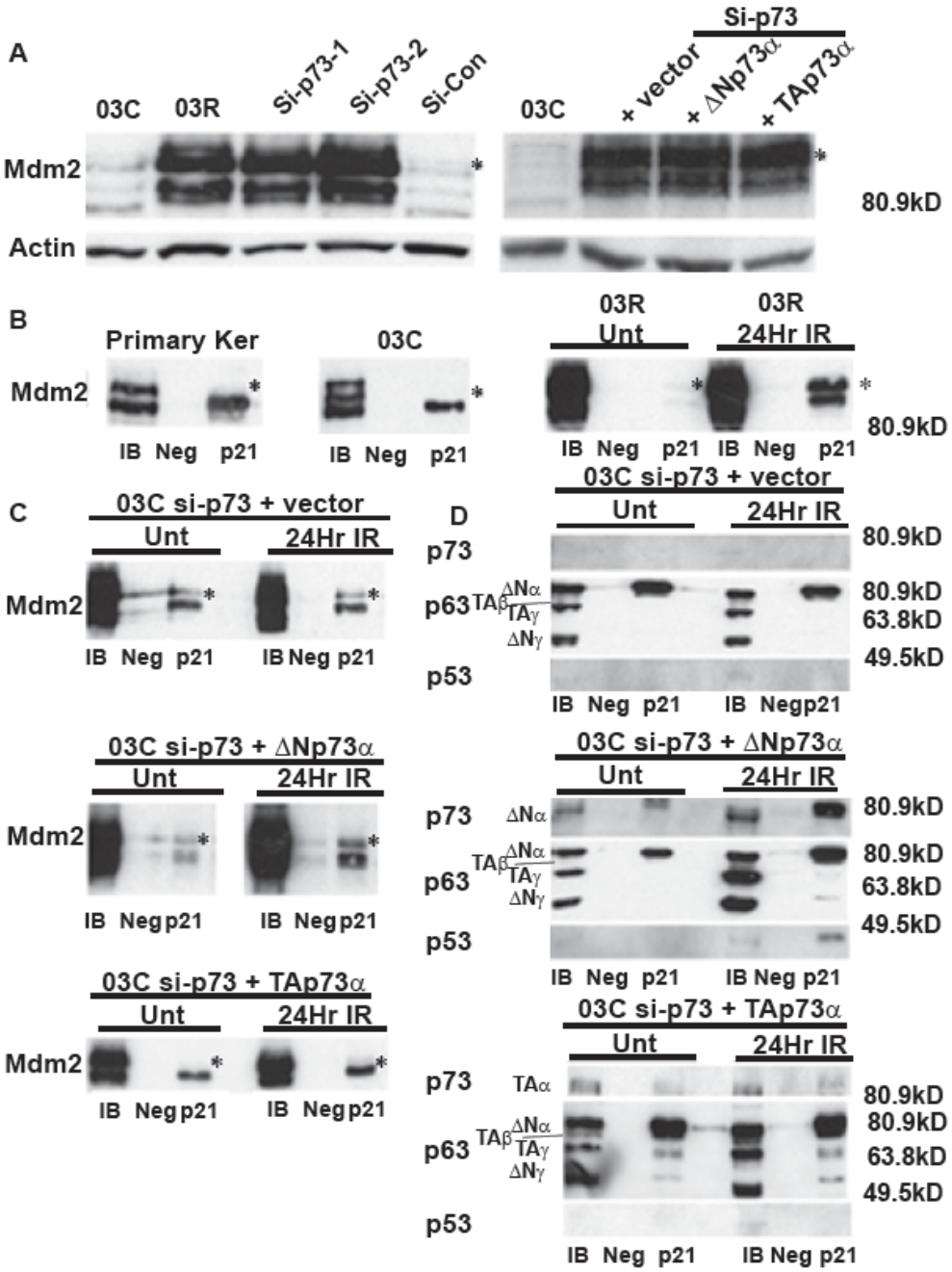

Figure 25: TAp73α expression is coordinated with removal of Mdm2 from p53:DNA complex and aiding DNA binding of TAp63 isoforms

Figure 25: Expression of TAp73α disrupts Mdm2 interaction with DNA-bound p53 as well as enhancing TAp63 isoform DNA binding. A) Immunoblot to detect Mdm2 expression in the mouse clonal epidermal carcinogenesis model. The 03R cells and derivatives of 03C si-p73 cells all express high levels of Mdm2 compared to the 03C precursors. The upper, starred band is Mdm2 while the lower band is most likely another Mdm family member, MdmX. B) DAI analysis of Mdm2 in complex with DNA-bound p53 in primary keratinocytes, initiated 03C cells, and malignant 03R cells. The primary keratinocytes and 03C cells do not exhibit Mdm2 in complex with DNA-bound p53 while the 03R cells show strong binding of Mdm2 in the complex, particularly after treatment with IR. C) DAI analysis of Mdm2 interaction with the DNA-bound p53 family members in 03C si-p73 + Vector, + ΔNp73α, or + TAp73α cells. The + Vector- or + ΔNp73α–expressing cells resemble the 03R cells in that the Mdm2 protein is found in complex with DNA-bound p53 family members. However, the 03C si-p73 + TAp73α cells resemble the primary keratinocytes and initiated 03C cells with Mdm2 not found in the DNA-bound complex. D) DAI analysis of p53, p63, and p73 isoform DNA binding capabilities in the same lysates used to detect Mdm2 in the DNA-bound complex. As in Figures 19 and 20, the presence of TAp73α aids DNA binding capabilities of lower molecular weight more transcriptionally active p63 isoforms while only the ΔNp63α isoform is bound to DNA in cells lacking p73 (+ Vector) or cells expressing ΔNp73α.

4.9 Cells expressing TAp73α are refractory to treatment with Mdm2-p53 inhibitor Nutlin-3 while cells lacking p73 or expressing ΔNp73α are sensitive

We have shown that the p73 protein is important in tumor suppression of SCC, in keratinocyte response to IR, in normalizing the DNA binding capabilities of p63 isoforms and in inhibiting the interaction of the p53 family inhibitor, Mdm2, with DNA-bound p53 family complexes. This correlates with increased basal levels of p53 downstream target mRNAs. Additional evidence for TAp73α ability to normalize squamous cell carcinogenic cells was the response of TAp73α-expressing cells to the Mdm2-p53 inhibitor Nutlin-3 (Figure 26). The p53 null NK1 cells, the initiated 03C cells, malignant 03R cells, and si-p73 cells + Vector, + ΔNp73α, and + TAp73α were treated with DMSO (Mock), Nutlin-3A (active enantiomer of the cis-imidazoline that inhibits Mdm2 interaction with p53), or Nutlin-3B (inactive enantiomer that does not inhibit Mdm2 interaction with p53). The NK1 cells were refractory to treatment with Nutlin-3A as expected because they do not express p53 and have normal low levels of Mdm2, therefore having abrogated the pathway through which Nutlin-3A acts. The initiated 03C cells were responsive to treatment with Nutlin-3A compared to Nutlin-3B, consistent with their expression of wtp53 and low levels of Mdm2. However, the malignant 03R cells and 03C si-p73 + Vector or + ΔNp73α cells were sensitive to treatment with Nutlin-3A, consistent with the over-expressed Mdm2 levels and the fact that Mdm2 was seen in complex with DNA-bound p53 family members. The 03C si-p73 + TAp73α cells, were comparable to the 03C cells in their response to Nutlin-3A, consistent with the ability of TAp73α to remove Mdm2 from p53 DNA-bound complexes.

In conclusion, we have shown that p73 plays an important role in tumor suppression of SCC and in keratinocyte cellular response to IR, but that p73 activity is dispensable for an apoptotic response to UVB. We uncovered multiple changes in DNA binding capabilities of p53 family members concomitant with loss of p73 protein expression in $p53^{+/+}$ initiated keratinocytes, some of which could be reversed upon reconstitution with TAp73α but not ΔNp73α, altering epidermal cell fate. A model and table summarizing our findings is provided in Figure 27.

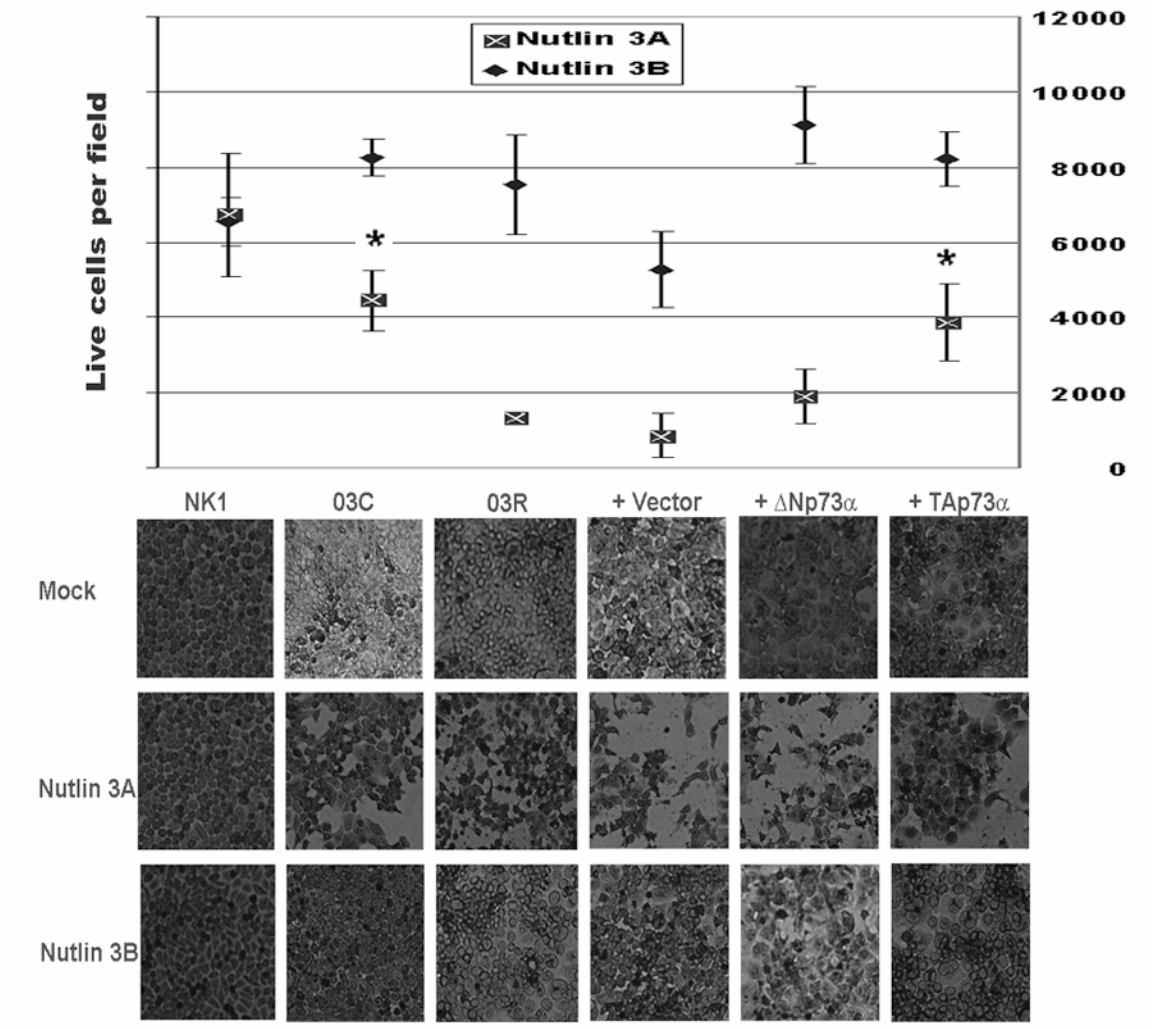

Figure 26: Cells expressing TAp73α are refractory to treatment with Nutlin-3A while cells lacking p73 expression or expressing ΔNp73α are sensitive

Figure 26: Cells expressing TAp73α are refractory to treatment with Mdm2-p53 inhibitor Nutlin-3A while cells lacking p73 expression or expressing ΔNp73α are sensitive. Cells as indicated were allowed to grow to 50% confluence following plating at equal densities. Cells were either mock treated (DMSO) or treated with 10 μM Nutlin-3A (active enantiomer) or 10 μM Nutlin-3B (inactive enantiomer) (Roche, dissolved in DMSO) once daily for 96 Hrs. then fixed in methanol and stained with Giemsa. Photographs were taken (200X) using Magnafire to show confluence of cells 96 Hrs. following treatment. Cells were counted using Imagepro software. The graph depicts the average (+/- SD) of five counted fields of cells treated with Nutlin-3A or Nutlin-3B. The p53 null NK1 cells were refractory to Nutlin-3A (p-value = 0.8670 comparing 3A-treated to 3B-treated) as was expected because they do not have increased Mdm2 levels and lack p53 expression thus have abrogated the pathway through which Nutlin-3 functions. Each of the other cell types was sensitive to treatment with Nutlin-3A compared to Nutlin-3B (p-value < 0.005 for each cell type). However, when each cell line was statistically analyzed (Student t-test) compared to the 03C cells as a "normal" (non-cancerous) baseline, the TAp73α–expressing cells (starred on the graph together with the 03C cells against which they were compared, p-value = 0.14) were more refractory to treatment with Nutlin-3A than the 03R cells (p-value < 0.01), 03C si-p73 + Vector cells (p-value < 0.01), or + ΔNp73α cells (p-value = 0.02).

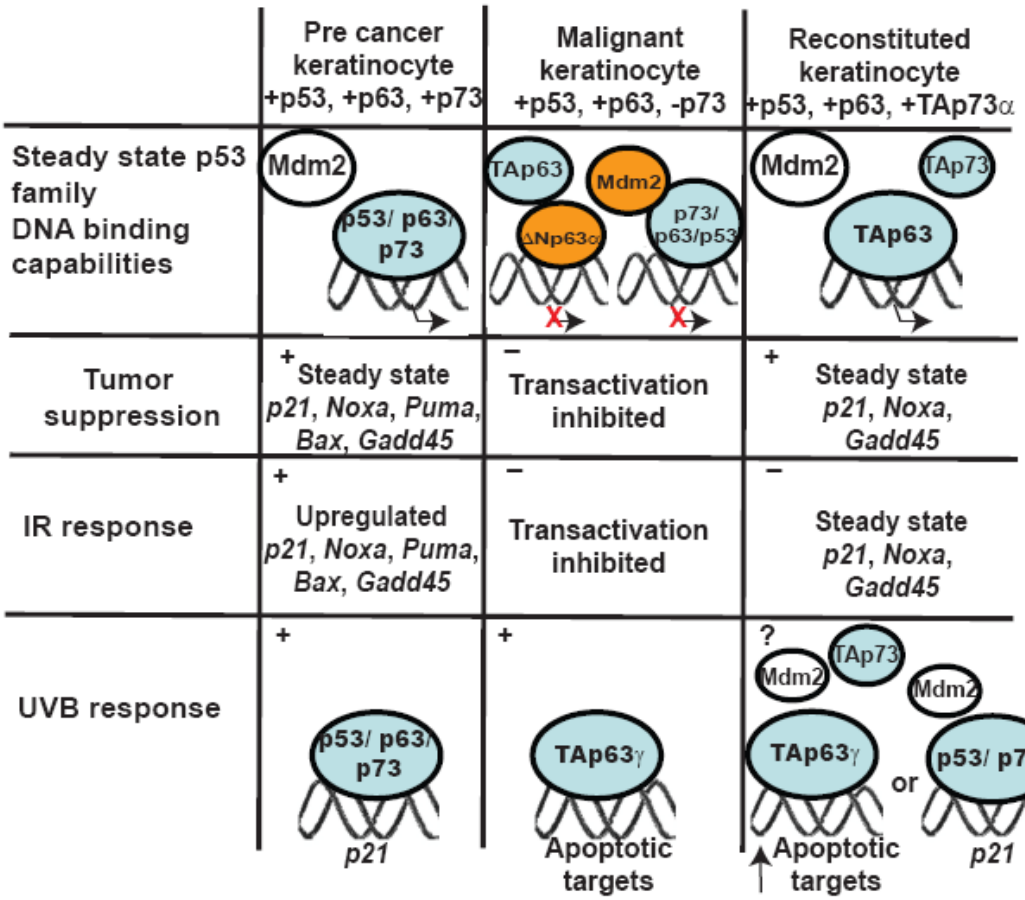

Figure 27: Models of p53 family activities in tumor suppression and cellular response to DNA damage. A table summarizing the findings presented in this current study. The first line depicts the changes in predominant p53 family DNA-binding capabilities in initiated cells expressing all three p53 family members versus malignant cells lacking p73 expression and the shift to partially restore DNA-binding complexes upon reconstitution of malignant cells with TAp73α. Blue ⬭ represents transcriptionally active isoforms of the p53 family members capable of binding to downstream target promoters while orange ⬭ represents inhibitors of transactivation. The downstream effects of these changes in DNA binding complexes are summarized showing that the cells lacking p73 expression form tumors and are deficient in IR response while reconstitution of these cells with TAp73α restores tumor suppression but not IR sensitivity. The p73 protein is not crucial for keratinocyte apoptotic response to UVB, but TAp63γ may mediate this function, causing an apoptotic response rather than G1 arrest response. While the role of p73 in UVB response is unknown, we speculate that expression of TAp73α in the malignant cells will enhance the cellular response to UVB by increasing the DNA binding capacity of TAp63γ to downstream promoters to mediate this response or aid p53/p73-mediated cell cycle arrest response, balancing the p63-mediated apoptosis.

5. Discussion:

5.1.1. Tumor suppressive functions of p73 in light of the other p53 family members

Because p73 is rarely mutated in human cancer [Moll and Slade, 2004], it is not widely considered a tumor suppressor in the classic Knudson definition [Knudson, 1971]. However, loss of heterozygosity (LOH) and biallelic methylation (gene silencing) of p73 has been reported in many cancers (Table 2 and [Stiewe and Putzer, 2002]). The majority of the early clinical studies to examine p53 family members in cancer focused on total expression levels showing that, surprisingly, p73 and p63 were frequently upregulated. Often, upon later examination with isoform-specific antibodies and RT-PCR, it was shown that, in fact, it was the ΔN isoforms of p73 and p63 that were upregulated. However, loss of p73 and p63 expression was frequently associated with increased tumor invasiveness, poor differentiation, chemoresistance, and poor prognosis in many human cancers regardless of p53 mutation status including esophageal cancer, bladder cancer, inflammatory breast cancer, thyroid cancer, SCC, and cervical cancer [Ahomadegbe et al., 2000; Araki et al., 2002; Ferru et al., 2005; Koga et al., 2003; Masuda et al., 2003; Matsumoto et al., 2004; Nenutil et al., 2003; Puig et al., 2003]. In well-differentiated human head and neck squamous cell carcinoma (HNSCC), similar to normal tissue, basal cells were immunopositive for p73, while in moderately-differentiated carcinomas p73 was ubiquitously expressed [Faridoni-Laurens et al., 2001], and in poorly differentiated SCCs, immunostaining was negative for p73 [Kamiya et al., 2004]. As in human tumors, while an initial increase in p73 immunostaining accompanied the early stages of DMBA-induced hamster oral SCC [Chen et al., 2002], the loss of p53 family member expression

in $p53^{+/-}$;$p63^{+/-}$, $p53^{+/-}$;$p73^{+/-}$, or $p63^{+/-}$;$p73^{+/-}$ mice was associated with increased tumor invasiveness [Flores et al., 2005]. It was shown that 45% of $p53^{+/-}$;$p73^{+/-}$ mice and 30% of $p63^{+/-}$;$p73^{+/-}$ mice developed metastatic tumor types including SCC that metastasized to the heart and lung. Only 5% of tumors from mice heterozygous for p53 alone metastasized. In these studies, expression levels of individual p73 isoforms were not distinguished. Collectively, these findings show that p73 loss is frequently associated with the later, more aggressive stages of carcinogenesis. However, the question remains about whether p73 can act as a tumor suppressor in early stages of conversion to malignancy and whether direct manipulation of p73 can affect tumor progression.

The current study demonstrates a tumor suppressive role for p73 showing that loss of p73 alone in initiated $p53^{+/+}$ keratinocytes expressing multiple p63 isoforms was sufficient to trigger malignant conversion to poorly differentiated SCC. Whether spontaneous or siRNA-induced, loss of p73 associated with rapid cellular growth characteristics, alterations in cellular morphology, and tumorigenesis. As shown in the Appendix, microarray analysis of biological duplicates of 03C si-p73-1 cells compared to 03C si-Con cells and 03R cells showed that the 03C si-p73-1 cells had undergone massive global changes in gene expression, making them genetically similar to the 03R cells. The microarray analysis performed in this study was limited because a second 03C si-p73 clone (si-p73-2) is needed to compare to the 03C si-p73-1 clone for confirmation of the massive gene expression changes observed. Further, the 03C si-p73 + TAp73α cells were not analyzed by microarray to determine whether the global genetic changes observed upon loss of p73 expression could be reversed upon reconstitution with TAp73α. Both of these additional microarray studies would be informative concerning

the specific role of p73 in malignant conversion and of TAp73α in tumor suppression. Nevertheless, the global gene changes observed upon silencing of p73 expression in the 03C cells suggest that a genetic "signature" was already established in the initiated 03C cells to predispose them to give rise to malignant cells producing poorly differentiated SCC, particularly upon loss of total p73 expression. It has previously been shown that the 05C (giving rise to the 05R producing moderately differentiated SCC) and the 09C (giving rise to the 09R producing benign papilloma) have different genetic signatures to give rise to cells with benign or malignant cell fates [Wang et al., 2002]. Analysis of the microarray data using the PNRP and DWD metagene profile methods showed that the 03C to 03C si-p73 conversion exhibited a global genetic change signature comparable to that of the 03C to 03R transition (data not shown) indicating that the silencing of p73 expression in the 03C cells was a sufficient trigger to lead to massive gene expression changes associated with malignant conversion to SCC.

To address isoform specificity of p73 in tumor suppression, we showed that recapitulation of TAp73α expression but not ΔNp73α expression was sufficient to restore more normal cellular growth characteristics *in vitro,* including growth in monolayers more like the initiated 03C cells and their precursors than the tumorigenic 03R cells. The TAp73α isoform acted as a tumor suppressor of SCC *in vivo,* while cells lacking p73 or expressing ΔNp73α formed tumors.

Our findings show that direct manipulation of p73 can alter epidermal cell fate, that loss of p73 can trigger conversion to SCC, and that reconstitution of TAp73α function can suppress tumor formation, supporting the notion that restoring TAp73α function is a rational target for molecular targeted therapy in SCC. Several studies have

shown that activation of p73 was sufficient to trigger apoptosis of cancer cells and even cause tumor regression in mice regardless of p53 status. This is particularly exciting considering that, even though restoration of p53 function lead to tumor regression *in vivo* [Kastan, 2007; Ventura et al., 2007], p53 is mutated in 50% of cancers, functionally inactivated in up to 90%, and therefore difficult to reactivate as a therapeutic target [Hofseth et al., 2004]. Adenovirus mediated transfer of p73 to pancreatic, colorectal, breast, lung, liver, and ovarian cancer cells attenuated cell growth even when the cells were resistant to wild type p53 gene therapy [Das et al., 2005; Rodicker and Putzer, 2003; Sasaki et al., 2001]. Further, reactivation of p73 by a p53-derived apoptotic peptide led to tumor regression *in vivo* and attenuation of cancer cell growth *in vitro* independent of whether p53 was wild type, mutant, or functionally inactivated [Bell et al., 2007]. However, certain specific mutant p53 molecules, altering the conformation of the p53 DNA binding domain, have been shown to bind to and inactivate p73 [Gaiddon et al., 2001; Strano et al., 2000]. Thus reactivation of p73 was effective for tumor suppression in our study in $p53^{+/+}$ cells lacking wild type p53 function [Knights et al., 2003], but may not be effective in the context of certain p53 mutations. In the current study we show that direct specific reconstitution of TAp73α in $p53^{+/+}$ malignant-converted cells suppresses poorly differentiated SCC *in vivo,* adding credence to the value of TAp73α activation as a potential therapeutic target in solid tumors of the epithelium.

5.1.2. DNA binding cooperation and competition between p53 family members for tumor suppression or carcinogenesis

In one mouse model, it was shown that it is not the p73 but the p63 protein that plays a significant role in protection against SCC. All SCCs (15/15) produced by $p63^{+/-}$

;$p73^{+/-}$ mice in this model had undergone loss of heterozygosity (LOH) of the other p63 allele but none of these tumors had undergone LOH of the other p73 allele [Flores et al., 2005]. In another independently derived mouse model, no $p63^{+/-}$ or $p63^{-/-}$ mice developed tumors of any kind [Keyes et al., 2006]. It was suggested that if any tumor suppressive function of p73 existed against SCC, based on the first mouse model, it must be dependent upon the presence of p63 [Rocco and Ellisen, 2006]. The rationale was that, since LOH of p73 had not occurred in the SCC tumors, the p73 protein's tumor suppressive function should have been intact if, indeed, it was capable of acting alone in suppression of SCC. Our data does not contradict this reasoning, but rather adds credence to it and shows how the p73 and p63 family members could be cooperating for this tumor suppressive function in SCC as will be discussed subsequently.

It has been proposed that the capability of individual p53 family members to induce common downstream target genes including cell cycle arrest response gene *p21*; DNA damage repair gene *Gadd45*; and apoptotic response genes *Noxa*, *Puma*, and *Bax* [el-Deiry et al., 1993; Kastan et al., 1992; Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda et al., 2000] is one method by which the TA isoforms of the p53 family members carry out tumor suppressive functions. However, there are also multiple layers of cooperation and competition between family members as discussed in the introduction and summarized in Figure 5. Our data suggest an obligate cooperation between TAp73α and lower molecular weight TAp63β, TAp63γ and ΔNp63γ isoforms for increased steady state DNA binding capabilities of the p63 isoforms. Loss of p73 expression either spontaneously at malignant conversion in the 03R cells or via targeting by siRNA in the initiated 03C cells lead to reduced DNA binding capabilities of the p63

isoforms which was at least partially reversible upon reconstitution of 03C si-p73 cells with TAp73α. TAp73α by itself is transcriptionally inactive [Alarcon-Vargas et al., 2000]. However, TAp63γ is the most transcriptionally active p63 isoform [Yang et al., 1998] and the ΔNp63γ isoform had some transactivational activity due to a second transactivation domain [Dohn et al., 2001; Ghioni et al., 2002]. This transactivational activity corresponded with the ability of ΔNp63γ to induce a low level of apoptosis in p53 null Saos2 cells. Further, cotransfection of TAp63γ in a 5:1 ratio with ΔNp63γ yielded an increase in transcriptional activity over that seen by TAp63γ alone using a reporter construct containing multiple p53 binding sites, but the mechanism for this cooperative interaction was not addressed [Yang et al., 1998]. In our study, when total p73 expression was lost, the TAp63β, TAp63γ, and ΔNp63γ isoforms exhibited reduced DNA binding capabilities and the p53 family target genes were not induced following DNA damage by IR. This data is summarized in the model and table given as Figure 27. As will be discussed subsequently, reconstitution of TAp73α into cells did not restore sensitivity to IR. However TAp73α did restore DNA binding capabilities of TAp63β, TAp63γ and ΔNp63γ isoforms and increased steady state levels of p53 target genes as well as tumor suppression. Thus, this cooperation between TAp73α and p63 isoforms for DNA binding capability provides one explanation for how p73 and p63 cooperate for tumor suppression of SCC. The mediation of p63 isoform DNA binding capabilities by TAp73α could be via direct protein-protein interaction with p63 isoforms at DNA, via interaction with a common activating cofactor such as Aspp2, or via sequestration of p63 inhibitors such as ΔNp63α or Mdm2, allowing lower molecular weight, more transcriptionally active p63 isoforms to gain advantage for DNA binding.

The p21 promoter was chosen as the probe for the DNA binding assays in this study because it contains a p53 binding site that all three p53 family members are capable of interacting with and because the p21 mRNA expression was not induced in 03R cells and 03C si-p73 cells following IR treatment as compared to the 03C cells that induced p21 following DNA damage by IR. Thus the alterations of DNA binding capabilities of the p53 family members to the p21 probe in 03R and 03C si-p73 cells could be directly linked to downstream biological defects in DNA damage response. DAI probes were also synthesized for the Mdm2, Noxa, Bax, and Puma p53 binding sites (see Materials and Methods) and the DNA binding capabilities of the p53 family members to these probes was tested using 03C and 03R cell lysate. While all three p53 family members were capable of strongly binding to the p21 and Mdm2 probes in the 03C cells, the 03R cells exhibited reduced DNA binding capabilities of all three p53 family members to both probes (data not shown for Mdm2 probe). In general, all three p53 family members bound with less affinity to the p53 binding sites present in the apoptotic gene probes (Noxa, Puma, and Bax) as compared to the p21 and Mdm2 probes in both the 03C and the 03R cells, consistent with reports that p53 binds with lower affinity to apoptotic target genes [Weinberg et al. 2005]. Differential binding of specific p63 isoforms (particularly TAp63γ) to the Noxa probe was observed following DNA damage by UVB in both the 03C and 03R cells and the 03R cells underwent apoptosis following UVB treatment. In further studies, it will be important to determine the DNA binding capability profiles of the p53 family members to the binding sites in genes involved in apoptosis, such as Noxa, Puma, and Bax, as well as downstream target genes involved in other cellular outcomes to determine p53 family isoform-specific responses to various types of DNA

damage rather than focusing solely on the promoter of p21. It has recently been discovered that p63, in addition to binding the canonical p53 binding site, preferentially binds to an alternate DNA binding site with sequence RRRCGTGYYY [Osada et al., 2005]. The global distribution of this alternate sequence in the genome has not yet been characterized. However, the alternate sequence was found to be present in the p21 promoter approximately 2 kb upstream of the transcriptional start site (as opposed to the canonical p53 binding site used in these studies located 2.3 kb upstream of the start site, Jayme Gallegos and Dr. Hua Lu, personal communication). It will be important to test the DNA binding capabilities of the p53 family members to such alternate sequences and, particularly, to study whether localization of combinations of p53 family members to the canonical and alternate DNA binding sequences contributes to the precise coordination of downstream target gene induction in response to cell stress and DNA damage. The DAI assay in combination with CHIP could be informative in this regard.

In addition to pro-transactivational cooperation, the p53 family member isoforms also exhibit dominant negative inhibition and competition for target promoters. For example, the ΔNp63α isoform was a strong suppressor of transactivation by TAp63γ [Yang et al., 1998]. The ΔNp63α isoform was found to be the predominant p63 isoform expressed in human SCC [Cui, He et al., 2005]. In recent studies, both direct inhibitory ΔNp63α/TAp73β protein-protein interactions and competitive inhibitory ΔNp63α binding to *Puma* and *Noxa* promoters were implicated in reduced p53 family-dependent apoptosis in SCC cells [Rocco, Leong et al., 2006]. Unlike normal epithelial cells, it was shown that SCC cells were dependent upon ΔNp63α expression for survival [Deyoung et al., 2006]. Our data suggest that in the absence of TAp73α protein

expression, ΔNp63α gains predominance over TAp63 isoforms in DNA binding capability, driving malignant progression of SCC and promoting tumor survival. Analysis of p53 family DNA binding capabilities in SCC-producing cells and in lysate from SCC tumors showed that only the ΔNp63α isoform remained robustly bound to the p21 probe, even though p53 and multiple p63 isoforms remained expressed in the cells and in the tumors. The presence of the TAp73α isoform may restore a functional balance between TA and ΔN p63 isoforms for tumor suppression, either through bringing more transcriptionally active p63 isoforms directly to DNA binding sites or through removing ΔNp63α from DNA binding sites, allowing the more transcriptionally active p63 isoforms to gain access to target promoters. Alternatively, TAp73α may facilitate DNA binding activities of p63 and p53 through interactions with other proteins apart from direct DNA binding, as suggested by the observed reduction of Mdm2 in the DNA-bound p53 family complexes in the presence of TAp73α. Testing the mechanisms through which TAp73α facilitates the DNA binding of lower molecular weight p63 isoforms will require parallel IP assays and isoform-specific ChIP as antibodies improve.

5.1.3. Response of p53 family members to DNA damage with IR and UVB and hierarchy of family members in coordinating cell cycle arrest and apoptosis

All three p53 family members can induce both cell cycle arrest and apoptosis in response to DNA damage [Dohn et al., 2001; Horn and Vousden, 2007; Jost et al., 1997]. In cancer, loss of p73 protein expression correlated with p21 down-regulation and increased tumor invasiveness in esophageal cancers regardless of p53 mutation status [Masuda et al., 2003]. Radioresistance in cervical cancer patients was associated with

TAp73 loss [Liu, Leung et al., 2004] and overexpression of TAp73α in p53-impaired cervical cancer cells enhanced radiosensitivity [Liu, Chan et al., 2006]. Thus the study of p73 in mediating the DNA damage response pathways is crucial for understanding how the use of current cancer therapeutics may be optimized or new therapies developed.

Our data show a requirement for p73 in mediating keratinocyte response to DNA damage induced by IR even in the presence of wild type p53. The loss of total p73 protein expression in initiated $p53^{+/+}$ keratinocytes associated with IR resistance as determined by enhanced cell survival in cell viability assays compared to mock treated controls, loss of G1 arrest, and loss of induction of p53 target genes *p21*, *Noxa*, P*uma*, *Bax*, and *Gadd45*. The initiated 03C cells that were sensitive to IR exhibited p53 induction, but did not show an induction of p73 protein expression following treatment with IR. Increased expression of p73 is not specifically required for p73 to mediate cellular response to radiation [Liu, Chan et al., 2006]. The increase of p73 expression following DNA damage has been shown to be cell cycle-dependent in that the p73 protein is increased only after progression through G1/S phase [Irwin et al., 2000; Wang and Ki, 2001] and TAp73 isoforms accumulate during S phase [Fulco et al., 2003]. Our data shows that the majority of the 03C cells were arrested in G1/S following IR treatment and so the lack of p73 induction is expected.

The only change observed in the p73 isoforms to explain the requirement of p73 for IR response was an enhanced DNA binding capability of the TAp73α isoform to the p21 promoter following IR treatment. The TAp73α isoform gained predominance over the ΔNp73α isoform in the DNA-bound state following IR in the non-transformed 291 cells or DNA-bound TAp73α was stabilized so that it was equivalent to DNA-bound

ΔNp73α in the initiated 03C cells. Loss of p73 protein expression associated with predominant DNA binding of ΔNp63α to the p21 probe regardless of whether the cells were treated with IR or not. Reconstitution of IR resistant cells with TAp73α or ΔNp73α isoforms was not sufficient to restore cellular sensitivity to IR, indicating these individual p73 isoforms are not, by themselves, capable of mediating the cellular IR response. The lower molecular weight p73 isoform expressed in the primary keratinocytes, 291 cells, and 03C cells, though not confirmed, was expected to be TAp73β, the most transcriptionally active p73 isoform. The identity of this lower isoform could not be confirmed by size comparison with known p73 isoforms because there are multiple p73 splice variants that could migrate at this same location. However, the siRNA targeted against p73 in the 03C cells silenced all isoforms of p73, including this lower molecular weight isoform. Though not tested, reconstitution of TAp73β into the 03C si-p73 cells, thereby restoring expression of the most transcriptionally active p73 isoform, may be sufficient to restore cellular response to IR. Further studies will show whether restoration of DNA damage responsiveness can be mediated by the addition of a single p73 isoform not tested in this study, ratios of p73 or p63 and p73 isoforms, interaction with regulatory cofactors such as p300 or Aspp proteins, or loss of p53 family inhibitory interactions such as with Mdm2 or ΔNp63α.

Recently, the DNA damage response of p53 was uncoupled from its tumor suppressor function in a mouse model of radiation-induced lymphoma [Christophorou et al., 2006]. It was shown that p53 mediated an acute DNA damage response immediately following IR but that this response was not responsible for suppression of tumors. Rather, p53 mediated its tumor suppressive function through induction of the $p19^{ARF}$ pathway

only in select cells. The tumor suppressive activities of other p53 family members may also be largely distinct from their DNA damage responsiveness. Our data indicate that TAp73α can mediate a tumor suppressive function in SCC even though it is incapable of restoring cellular response to IR. Further studies of p53 family activities at discrete, progressive stages of tumorigenesis should be informative in this regard, uncovering the mechanisms through which p73 and p63 isoforms exert their tumor suppressive activities.

Though the p73 protein is reportedly induced following treatment with UV [Lin et al., 2004], its role in mediating UV response has not been well studied. Our data indicate that the p73 protein is not required for keratinocyte sensitivity to UVB. Malignant cells that had lost p73 expression and become resistant to IR remained sensitive to treatment with UVB. This was characterized by reduced cellular viability in colony forming assays of cells treated with UVB compared to mock treated controls, and induction of p53 family target genes. In past studies, the ΔNp63α isoform was shown to be phosphorylated and downregulated in response to UVB in keratinocytes [Liefer et al., 2000; Westfall et al., 2005] and also exhibited a reduced affinity to p53 target gene binding sites due to phosphorylation [Papoutsaki et al., 2005]. Meanwhile, the TAp63γ isoform was upregulated in response to UV treatment [Katoh et al., 2000]. We did not observe changes in protein expression levels of the p63 isoforms in our model system following treatment with UVB, but did observe enhanced DNA binding capability of the TAp63γ isoform to both the p21 and Noxa promoter probes even in the absence of p73 protein expression. The keratinocytes did not arrest in G1 following treatment with UVB. Instead, there were fewer cells in the G1 and G2 phases of the cell cycle, more cells in the S phase, the apoptotic target genes *Noxa* and *Puma* were induced, and caspase-3 activity

increased, indicating an apoptotic response to UVB. In the 03C si-p73 cells, the *p21* and *Gadd45* targets were also induced, but no G1 arrest was observed. Gadd45 is involved in regulating NER following DNA damage by UVB [Smith et al., 1996; Zhan et al., 1996], but Gadd45 must be released from p21 in order to carry out this function [Maeda et al., 2005]. Thus, in 03R and 03C si-p73 cells, the expression of *p21* and *Gadd45* mRNA but failure to undergo G1 arrest following treatment with UVB may indicate aberrant DNA damage response.

One role of keratinocytes is to provide a protective barrier against DNA damage derived from exposure to the sun. In humans, the apoptotic keratinocyte response to UVB is carefully balanced with cell survival. If the decision to die is made prematurely, the proliferative basal layer of keratinocytes could be lost, thereby abrogating normal skin homeostasis. However, if severely damaged cells escape apoptosis and continue to proliferate, malignancy could result. Thus the balance between survival and apoptosis in normal keratinocytes is crucial and is mediated by multiple pathways [Van Laethem et al., 2005]. Though not tested in the current study, it is possible that the loss of p73 protein expression may predispose keratinocytes to p63-mediated apoptosis even where the G1 arrest response would be preferable. It remains to be tested whether the presence of TAp73α may further enhance the DNA binding capability of TAp63γ to apoptotic downstream target promoters in response to UVB or repress the apoptotic activities of p63 and balance them by enhancing the G1/S arrest response.

We propose a hierarchy of p53 family member mediation of DNA damage response where p73 is required for p53/p73 mediated G1/S arrest response to DNA damage but the p63 protein can mediate an apoptotic response to DNA damage by UVB

in the absence of functional p73. Due to the basic C terminal DNA binding domain unique to p53, we propose that p53, when present, is the most rapid responder for transactivation of downstream target genes (either for apoptosis or G1 arrest) in response to DNA damage [Liu and Kulesz-Martin, 2006; Liu, Lagowski et al., 2004]. This rapid response may or may not depend on the expression of p73 and p63 depending on the type and severity of DNA damage and the tissue involved. For example, the p73 and p63 proteins were required for p53 transcription-dependent apoptotic response to DNA damage with doxorubicin and IR in mouse embryonic fibroblasts (MEFs), and in the developing central nervous system (CNS) [Flores et al., 2002], but not in T cells [Senoo et al., 2004]. In wild type MEFs, ChIP assays showed that after DNA damage by doxorubicin, both p53 and p63 were associated with *Mdm2*, *p21*, *Bax*, *Perp*, and *Noxa* promoters, but in $p63^{-/-}$;$p73^{-/-}$ MEFs, p53 was not associated with apoptotic promoters. The p63 protein was present at apoptotic promoters in $p53^{-/-}$ MEFs, leading the authors to suggest that p63 could mediate apoptotic response if p53 was defective. However, the DNA binding capabilities of specific p63 isoforms or of p73 were not evaluated due to lack of specific antibodies required for ChIP. Thus it remains unclear whether the p63 protein bound at apoptotic promoters was a TA or a ΔN isoform. Based on our data, we propose that in response to IR, expression of the p73 protein is required to induce cell cycle arrest in keratinocytes, even those expressing wild type p53, such as the 03R and 03C si-p73 cells. This hypothesis could be further tested by silencing p73 expression in the p53 null NK1 cells followed by transfection with wild type p53 and treatment with IR to determine whether p73 is required for the G1/S arrest in keratinocytes in the presence and absence of p53. In the epidermal model of carcinogenesis, loss of p73 expression

abolished the G1/S arrest response and abrogated induction of both cell cycle arrest and apoptotic p53 target genes in response to IR. In this scenario, it is the inhibitory ΔNp63α isoform that is predominantly bound to DNA and this contributes both to malignant progression and resistance to DNA damage by IR. However, in response to UVB in the keratinocytes, the TAp63γ protein can mediate a "last ditch effort" apoptotic response to DNA damage involving DNA binding to apoptotic promoters and enhanced expression of apoptotic target genes. Cells in this scenario would not arrest in G1, but would die following accumulation in the S phase of the cell cycle. Treatment of cells with other types of DNA damaging agents such as doxorubicin, cisplatin, and etoposide may help elucidate the requirement of the individual p53 family members and how they cooperate with one another in the hierarchical response. The hypothesized hierarchy of p53 family responses to DNA damage is summarized in Figure 28A.

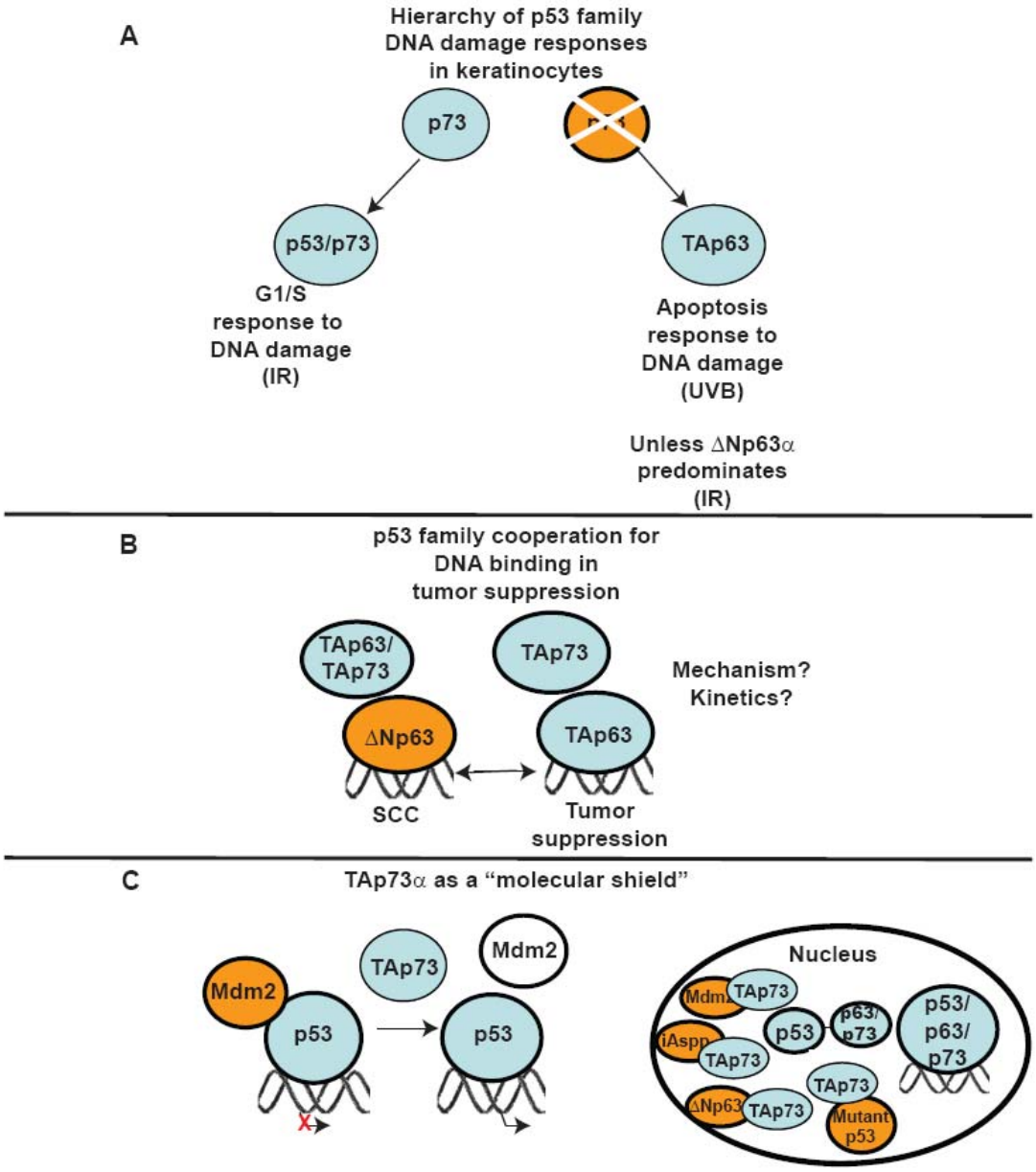

Figure 28: Models of hypotheses generated from the current study

Figure 28: Models of hypothesis generated from the current study. A) Hierarchy of p53 family DNA damage responses in keratinocytes in the presence or absence of p73. The hypothesis is that in the presence of p73, cooperation between p53 and p73 mediate a G1/S arrest response to DNA damage by IR. When p73 expression has been lost, the ΔNp63α isoform predominates as the DNA bound form, and restoring single isoforms of p73 does not restore sensitivity to IR. However, TAp63γ can mediate a "last ditch effort" apoptotic response to UVB. Reconstitution of malignant cells with TAp73α may enhance the TAp63γ-mediated apoptotic response by promoting TAp63γ DNA binding to downstream promoters or by sequestering p53 family inhibitors such as ΔNp63α. Alternatively, the presence of TAp73α may enhance a p53/p73-mediated G1 arrest response to damage by UVB, decreasing the TAp63γ-mediated apoptotic response. B) Data presented in this thesis indicated that loss of p73 expression reduced DNA binding capabilities of lower molecular weight p63 isoforms and that the inhibitory ΔNp63α isoform was preferentially bound to DNA *in vitro* and *in vivo* in SCC. Expression of TAp73α restored lower molecular weight p63 isoform DNA binding capabilities. Possible mechanisms for TAp73α-mediated increases in p63 isoform DNA binding capabilities include direct protein-protein p73:p63 interactions at DNA, interaction between TAp73α and TAp63 isoforms mediated through a common cofactor, or sequestering of ΔN p53 family isoforms away from DNA by TAp73α to enhance the opportunity for TAp63 DNA binding. The mechanism and kinetics of this cooperative DNA-binding restoration remain to be explored. C) Expression of the TAp73α protein correlated with removal of inhibitory Mdm2 from DNA-bound p53. Since TAp73 has been shown to be associated with multiple p53 family inhibitors we hypothesize that TAp73α acts as a molecular shield to localize p53 family inhibitors away from TA p53 family isoforms in the nucleus. Such a shield would act to enhance the DNA binding capabilities and the transactivational potency of the TA p53 family member isoforms to maintain normal cellular activities, tumor suppression, and DNA damage responsiveness.

5.1.4. TAp73α as a molecular shield against inhibitors of p53 family members

Interplay between p53 family members can occur through protein-protein interactions [Davison et al., 1999; Gaiddon et al., 2001; Lang et al., 2004; Muller et al., 2005; Olive et al., 2004], protein-DNA interactions [Cui, Nguyen et al., 2005; Stiewe, Theseling et al., 2002], or shared interactions with non-p53-family proteins such as Aspp1 or Aspp2, Mdm2, or p300 [Bergamaschi et al., 2004; Braithwaite et al., 2006; Koutsodontis et al., 2005; Maisse et al., 2003]. We have shown both cooperative and competitive interactions between p53 family members involved in tumor suppression and DNA damage response. We propose that the TAp73α protein has an additional role in aiding the TA p53 family members by acting as a "molecular shield" through direct inhibition of p53 family inhibitors such as Mdm2 and ΔNp63α, sequestering them away from p53 family members in the nucleus and thus enhancing the transactivational capacity of the p53 family. Our data suggests that, in the presence of TAp73α, inhibitory interactions of Mdm2 with DNA-bound p53 family members are decreased and pro-transactivation DNA binding capabilities of TAp63β, TAp63γ, and ΔNp63γ are enhanced. The presence of TAp73α seems to normalize the p53 family DNA binding capabilities in malignant cells so that the DNA-bound isoforms are more similar to those found in initiated or non-transformed keratinocytes and Mdm2 does not interact in the complex. This can explain why the keratinocytes expressing TAp73α were refractory to treatment with the Mdm2-p53 inhibitor Nutlin-3A while ΔNp73α-expressing cells or cells lacking p73 were sensitive. In the keratinocytes expressing TAp73α, Mdm2 was no

longer in inhibitory DNA-bound complexes with p53 and so the treatment of these cells with Nutlin-3A was redundant.

The p73 protein interacts with multiple inhibitors of p53 family members such as Mdm2, MdmX, mutant p53, ΔNp63, ΔNp73, cMyc, and iASPP [Bergamaschi, Samuels et al., 2003; Gaiddon et al., 2001; Grob et al., 2001; Michael and Oren, 2002; Rocco, Leong et al., 2006; Wang, Arooz et al., 2001; Watanabe et al., 2002]. Unexpectedly, p73 and p63 are stabilized by interactions with Mdm2 while p53 is degraded. Mdm2 and TAp73α colocalized in the nucleus, reducing the transactivational activity of p73 [Gu et al., 2001]. In a separate study, adenovirus-mediated p73 overexpression in neuroblastoma cell lines lead to nuclear accumulation of p53, upregulation of p21, and induced growth arrest in neuroblastoma cell lines where p53 had previously been inactive [Goldschneider et al., 2003]. Though not addressed in either of these studies, it would be interesting to see whether colocalization of p73 with Mdm2 competitively keeps Mdm2 from ubiquitylating p53 and targeting it for nuclear export, thereby allowing increased p53 association with target promoters. Alternatively, the p73 protein may indirectly activate p53 through association with an Mdm2 inhibitor, $p19^{ARF}$ (known to interact with all three p53 family members, see Table 3). It has been shown that the $p19^{ARF}$ protein can sequester Mdm2 away from p53, thereby providing one mechanism though which Mdm2-mediated p53 turnover is avoided and partially explaining how p53 mediates its tumor suppressive function apart from its DNA damage response function [Christophorou et al., 2006; Sherr and Weber, 2000]. Interestingly, the tumorigenic 03R cells in the clonal model of epidermal carcinogenesis exhibited a 4-fold increase in $p19^{ARF}$ expression compared to 03C cells (Chad Knights, unpublished data). Mdm2 and $p19^{ARF}$

colocalized in the 03R cell nuclei (unpublished), but Mdm2 was still capable of inhibitory interactions with p53 (data presented in this current study as well as [Knights et al., 2003]). It would be interesting to determine if restoring TAp73α expression in the 03R cells would trigger a $p19^{ARF}$-mediated relocalization of Mdm2, thus allowing reactivation of p53. Indirect immunoflourescence of p73/$p19^{ARF}$/Mdm2 complexes and subcellular localization could be informative. It will be important to follow up on the idea that p73 can act as a "molecular shield" to sequester p53 family inhibitors away from DNA-bound p53 family members. Further elucidation of the relationships between the p53 family members and their interacting proteins will be crucial for understanding the process of malignant transformation, cancer progression, and for optimization of p53 family member responses to anti-cancer therapeutics.

6. Future Studies and Perspectives:

The data generated in this current study lead to multiple new questions concerning the interplay between p53 family members in mediating cell fate. Some of these questions are discussed in the following section together with ideas for experimental approaches to address these questions. A schematic overview of this section has been provided as Figure 28.

6.1.1. What is the hierarchy of p53 family coordination of cell cycle arrest versus apoptosis in tumor suppression and response to DNA damaging agents?

We propose that the p53 family member isoforms exhibit a hierarchy of actions for coordinating normal cellular functions where the DNA damage response and tumor suppressive functions of the p53 family members are not directly linked. In this hierarchy, the p73 protein is required for p53/p73 mediated G1/S response to DNA damage and the TAp73α isoform is required for optimum DNA binding capability of transcriptionally active p63 isoforms to promote tumor suppression. Further, in this model, both the tumor suppressive and DNA damage functions of the p53 family members are enhanced by p73-mediated sequestering of p53 family inhibitors such as Mdm2. This idea is based on the fact that the loss of p73 expression in $p53^{+/+}$ initiated cells led to tumorigenesis and loss of sensitivity to IR while reconstitution of malignant cells with TAp73α, but not ΔNp73α, restored tumor suppression, promoted DNA binding of transcriptionally active p63 isoforms, removed the p53 inhibitor Mdm2 from DNA-bound p53 family members, rendered the cells refractory to treatment with Nutlin-3A, but did not restore sensitivity to IR, uncoupling TAp73α-mediated tumor suppression from

DNA damage response. It is reasonable to consider that restored levels of TAp73α expression may, through protein-protein interactions with TAp63 isoforms as opposed to direct p73 DNA binding, activate a basal p53 family-mediated stress response necessary for tumor suppression. Indeed, basal levels of p53 downstream target mRNAs increased in malignant 03C si-p73 cells expressing TAp73α compared to cells that had lost p73 expression or expressed ΔNp73α. However, to restore IR sensitivity to malignant cells may require a more precise balance of expression levels of p73:p63 isoforms, enhanced interaction with specific activating cofactors, or reduced interaction with inhibitory cofactors. For example, the transcriptionally active TAp73β isoform or higher levels of both TAp73α and TAp63β or γ may be needed to fully compete away the inhibitory ΔNp63α isoform from target gene promoters to allow DNA binding of transcriptionally active p53 family isoforms, cofactors such as Aspp2 may be required to interact with the family members, or Mdm2 may need to be sequestered from p53 family members.

While p73 appears to be crucial for p53 family tumor suppression and IR response, p73 is not required for response to UVB in malignant keratinocytes. We hypothesize that in the hierarchy of p53 family functions, the p73 and p53 proteins cooperate to mediate a preferential G1/S arrest response as was observed in initiated 03C cells in response to IR. However, in the absence of p73, cells rely on the only remaining fully functional p53 family member, p63 to bind and transactivate *Noxa*, *Puma* and other apoptosis-related genes. We observed that the 03R or 03C si-p73 cells did not accumulate in G1 or G2 phases of the cell cycle, but progressed to S phase following treatment with UVB and that the downstream genes that were induced were preferentially apoptotic. In order to determine the importance of p63 in mediating apoptosis following DNA damage

in cells lacking p73, total p63 could be silenced using siRNA in the 03R cells or in the 03C si-p73 cells followed by treatment with UVB to determine if the loss of p63 renders the cells UVB resistant. Addition of TAp63β or γ isoforms to cells lacking p63 would then allow determination of which isoforms were crucial for mediating the UVB response. Alternatively, isoform specific siRNA against TAp63γ in the 03R cells or 03C si-p73 cells could elucidate the importance of this specific p63 isoform in mediating UVB response (isoform specific siRNAs against p63 are being synthesized in the laboratory of Dr. Alea Mills, personal communication).

Further studies are needed to determine if p73 has a role in aiding p63 isoforms in response to UVB. When the TAp73α isoform was reconstituted to tumorigenic cells that had previously lost p73 expression and exhibited aberrant p63 DNA binding capability, TAp73α aided TAp63 isoform DNA binding. If TAp63β and γ isoforms mediate cellular response to UVB then the presence of TAp73α may aid DNA binding of these p63 isoforms and therefore increase cellular apoptotic response to UVB. Alternatively, the presence of TAp73α could restore the p53/p73 mediated G1/S arrest response to balance or repress the p63-mediated apoptotic response. The kinetics and mechanisms of these interactions between p63 and p73 isoforms for mediating cellular responses warrant further study.

6.1.2. What are the mechanisms by which TAp73α aids p63 isoform DNA binding capabilities?

The TAp73α isoform could aid lower molecular weight p63 isoform DNA binding capabilities through multiple mechanisms such as pre-loading on the DNA and subsequent recruitment of p63 to the DNA, through protein-protein interactions to keep

inhibitory ΔNp63 or ΔNp73 isoforms from competitively binding DNA, or through interactions with enhancers such as Aspp2 or p300. The DAI assay is informative for determining DNA binding capabilities of the p53 family members and their co-regulators to a specific DNA probe. However, the kinetics and mechanisms must also be addressed by immunoprecipitation with isoform-specific antibodies and by ChIP to show the order in which the p73 and p63 proteins interact with downstream promoters in the context of chromatin following specific types of DNA damage. The data generated using the DAI assay indicate that steady state levels of p63 DNA binding to the p21 probe were enhanced by the presence of TAp73α. The enhanced lower molecular weight p63 isoform DNA binding capabilities corresponded with increased basal levels of downstream target mRNA. The ChIP assay with isoform-specific antibodies could be used to show whether TAp73α must be present at the DNA to recruit TAp63 isoforms or whether TAp73α must exit the DNA, perhaps removing inhibitory ΔNp63α as it goes, to allow loading of the TAp63 isoforms. Further analysis of the DNA binding capabilities of p53 family members at other downstream target promoters will also elucidate a more complete picture of how p63 and p73 cooperate for tumor suppression. Once the basal activities of p73 and p63 isoforms cooperating at DNA have been addressed, the even more challenging questions of isoform-specific mediation of response to DNA damage can be explored.

6.1.3. Can TAp73:TAp63 cooperative interactions be utilized to promote cellular response to DNA damaging agents?

Knowledge of the differential responses of keratinocytes in the mouse model of carcinogenesis to DNA damage by IR versus UVB could provide an advantage in

experimental design to address kinetics and mechanisms of TAp73α aiding TAp63 DNA binding following DNA damage. TAp63γ appeared to mediate an apoptotic response to UVB in malignant keratinocytes even in the absence of p73 isoforms. Keratinocytes lacking p73 but expressing TAp63 isoforms could therefore provide a minimal experimental system to address the effects of adding back TAp73α to promote cell cycle arrest versus apoptotic response to DNA damage with IR versus UVB. We hypothesize that an untested p73 isoform, such as TAp73β, a specific balance of p63 and p73 isoforms, or interaction with additional cofactors must be crucial for cellular response to IR as silencing p73 abrogated cellular response to IR but adding back TAp73α or ΔNp73α isoforms did not restore IR sensitivity. To address this hypothesis, TAp73β and/or ratios of TAp73:TAp63 isoforms can be transfected into cells to determine which ratios are required for optimal removal of ΔNp63 from DNA and subsequent DNA binding of transcriptionally active isoforms for transactivation of target genes. It will be crucial to understand the kinetics and mechanisms behind which p63 and p73 isoforms interact in order to take full advantage of these cooperative interactions for development of therapeutic targets and for optimization of cellular responses to current anti-cancer agents. If isoform-specific ratios are not sufficient for reactivation of cellular response to DNA damage, this could indicate that prerequisite p53 family interactions with enhancers such as Aspp2, Abl, or p300 were interrupted together with loss of p73 expression at malignant conversion or, alternatively, that loss of p73 allowed permanent inactivation of the other p53 family members by dominant inhibitors.

6.1.4. Can TAp73α act as a molecular shield against p53 family inhibitors to allow DNA binding of transcriptionally active p53 family members?

The p73 protein interacts with multiple inhibitors of p53 family members such as Mdm2, MdmX, mutant p53, ΔNp63, ΔNp73, cMyc, and iASPP [Bergamaschi, Samuels et al., 2003; Gaiddon et al., 2001; Grob et al., 2001; Michael and Oren, 2002; Rocco, Leong et al., 2006; Wang, Arooz et al., 2001; Watanabe et al., 2002]. Our hypothesis, as depicted in Figure 28C, is that non-DNA bound p73 can shield transcriptionally active p53 family members by sequestering inhibitors, enhancing the capabilities of p53 family members to transactivate downstream targets. The DAI assay is useful for studies to address this hypothesis because proteins can be separated into DNA-bound and non-DNA bound fractions. Experimentally, cell lysate can be incubated with the biotin-labeled DNA probe of interest, the non-DNA bound fraction of proteins removed prior to the washing of the streptavidin beads, and then immunoprecipitation assays performed on the non-DNA bound fraction using, for example, p73 or Mdm2 antibodies or antibodies for other p53 family inhibitors. In this way, the fraction of p53 family members and their inhibitors that is found in the DNA-bound complex can be analyzed side by side with the immunoprecipitated non-DNA complexes by immunoblotting.

Another way to test the validity of the hypothesis that TAp73α may act as a molecular shield against Mdm2 and an enhancer of transcriptional activity of the more transcriptionally active p63 isoforms would be to perform mutation analysis studies of TAp73α to mutate the Mdm2 binding domain, the oligomerization domain (responsible for oligomerization with p63), or both domains simultaneously followed by testing the DNA binding capabilities of the p53 family members and the presence of Mdm2 in the DNA-bound complex. The mutated TAp73α constructs could be transfected into the 03C si-p73 cells and DNA bound vs. unbound complexes analyzed by DAI and IP to

determine whether p73 interactions with Mdm2 are required for the restored DNA binding capabilities of the lower molecular weight p63 isoforms, whether oligomerization of p63 and p73 are required for enhanced p63 isoform DNA binding capability, or whether the enhanced DNA binding capabilities of the lower molecular weight p63 isoforms are a result of p73 interaction with p53 family inhibitors to allow access of transcriptionally active p53 family members to the DNA target sequences.

Finally, immunoflourescence to show colocalization of proteins and qPCR to coordinate colocalization with downstream biological function could elucidate the function of p73 as a molecular shield, a finding that may lead to novel therapeutics for activating transcriptionally active p53 family members.

6.1.5. What are some strategies for treatment of SCC and other epidermal tumors by reactivating transcriptionally active p53 family members?

Restoration of p53 function lead to tumor regression *in vivo* [Kastan, 2007; Ventura et al., 2007] and thus new strategies are actively being sought to utilize the tumor suppressive function of p53 as a molecular target for cancer therapeutics [Levesque and Eastman, 2007]. Adenovirus-mediated gene therapy to target active p53 to tumors has been marginally effective, but nonetheless phase one clinical trials are underway [Fujiwara et al., 2006]. However, p53 is mutated in 50% of cancers, functionally inactivated in up to 90%, and therefore difficult to reactivate as a therapeutic target [Hofseth et al., 2004]. Some strategies for reactivation of p53 are to stabilize mutant p53 in a wild type conformation or to chaperone p53 DNA binding using small molecules such as Prima-1, Mira-1, or CP-31298 [Levesque and Eastman, 2007]. Where p53 is not mutated, but inactivated by interaction with the inhibitor Mdm2, small molecules such as

the Nutlins or RITA are being developed for therapeutic use [Issaeva et al., 2004; Vassilev, 2005].

The p73 and p63 family members of p53 are rarely mutated in human cancer and therefore may prove to be more effective targets for anticancer therapy than p53. However, greater understanding of p73 and p63 isoform function, tissue specificity, and cooperative or competitive interaction is needed before these family members can be used as therapeutic targets or the delicate balance between isoforms may be disrupted with detrimental effects. Further, certain specific mutant p53 molecules, altering the conformation of the p53 DNA binding domain, have been shown to bind to and inactivate p73 and p63 [Gaiddon et al., 2001; Strano et al., 2002; Strano et al., 2000]. Thus reactivation of p53 family members for therapeutic effect may not be effective in the context of certain p53 mutations. Current strategies for activation of p73 have included adenovirus-mediated transfer of p73 to pancreatic, colorectal, breast, lung, liver, and ovarian cancer cells which attenuated cell growth even when the cells were resistant to wild type p53 gene therapy [Das et al., 2005; Rodicker and Putzer, 2003; Sasaki et al., 2001]. Recently, reactivation of p73 by a p53-derived apoptotic peptide (including 37 evolutionarily conserved amino acids taken from the DNA binding domain of the p53 protein) led to tumor regression *in vivo* independent of whether p53 was wild type, mutant, or functionally inactivated [Bell et al., 2007]. This was reportedly due to the p53-derived apoptotic peptide disrupting p73 interaction with the p53 family inhibitor iASPP to allow reactivation of p73 regardless of p53 status. If our model is correct that TAp73α acts as a molecular shield to inhibit the inhibitors of the p53 family members and allow enhanced DNA binding and activity of transcriptionally active isoforms, then specifically

increasing TAp73α expression in cancer cells should have therapeutic effect. Indeed, in the clonal model of epidermal carcinogenesis, increasing TAp73α expression in malignant cells led to tumor suppression *in vivo*. However, activation of combinations of p53 family isoforms may be required to recapitulate full function in concert with DNA damaging agents, as simply restoring one isoform of p73 to malignant cells was not sufficient to restore sensitivity to IR. Since p63 was capable of mediating an apoptotic response to UVB in malignant cells in the absence of p73, it would be interesting to determine if UVB or oxidative stress-inducing UVB mimetics such as 4-nitroquinoline-1-oxide (4-NQO) could be utilized to force tumorigenic cells lacking p73 expression to undergo p63-mediated apoptosis. Utilization of TAp73α activity and TAp73/TAp63 cooperative interactions for targeted therapy will require further studies of the p53 family inhibitors that TAp73α interacts with such as iASPP, Mdm2, mutant p53, and the ΔN p53 family isoforms, as well as a more complete understanding of the mechanisms through which TAp73α aids DNA binding and activity of transcriptionally active p53 family isoforms.

7. Summary:

Overall, our studies show that, whether sporadic or siRNA induced, loss of p73 in initiated $p53^{+/+}$ keratinocytes was associated with loss of cellular responsiveness to IR and conversion to poorly differentiated SCC. The direct manipulation of p73 by specific silencing in initiated cells and the ability to trigger malignant conversion provide evidence for a causative role of p73 in cancer etiology rather than a strictly secondary selective effect during tumorigenesis. Reconstitution of TAp73α, but not ΔNp73α, restored tumor suppression *in vivo*, focusing on TAp73α as a tumor suppressive isoform. These results imply a role for p73 in epithelial cell fate. Further, we provide evidence for cooperation between TAp73α and transcriptionally active p63 isoforms in DNA binding capability and mediation of downstream target gene expression. The loss of p73 expression associated with reduced steady state DNA binding capabilities of p63 isoforms and reduced transactivation of p53 family downstream target genes in response to IR compared to cells that expressed p73. Reconstitution with TAp73α, but not ΔNp73α, restored steady state levels of p63 isoforms binding to DNA and increased steady state levels of p53 target genes even though expression of TAp73α did not restore cellular sensitivity to IR. The p73 protein was not required for keratinocyte response to UVB, however, suggesting a hierarchy of p53 family response to DNA damaging agents and a distinct role of p63 for keratinocyte response to UVB not requiring cooperative interaction with p73. In the absence of p73, a balance was tipped toward preferential DNA binding of ΔNp63α, promoting cancer cell survival and loss of responsiveness to IR. Also in the absence of p73, the p53 family inhibitor Mdm2 was found in complex with the DNA-bound p53 family members. Addition of TAp73α to malignant cells

restored initiated cell morphological and phenotypic characteristics and normalized the p53 family DNA binding profile by removing Mdm2 from the DNA complex, restoring the DNA binding capabilities of the more transcriptionally active lower molecular weight p63 isoforms, and therefore rendering the cells refractory to the Mdm2-p53 inhibitor Nutlin-3 as seen in normal (non-malignant) keratinocytes. We thus propose that TAp73α may act as a molecular shield against inhibitors of p53 family members such as ΔNp63α and Mdm2, allowing the transcriptionally active p53 family members to function for tumor suppression and response to DNA damage. Further studies of the functional role of p73 in the context of other endogenous family member isoforms will not only increase our understanding of the role of the p53 family in the distinct stages of tumor development but should lead to better predictions of patient prognosis and more effective treatments for epithelial-derived tumors, particularly where deregulation or loss of p63 and p73 expression is associated with increased tumor invasiveness and poor patient prognosis

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Appendix

Appendix Figure 1: The 03C si-p73-1 cells are genetically similar to the malignant 03R cells and divergent from the parental 03C cells. Microarray analysis of global gene expression changes associated with p73 loss in malignant conversion. Total RNA from biological replicates of the 03C si-Con cells, 03C si-p73-1 cells, and 03R cells was isolated using TRIzol followed by purification with the RNeasy mini RNA extraction kit (Qiagen). For each of the replicates, RNA was isolated independently from 2 plates, and 10 μg (derived from pooling equal aliquots of the isolated RNA) was submitted to the OHSU Cancer Institute Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resource for processing. Affymetrix mouse GeneChip® 430 2.0 (Affymetrix, Inc.) was used for hybridization, and data was analyzed using Microarray Suite (MAS) version 4.0 or 5.0 (Affymetrix) and Excel (Microsoft, Bellevue, WA). The graphs show pair-wise comparisons between each combination of samples. Blue dots represent specific gene expression changes, so the plots depict global gene expression changes comparing cell samples as listed (e.g. A sample compared to B sample = A:B). The red line is a value equal to "1" and thus blue dots above the red line are increased mRNA expression levels and blue dots below the red line are decreased mRNA expression levels when comparing the two samples. A striking similarity existed between the 03C si-p73-1 cells and the malignant 03R cells (global gene expression values clustered near the red line) while both the 03C si-p73-1 and 03R cells were divergent from their parental initiated 03C clone (global gene expression values diverged from the red line). The 03C si-p73-1 cells were also similar to the 03R cells in cell growth characteristics *in vitro*, in tumorigenesis *in vivo*, and in both resistance to treatment with IR and sensitivity to treatment with UVB *in vitro*. The 03C si-p73-2 cells were not subjected to the same microarray analysis comparisons, nor were the 03C si-p73 cells expressing Vector, ΔNp73α or TAp73α. Selective pressure during the cloning of the 03C si-p73-1 cells from the initiated 03C cells likely allowed the global genetic changes associated with loss of total p73 protein expression, while the 03R cells were never subjected to single cell cloning and thus exhibit some level of heterogeneity. The results obtained here suggest that a genetic "signature" was already established in the initiated 03C cells to predispose them to give rise to malignant cells with global gene expression changes producing poorly differentiated SCC, particularly upon loss of total p73 expression. It has previously been shown that the 05C (giving rise to the 05R producing moderately differentiated SCC) and the 09C (giving rise to the 09R producing benign papilloma) have different genetic "signatures" to give rise to cells with benign or malignant cell fates (Wang, Liu et al. 2002).

Wang, Z., Y. Liu, et al. (2002). "Gene expression profiling of initiated epidermal cells with benign or malignant tumor fates." Carcinogenesis **23**(4): 635-43.

Appendix Figure 1: Microarray analysis of global gene expression changes associated with p73 loss in malignant conversion