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Regulation of the Transcription and Subcellular Localization of the Tumor Suppressor PTEN by ΔNp63α

Mary Kathryn Leonard
Wright State University

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Regulation of the Transcription and Subcellular Localization of the Tumor Suppressor PTEN by ΔNp63α.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By

Mary Kathryn Leonard
B.S, Linfield College 2007

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Wright State University
WRIGHT STATE UNIVERSITY
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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Mary Kathryn Leonard ENTITLED Regulation of the Transcription and Subcellular Localization of the Tumor Suppressor PTEN by ΔNp63α BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

______________________________
Madhavi Kadakia, Ph.D.
Dissertation Director

______________________________
Gerald M. Alter, Ph.D.
Director, Biomedical Sciences Ph.D. Program

______________________________
Andrew Hsu, Ph.D.
Dean, School of Graduate Studies

Committee on Final Examination

______________________________
Madhavi Kadakia, Ph.D.

______________________________
Steven J. Berberich, Ph.D.

______________________________
Thomas L. Brown, Ph.D.

______________________________
Paula A. Bubulya, Ph.D.

______________________________
Michael Leffak, Ph.D.
Abstract

Leonard, Mary Kathryn. Biomedical Sciences Ph.D. Program, Wright State University 2012. Regulation of the Transcription and Subcellular Localization of the Tumor Suppressor PTEN by ΔNp63α.

Non-melanoma skin cancers (NMSCs) are the most common form of cancer in the United States with an estimated 3.5 million new cases each year. Surgical excision is the main treatment for NMSC, but leaves the potential for disfiguring scars and does not fully reduce the risk of recurrence since the surrounding tissue is also sun damaged and may contain tumor-promoting mutations. By understanding the molecular etiology of NMSC we may be able to expand treatment options to more than just resection. Normal epidermal development is dependent upon the expression of the transcription factor p63. Amplification of the ΔNp63α isoform is also frequently observed in NMSC, making it an excellent candidate for understanding the etiology of NMSC.

This dissertation focuses on how ΔNp63α influences keratinocyte proliferation by regulating phosphatase and tensin homologue deleted on chromosome ten (PTEN). The tumor suppressor PTEN has been called “the second guardian of the genome” for its widespread role in preventing tumor formation and is second only to p53 in the frequency of observed mutations or loss in human cancers. The studies presented in this dissertation demonstrate that ΔNp63α is able to maintain the proliferative potential of keratinocytes by activating Akt through transcriptional repression of PTEN. The inhibition of PTEN by ΔNp63α was independent of any repressive effects of ΔNp63α towards other p53 family
members. Since the discovery of nuclear PTEN the tumor suppressive functions of PTEN have expanded to include induction of cell cycle arrest, accentuation of apoptotic signaling and maintenance of chromosome stability. ΔNp63α was also shown to inhibit the nuclear localization of PTEN, which may further the increase the proliferative potential of basal layer keratinocytes. Inhibition of NEDD4-1 by ΔNp63α was implicated in reducing the ubiquitination of PTEN, thereby preventing its import into the nucleus. The studies presented here also identify a novel pool of PTEN that is localized to centrosomes only during mitosis. Coordinated control of both Akt and PTEN regulate centrosome composition and integrity during mitosis and provides insight into how PTEN functions as a multifaceted tumor suppressor. The importance of the ΔNp63α/PTEN/Akt signaling loop in epidermal biology was highlighted by the significant disruption of ΔNp63α and PTEN levels in NMSC. Altogether, these studies provide important molecular insight into the control of keratinocyte proliferation by the ΔNp63α-PTEN-Akt signaling loop.
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I. Introduction

A. Non-melanoma skin cancers.

Non-melanoma skin cancers (NMSCs) are the most common form of cancer in the United States with an estimated 3.5 million new cases each year (Rogers et al., 2010). Exposure to ultraviolet B (UVB) light is the leading cause of NMSC and changes in social behavior including increased usage of tanning beds have contributed to the rise in the NMSC (Markovic et al., 2007). Basal cell carcinomas (BCC) represent ~60% of all NMSCs, while squamous cell carcinomas (SCC) represent ~40% (Society, 2010). The treatment cost of NMSC to Medicare alone is $285 million per year, making it the fifth most expensive cancer to treat with few treatment options other than surgical resection (Housman et al., 2003; Joseph et al., 2001). Both BCC and SCC arise from keratinocytes of the epidermis, but have distinct phenotypes and aggressive behaviors.

Basal cell carcinomas are thought to arise from the basal layer of the epidermis and are rarely metastatic, usually only showing local invasion. The keratinocyte subpopulations of origin for squamous cell carcinoma are hotly debated, but have phenotypes akin to the spinous layer of the epidermis (Figure 1). Although the propensity for metastasis is generally low (< 5%) for all NMSC, SCC can become metastatic, often growing along nerve sheaths within the dermis (termed perineural invasion) (Czarnecki et al., 1994). Removal of affected tissue is the primary and most effective treatment for both BCC and SCC, although the rare cases of metastatic NMSC require radiation or platinum based therapies in addition to surgery. Mohs Micrograph Surgery (MMS) is the most
effective treatment option, especially for high risk NMSC, with 5 year cure rates of ~98% (Rowe et al., 1989a; Rowe et al., 1989b). Due to the time consuming nature and advanced training required to perform MMS, simple excision is the main treatment of NMSC. Standard curettage or resection surgeries can also leave potentially disfiguring scars, but do not fully reduce the risk of recurrence since the surrounding tissue is also sun damaged and may contain tumor-promoting mutations. In fact, patients with BCC and SCC are at a 19% and 30% respective risk of developing a secondary primary cancer and are 2.4- to 3-fold more likely to later develop melanoma (Krueger and Williams, 2010).

The increasing frequency of skin cancers combined with the lack of treatment options exemplifies the need for increased research into the etiology of skin cancer. By understanding the development of NMSC we may be able to expand treatment options to more than just resection. To understand the etiology of NMSC we must first identify molecular pathways involved in keratinocyte proliferation, as these are cells of origin for both BCC and SCC. Commitment to the keratinocyte lineage is dependent upon the expression of the transcription factor p63 (Mills et al., 1999; Yang et al., 1998; Yang et al., 1999). The frequent overexpression of p63 in NMSC and the requirement of p63 for the development of the epidermis make it an excellent candidate for elucidating the molecular underpinnings of NMSC (Bircan et al., 2006; Choi et al., 2002; Di Como et al., 2002; Mills et al., 1999; Sakiz et al., 2009; Yang et al., 1998; Yang et al., 1999). If we know how p63 influences keratinocyte development and proliferation we could potentially create preventative therapies and improve current NMSCs treatment strategies. This dissertation seeks to determine how p63 influences keratinocyte proliferation in the context of NMSC development.
Figure 1: Structure of human skin. (A) Hematoxylin and eosin (H&E) staining of normal adult skin. The locations of the sub-layers of two main components of skin, the epidermis and dermis, are designated by braces. (B) H&E staining of the two most common types of non-melanoma cancers that arise from the epidermis: basal cell (left) and squamous cell (right) carcinomas.
**Figure 2: Structure of p63.** A) Schematic of the p63 gene showing alternative promoters at the 5’-terminus and splice variations (α, β, γ, δ, and δ) of the 3’-terminus. Exons comprising functional domains are color coded as follows: dark blue, full length transactivation (TA) domain; purple, ΔN-specific TA domain; green, DNA binding domain; red, oligomerization domain (OD); light blue, sterile alpha motif (SAM); and yellow, transcription inhibitory domain (TID). (B) Diagram of the p63 protein isoforms and functional domains generated from Trp63 gene.
B. Structure of p63.

Initially discovered in 1999 because of high sequence homology to the tumor suppressor p53, the transcription factor p63 was quickly identified as a major contributor to epithelial morphogenesis rather than a canonical tumor suppressor like p53 (Mills et al., 1999; Yang et al., 1998; Yang et al., 1999). Analysis of the *Trp63* gene found that there are at least nine possible p63 isoforms, depicted in Figure 2, due to alternative promoter usage and 3’ terminal splicing (Mangiulli et al., 2009; Yang et al., 1998). Isoforms using the P1 promoter, referred to as TAp63 isoforms, contain the full length amino-terminal transactivation (TA) domain while use of the internal P2 promoter yields isoforms with a unique, truncated transactivation domain known as ΔNp63. A third form of p63, unique to spermatozoa of great apes, is generated from a cryptic promoter upstream of P1 to yield the so-called germ-cell TAp63 (GTAp63) (Beyer et al., 2011). Expression of the full carboxy-terminus of p63 generates p63α isoforms (TAp63α or ΔNp63α) while alternative splicing yields the sequentially shorter p63β or p63γ isoforms (TAp63β, TAp63γ and ΔNp63β and ΔNp63γ) (Yang et al., 1998). Recently, two additional isoforms of ΔNp63, ΔNp63δ and ΔNp63ε, have been identified (Mangiulli et al., 2009). Delta and epsilon isoforms of TAp63 have not yet been detected in vivo.

All p63 isoforms share the same DNA binding domain (DBD). Due to the high sequence homology (65%) to the DBD of p53, p63 proteins are capable of binding to the p53 responsive elements in the promoters of target genes (Yang et al., 1998). Like p53, p63 is only transcriptionally active when bound as tetramers that are linked through the oligomerization domain (OD) (Brandt et al., 2009; Joerger et al., 2009; Natan and Joerger). Because the OD is shared by all p63 isoforms, p63 can form homo-tetramers of
a single isoform or hetero-tetramers of TAp63 and ΔNp63 (Joerger et al., 2009). The transcription inhibitory domain (TID) of TAp63α physically interacts with the full-length transactivation domain to prevent TAp63α dimers from joining to form a transcriptionally active tetramer (Deutsch et al., 2011; Serber et al., 2002). While the ΔNp63α isoform also contains a TID, the truncated transactivation domain cannot interact with the TID thus allowing the ΔNp63α isoform free to constitutively form tetramers (Deutsch et al., 2011).

Both TAp63α and ΔNp63α also contain a sterile-alpha-motif (SAM) within the carboxy-terminus which is not found in any of the shorter isoforms (Thanos and Bowie, 1999). The SAM domain is thought to aid in protein-protein interactions with mutations in this domain being associated with Ankyloblepharon–Ectodermal dysplasia–Clefting syndrome (Rinne et al., 2009; Thanos and Bowie, 1999).

C. Role of p63 isoforms in skin development.

Proper development and maintenance of a stratified epidermis requires the correct expression of the transcription factor p63 (Mills et al., 1999; Yang et al., 1998; Yang et al., 1999). Unlike its sister protein p53, p63 has a well-defined role in development. Mice deficient of all p63 isoforms fail to form a stratified epidermis, causing severe dehydration and ultimately death, in addition to failed formation of limbs, craniofacial bones and mammary or prostate tissue (Mills et al., 1999; Yang et al., 1999). While ΔNp63α is the predominant isoform in adult epithelia, with the exclusion of germ cells, the contribution of the different isoforms during skin development is complex (Beyer et al., 2011; Di Como et al., 2002; Suh et al., 2006).

As early as day embryonic day 7.5 expression of the TAp63 isoforms can be detected in mouse embryonic ectoderm (Koster et al., 2004). However, by embryonic day
9.5 the ΔNp63α isoform becomes the most predominant isoform and continues to drive the expansion of the epidermis into a fully differentiated and stratified tissue (Koster et al., 2004; Yang et al., 1998). Despite the differences in the onset of expression, the exact roles of the TAp63 and ΔNp63 isoforms during development are somewhat controversial. Using thymic epithelia from wildtype and p63-/- mice as a model system, Senoo et al. found that ΔNp63α is critical for proliferation of epithelial cells, but not for lineage commitment or differentiation (Senoo et al., 2007). Koster et al. found that the TAp63 isoforms were critical for epithelial commitment and the ΔNp63 isoforms were required for proliferation and terminal differentiation of the epidermis (Koster et al., 2004; Koster et al., 2009; Senoo et al., 2007). Complicating the story still, the Aberdam group found that ΔNp63, not TAp63, was essential for epidermal commitment using an in vitro embryonic stem cell system (Medawar et al., 2008). According to this same study, however, ΔNp63 may not be necessary for commitment of the ectoderm to other epithelial lineages (Medawar et al., 2008).

The isoform controversy is somewhat resolved by the re-evaluation of the initial strains of p63-knockout mice as well as the development of p63 isoform specific knockout mice. Soon after the discovery of the p63 gene, two groups simultaneously generated p63 knockout mice using different stem cell technologies (Mills et al., 1999; Yang et al., 1999). The McKeon laboratory generated a true p63 knockout mouse by replacing exons 6-8, which encompass the shared DNA binding domain, with a neomycin cassette thus ablating all isoforms of p63 protein (Yang et al., 1999). The “knockout” strain developed by Mills et al, dubbed Bdrm2, employed a gap-repair mechanism that truncated Trp63 after exon 10 through the insertion of a vector homologous to exons 5-10.
(Mills et al., 1999). This Bdrm2 mutant strain resulted in deletion of the alpha, beta and gamma isoforms of p63, which were the only isoforms known at the time. While both the McKeon laboratory’s and the Bdrm2 p63 homozygous knockout mice are nearly phenotypically identical in that the mice lack appendages, secondary sex organs and have severely retarded craniofacial development, it was later found that Bdrm2-p63 mouse still produced epidermal “micro-rafts” as well as positively staining for p63 in the simple epithelia of the internal mucosal tissues. The Bdrm2-mutation failed to disrupt the expression of ΔNp63δ and ΔNp63ε, thus allowing for a minute amount of epithelial development (Romano et al., 2009). These micro-rafts are sections of fully stratified epidermis, but are rare, small (negligible to the naked eye), and insufficient to prevent neo-natal death due to dehydration. Contrary to in vitro studies by Koster and Senoo, the re-evaluation by Wolf et al. of the Bdrm2-p63 strain of mice implicated that the ΔNp63 isoforms may be sufficient to drive epidermal commitment, proliferation and differentiation, but that the ΔNp63δ and ΔNp63ε isoforms are relatively weak regulators of these processes (Koster et al., 2004; Senoo et al., 2007; Wolff et al., 2009). It would be another decade before this hypothesis was tested through the generation of isoform specific p63 knockout mice.

Again, two groups simultaneous developed TAp63 specific knockout mice using two different embryonic stem cell ablation technologies. While there exist differences in the wound healing ability of the two TAp63-/- mice strains, namely that the strain developed by Dr. Elsa Flores developed cutaneous ulcers while the strain from the McKeon group showed no such skin fragility, both TAp63-/- mice strains were viable with fully developed skin, secondary sex organs and appendages (Su et al., 2009; Suh et
al., 2006). Since the TAp63-/- mice did not recapitulate any of the phenotypes of the global p63-/- mice, it would suggest that it is truly the ΔNp63 isoforms that are necessary for epithelial proliferation and epidermal commitment and differentiation.

The importance of the ΔNp63 isoforms in the epithelial development is further highlighted by the recent production of ΔNp63-/- mice through replacement of the ΔNp63 specific 3’ exon with a GFP cassette (Romano et al., 2012). Disruption of the ΔNp63 isoforms did not affect the expression or function of TAp63, but severely disrupted epithelial development. The ΔNp63-/- mice, similar to the global p63-/- strains, failed to fully develop limbs or secondary sex organs and had major craniofacial abnormalities. Unlike the p63-/- mice, the ΔNp63-/- produced a single layer, primordial epidermis over much of the animal. The primordial epidermis, however, was non-functional due to disorganized differentiation, deficient proliferation, and lack of any barrier function. While neither TAp63-/- or ΔNp63-/- mice fully recapitulated the phenotype of the global p63-/- mice strains, the ΔNp63-/- strain had very similar defects in epithelial morphogenesis suggesting that the ΔNp63 isoforms are the master regulators of epithelial commitment and epidermal development with the TAp63 isoforms performing a supportive role in maintaining epidermal homeostasis during wounding.

While there remains much to be teased apart about the roles of TAp63 versus ΔNp63 isoforms during epidermal development, it is well accepted that ΔNp63α is the predominant isoform in most adult epithelial tissues. In fact, TAp63 is only readily detected in germ cells (TAp63α in ovaries or GTAp63 in spermatozoa) where it serves to induce apoptosis and preserve the fidelity of the germ line upon genotoxic insult (Beyer et al., 2011; Suh et al., 2006). ΔNp63α, on the other hand, is detected in the basal layer of
most epithelia with the highest expression in the basal layer of the epidermis and outer root sheath of hair follicles (Figure 3) (Di Como et al., 2002). Since NMSC primarily affect individuals over the age of 50 and ΔNp63α is the major physiologic isoform in adult tissue, emphasis will be put on examining the molecular mechanisms of ΔNp63α in keratinocyte and NMSC biology.
Figure 3: ΔNp63α is restricted to the basal layer of healthy skin. The localization of p63 in mouse skin was determined by co-localization with epidermal markers of differentiation. The left panel shows co-localization with the basal layer marker keratin 5 (K5, green). No co-localization of p63 with the spinous layer keratin 1 (K1, middle panel), nor the granulosum specific marker involucrin (Inv, right panel) was observed. Formalin fixed paraffin embedded skins sections and all antibodies used to differentially stain the epidermis, including the ΔNp63 isoform specific antibody RR14, were donated by Dr. Satrijit Sinha, SUNY Buffalo.
D. Transcriptional activity of ΔNp63α.

ΔNp63α levels are highest in the basal layer of the epidermis where it serves to maintain the proliferative nature of the basal layer stem cells by repressing the expression of many pro-apoptotic genes while enhancing expression of pro-survival genes (Barbieri et al., 2005; Senoo et al., 2002; Westfall et al., 2003; Yang et al., 1998). ΔNp63α controls gene expression by binding to either p53 or p63-specific responsive elements in the promoter of target genes (Osada et al., 2005). The exact p63-specific consensus sequence is more ambiguous than the p53 response element. Luciferase reporter studies with TAp63γ found that a guanine in the fifth position of the core, along with a several mismatches in the flanking purine (RRR) and pyrimidine (YYY) sequences of the canonical RRRCWWGY p53 response element, distinguishes the p63 responsive element from that of p53 (Osada et al., 2005). Studies by Ortt and Sinha, however, determined the p63 consensus sequence to be CA(T)TG core and AT-rich 5′ and 3′ flanking sequences when recombinant ΔNp63α was used in electromobility shift assays (Ortt and Sinha, 2006). Crystallographic studies between DNA and the p63 DBD support the conclusion by Ortt that the preferred p63 consensus sequence is a core CA(T)TG flanked by AT-rich regions (Chen et al., 2011). Despite their differences, the studies by Osada et al. and Ortt and Sinha both demonstrated a large heterogeneity in sequences to which p63 proteins can bind; imbuing p63 proteins with the ability to bind an extensive cohort of DNA elements (Ortt and Sinha, 2006; Osada et al., 2005).

The ability of ΔNp63α to bind to p53 and p63 specific responsive elements allows ΔNp63α and p53 to differentially affect the expression of the same genes. For example, p53 induces the expression of p21 to induce cell cycle arrest, while ΔNp63α suppresses
the expression of p21 to maintain proliferation (el-Deiry et al., 1993; Westfall et al., 2003). Additionally, analysis of the p21 promoter demonstrated that when co-expressed, ΔNp63α can act in a dominant negative manner to prevent the pro-apoptotic transcriptional activities of p53 and the TAp63 isoforms by competing for the same binding sites (Yang et al., 1998). ΔNp63α can also form heterotetramers with the TAp63 isoforms to inhibit the growth suppressive activities of TAp63 (Joerger et al., 2009).

E. ΔNp63α in cancer.

Amplified levels of ΔNp63α have been observed in multiple types of squamous cell and basal cell carcinomas (Bircan et al., 2006; Choi et al., 2002; Di Como et al., 2002; Hu et al., 2002; Park et al., 2000; Parsa et al., 1999; Sakiz et al., 2009). Overexpression of ΔNp63α in SCC most commonly occurs through genomic amplification (Hibi et al., 2000). More invasive, poorly differentiated SCC show decreased expression of p63 as compared to well-differentiated, less invasive cancers (Fukushima et al., 2009; Koga et al., 2003; Morita et al., 2005; Sakiz et al., 2009; Urist et al., 2002). It is currently unclear to what extent, ΔNp63α plays during early tumor development, but its function may be unnecessary, or even detrimental, to later stages of tumor progression and invasion as evidenced from its absence in more invasive cancers. Further solidifying the anti-invasive role of ΔNp63α, it is known that p63 represses the expression of many genes involved with invasion while inducing the expression of genes involved with repression of invasion, such as the vitamin D receptor (Barbieri et al., 2006; Kommagani et al., 2009). Furthermore, silencing of p63 increases the transforming potential of p53 null mouse embryonic fibroblasts (Lang et al., 2004).
Consistent with the idea of ΔNp63α being a weak oncogene, several transgenic mouse models demonstrated that overexpression of ΔNp63α alone resulted in hyperplasia and stem cell loss but were insufficient to generate tumors (Romano et al., 2009; Romano et al., 2010). However, Keyes et al. found that allograft studies utilizing primary keratinocytes overexpressing ΔNp63α and oncogenic Ras could form locally invasive SCC, implying that ΔNp63α can be oncogenic (Keyes et al.).

The frequency of ΔNp63α amplification combined with the inability of ΔNp63α alone to initiate tumor formation would suggest the reliance on additional oncogenic pathways in the development of NMSC. The Akt pathway is one of the most frequently activated proliferation pathways in human cancers. Hyperactivation of Akt is seen in both BCC (Paolini et al., 2011) and SCC (Hafner et al., 2010). Interestingly, activation of the Akt pathway in SCC was not found to be associated with mutations in Akt itself or upstream kinases, suggesting alternative means of Akt activation in NMSC (Hafner et al., 2010).

F. ΔNp63α and the PTEN/ Akt pathway.

In addition to repressing the expression of many pro-apoptotic genes while enhancing expression of pro-survival genes, ΔNp63α is also able to stimulate the activation of Akt, a powerful regulator of cell growth and survival (Barbieri et al., 2005; Ogawa et al., 2007; Senoo et al., 2002; Westfall et al., 2003; Yang et al., 1998). By phosphorylating a myriad of target proteins, the kinase Akt facilitates survival, enhances energy metabolism and prevents apoptosis (Figure 4). For example, phosphorylation of
**Figure 4: Schematic of the canonical Akt pathway.** PI3K phosphorylates PIP$_2$ to generate PIP$_3$ at the plasma membrane. Akt binds PIP$_3$, allowing for phosphorylation and activation of Akt. Once active, Akt can phosphorylate XIAP to inhibit apoptosis and inactivate p21 and GSK3β by phosphorylation to increase cell cycle progression and energy consumption, respectively. Activating phosphorylations are shown in gold, while inhibitory phosphorylations are depicted in red. PTEN inhibits the activation of Akt by dephosphorylating PIP$_3$ and preventing its recruitment and activation to the plasma membrane.
BAD by Akt prevents apoptosis while phosphorylation of the E3 ubiquitin ligase X linked inhibitor of apoptosis (XIAP) enhances the anti-apoptotic effects of XIAP (Dan et al., 2004; Datta et al., 1997). Inhibitory phosphorylation of glycogen synthase 3 beta (GSK3β) by Akt serves to increase energy consumption and attenuate the inhibitory effects of GSK3β on the oncogene β-catenin (Cross et al., 1995; Liu et al., 2002). Akt can also enhance cell cycle progression by enhancing cyclin D1 levels through inhibiting p21 and p27 (Shin et al., 2002; Zhou et al., 2001).

Activation of Akt in turn also enhances ΔNp63α levels, suggesting that Akt and ΔNp63α work in a positive feedback loop to jointly promote proliferation and survival (Segrelles et al., 2006). It remains unclear, however, as to how ΔNp63α is able to trigger the activation of Akt or how Akt stabilizes ΔNp63α.

Canonical activation of Akt occurs through recruitment of Akt to the plasma membrane whereby it becomes phosphorylated at serines 308 and 473 by PDK1 and mTORC2, respectively (Sarbassov et al., 2005; Stokoe et al., 1997). Akt translocates to the plasma membrane by binding to phosphatidylinositol 3,4,5-trisphosphate (PIP3) via its pleckstrin homology domain (Burgering and Coffer, 1995; Franke et al., 1995). Phosphatidylinositol 3-kinases (PI3K) produce PIP3 by phosphorylating phosphatidylinositol 3,4-bisphosphate (PIP2) (reviewed in (Engelman et al., 2006). Gradients of PIP3, and thus Akt activation, are normally kept in check by the dual protein-lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten). PTEN removes the 3’ position phosphate from PIP3, essentially quenching the signal required for the activation of Akt (Maehama and Dixon, 1998).
Germline mutations of PTEN lead to a spectrum of hyperproliferative disorders, often characterized by hamartomas and cancer development that exemplify the need for functional PTEN in growth regulation (Blumenthal and Dennis, 2008). At the transcriptional level PTEN is regulated by the p63 family members, p53 and p73. Both p53 and TAp73 induce PTEN to reduce proliferation and prevent cancers (Stambolic et al., 2001; Vella et al., 2009). ΔNp73, similar to the ΔNp63 isoforms in their ability to repress the TA isoforms, can repress transcription of PTEN to enhance proliferation in thyroid cancer cells (Vella et al., 2009). A canonical p53 responsive element in the pten promoter allows p53 to bind to and enhance transcription of PTEN (Stambolic et al., 2001). Interestingly, p73 controls PTEN transcription through a response element separate from that of p53 in the pten promoter (Vella et al., 2009). Because of the similarities in the DNA binding domain of the p53 family members, and the presence of both a p53 and a p73 response element in the pten promoter, ΔNp63α could bind the pten promoter to reduce PTEN expression and promote Akt activation and proliferation (Ogawa et al., 2007; Yang et al., 1998). Moreover, it is known that ΔNp63α is overexpressed while PTEN is down regulated in SCC (Odar et al., 2012).

G. Post-Translational Modification of PTEN.

In addition to being regulated at the transcriptional level, PTEN is subject to many post-translational modifications that control its stability, enzymatic activity and subcellular localization. Unmodified PTEN is a 403 amino acid protein comprised of a PIP\textsubscript{2} binding module at the amino-terminus followed by the phosphatase domain, a large C2 domain and a PDZ binding motif at the C-terminus as depicted in Figure 5.
Figure 5: Schematic of common post-translational modifications of PTEN. The functional domains of PTEN are color coded as follows: PIP$_2$ binding module (PBM) in blue, phosphatase domain in orange, C2 domain in green, PDZ binding motif in red. Sites of and types of common covalent modifications and the enzymes responsible are shown in gray.
(Georgescu et al., 1999). While the C2 domain is thought to confer membrane targeting to PTEN it has also been shown to be involved in protein-protein interactions along with the PDZ binding motif (Georgescu et al., 1999; Georgescu et al., 2000; Shen et al., 2007).

The phosphatase activity of PTEN can be diminished by multiple types of post-translational modifications. Enzymatic activity of PTEN can be directly squelched by oxidation of cysteine 124, the key to nucleophilic removal of phosphate groups, by a reactive oxygen species (ROS) (Cho et al., 2004; Ross et al., 2007). Phosphorylation of a cluster of serine and threonine residues between positions 366-385 are also known to regulate the stability and activity of PTEN. When phosphorylated, the C-terminal tail bends back to create a “closed” conformation that inhibits the degradation of PTEN (Al-Khouri et al., 2005; Georgescu et al., 1999; Maccario et al., 2007). This phosphorylation-induced close conformation, while stabilizing the protein, can also inhibit the phosphatase activity of PTEN by up to 30% (Torres and Pulido, 2001). A myriad of kinases have been shown to phosphorylate PTEN at different regions, all leading to increased stability at the expense of phosphatase activity. Phosphorylation by casein kinase 2 (CK2) at serines 370 and 388 primes PTEN for phosphorylation by GSK3β (Al-Khouri et al., 2005). Phosphorylation of the C2 domain by ROCK1 has been shown to be important in blocking chemotaxis by inhibiting the phosphatase activity of PTEN in macrophages exposed to chemoattractants (Vemula et al., 2010).

Phosphorylation can also increase protein stability by affecting the deposition of other covalent modifications. Phosphorylation by Rak at serine 336 has been shown to prevent PTEN degradation by blocking the interaction and subsequent ubiquitination by
the E3 ligase neuronal precursor cell-expressed developmentally downregulated 4 (NEDD4-1) (Wang et al., 2007; Yim et al., 2009).

Poly-ubiquitination of PTEN at lysine 13, and more predominantly, at lysine 289 can lead to proteasome mediated degradation of PTEN (Trotman et al., 2007). Several E3 ubiquitin ligases, in addition to NEDD4-1, have been shown to regulate the stability of PTEN. Most of the ligases responsible for PTEN ubiquitination are part of the NEDD4 family of Homologous to the E6-AP Carboxyl Terminus (HECT) E3 ubiquitin ligases. These include NEDD4-1, WW domain containing protein 2 (WWP2) and chaperone-associated E3 ligase (CHIP) (Ahmed et al., 2012; Maddika et al., 2011; Wang et al., 2007). The Really Interesting New Gene (RING) E3 ligase XIAP also down regulates PTEN stability via ubiquitination (Van Themsche et al., 2009).

Addition of a single ubiquitin moiety to PTEN at lysines 13 and 289, termed mono-ubiquitination, serves as a nuclear translocation signal for PTEN rather than a destruction signal like poly-ubiquitination (Ahmed et al., 2012; Trotman et al., 2007; Van Themsche et al., 2009). Of the E3 ligases known to ubiquitinate PTEN, only NEDD4-1, XIAP and CHIP have been demonstrated to influence the import of PTEN into the nucleus (Ahmed et al., 2012; Trotman et al., 2007; Van Themsche et al., 2009). Interestingly, NEDD4-1 has also been demonstrated to target ΔNp63α for destruction and could theoretically sequester NEDD4-1 away from PTEN (Bakkers et al., 2005). ΔNp63α also positively regulates heat shock protein 70 (HSP70) and Akt, which are important in the recruitment and activation of CHIP and XIAP, respectively (Ahmed et al., 2012; Dan et al., 2004; Wu et al., 2005). By influencing the recruitment and/or function of NEDD4-
XIAP or CHIP, ΔNp63α could indirectly control the ubiquitination of PTEN and thereby regulate the stability and subcellular localization of PTEN.

**H. Nuclear PTEN.**

Nuclear PTEN is predominantly found in arrested or differentiated cells while loss of nuclear PTEN is associated with melanoma progression (Gimm et al., 2000; Ginn-Pease and Eng, 2003; Jacob et al., 2009; Whiteman et al., 2002). Thus it is of potential therapeutic interest to understand how PTEN translocates into the nucleus and what the natural abundance of PTEN is in the nuclei of skin cells.

PTEN harbors a cytoplasmic localization sequence near the N-terminus that keeps the vast majority of the protein in the cytoplasm (Denning et al., 2007). However, multiple mechanisms, including mono-ubiquitination, have been demonstrated to regulate nuclear import of PTEN (Trotman et al., 2007). PTEN also contains four non-classical nuclear localization signals that can mediate its interaction with the major vault protein to facilitate nuclear import (Chung et al., 2005). Other studies suggest that PTEN may simply diffuse through the nuclear pore complex (Liu et al., 2005a). At only 47 kilodaltons, PTEN is a small enough to pass through nuclear pores which typically restrict proteins larger than 60 kilodaltons (Boulikas, 1993; Peters, 1986).

Retention of PTEN within nucleus is a balance between nuclear import and export. The subcellular distribution of PTEN has been demonstrated to be cell cycle dependent, which is consistent with the strongest observations of nuclear PTEN in G1 arrested or quiescent cells (Ginn-Pease and Eng, 2003; Jacob et al., 2009; Liu et al., 2007). PTEN is exported out of the nucleus upon activation of the PI3K/Akt pathway at the beginning of S-phase (Liu et al., 2007). Active Akt mediates the nuclear export of
PTEN by leading to the downstream activation of S6K1 and S6K2 that then physically bind and shuttle PTEN out of the nucleus (Liu et al., 2007). The nuclear export of PTEN by S6 kinases can be blocked by the activation of LKB1 and AMPK upon stress signaling (Liu et al., 2011a).

Once in the nucleus PTEN appears to have many functions independent of those associated with its cytoplasmic localization. Nuclear PTEN is important for inducing cell cycle arrest and maintaining chromosome stability and these processes may be, at least in part, independent of its phosphatase activity and thus of Akt inhibition (Chung and Eng, 2005; Gimm et al., 2000; Jacob et al., 2009; Liu et al., 2005b; Liu et al., 2011b; Shen et al., 2007). Direct physical interaction of PTEN, regardless of the enzymatic ability of PTEN, has been shown to enhance the growth regulatory processes of multiple proteins. PTEN can shield p53 and p73 from MDM2 mediated degradation thereby enhancing induction of apoptotic genes (Lehman et al., 2011; Mayo et al., 2002). Nuclear PTEN has also been implicated in maintaining chromosome stability through co-localization with p73 and Rad51 at sites of DNA damage and through aiding in proper kinetochore attachment via CENP-C (Gupta et al., 2009; Lehman et al., 2011; Shen et al., 2007).

I. Centrosome Associated Functions of PTEN.

Loss of PTEN is associated with increased growth rates, invasion, chromosomal instability and aneuploidy (Ehlers et al., 2008; Levine et al., 1998; Li et al., 2009; Puc et al., 2005). PTEN contributes to genomic integrity by enhancing kinetochore attachment and Rad51 activity, but may also do so by regulating the mitotic machinery (Gupta et al., 2009; Shen et al., 2007). PTEN null mouse embryonic fibroblasts (MEFs) escape taxol induced mitotic arrest faster than wildtype cells, suggesting that PTEN is involved in the
mitotic spindle checkpoint (Gupta et al., 2009). Constitutive pro-growth signaling, via Akt activation, was shown to display high levels of chromosome instability and centrosomal amplification that could be reversed by overexpression of PTEN (Li et al., 2009; Nam et al., 2010). Basal Akt activity, and therefore PTEN status, has also been shown to be important in normal mitotic progression as pharmacologic inhibition of Akt resulted in centrosome defects and bi-polar spindle formation (Liu et al., 2008).

Defective centrosome amplification (i.e. more than two centrosomes per cell) is frequently seen in both solid and hematological human cancers, while it rarely occurs in normal cells (Chng et al., 2006; Pihan et al., 2003). Previous studies indicate centrosome amplification may even be a causative agent in tumorigenesis (Chng et al., 2006; Pihan et al., 2003). Centrosomes are commonly called the microtubule-organizing complex and are comprised of two orthogonally arranged centrioles surrounded by a dense lattice of proteins constituting the peri-centriolar material (PCM). The recruitment of γ-tubulin to the heart of the proximal ends of centrioles is critical for centrosome maturation, formation of a bipolar spindle and successful progression through mitosis (Fuller et al., 1995; O'Toole et al., 2012).

Organization and maturation of centrosomes is a complex and dynamic process that involves numerous proteins, many of which are regulated in a cell cycle dependent manner. For instance, recruitment of γ-tubulin to mitotic centrosomes requires the phosphorylation of pericentrin (PCNT) by polo-like kinase 1 (PLK-1) (Haren et al., 2009; Lee and Rhee, 2011). Gamma-tubulin then feeds back into the development of mature centrosomes, in part, by enhancing the recruitment of other PCM proteins, including PLK-1 (Lee and Rhee, 2011). PTEN has previously been shown to influence PLK-1
degradation in a phosphatase independent manner by binding to the anaphase promoting complex (APC) and enhancing the targeting of PLK-1 for destruction (Song et al., 2011). Through the regulation of PLK-1, PTEN may be able to influence the recruitment of other centrosome associated proteins. In fact, aberrant PTEN expression is correlated with up-regulation of many centrosome associated proteins in endometrial cancers (Matsushima-Nishiu et al., 2001) as well as poor prognosis in breast cancers (Saal et al., 2007). Together, this suggests that a balance between PTEN and Akt plays a key role in centrosome function.

**J. Rationale and Hypothesis.**

PTEN is the second most commonly lost or mutated tumor suppressor in all of human cancers (Hollander et al., 2011). Loss of PTEN is associated with increased growth rates, invasion, chromosome instability and aneuploidy (Levine et al., 1998; Puc et al., 2005). Determining the mechanisms by which PTEN is regulated will contribute to understanding how PTEN can play an instrumental role in so many vital processes. PTEN is under the transcriptional control of p63 family members p53 and p73 (Stambolic et al., 2001; Vella et al., 2009). The ability of the p53 family to share responsive elements, allows for the possibility that ΔNp63α may also transcriptionally regulate PTEN (Osada et al., 2005). Furthermore, ΔNp63α and PTEN both regulate cell proliferation by mediating the activity of Akt; PTEN down regulates growth by inhibiting Akt (Maehama and Dixon, 1998; Stambolic et al., 1998) activation while ΔNp63α stimulates growth by enhancing Akt activation (Ogawa et al., 2007). ΔNp63α levels are also enhanced by activation of Akt, suggesting that ΔNp63α, PTEN, and Akt form a critical regulatory loop to control keratinocyte proliferation (Segrelles et al., 2006).
ΔNp63α may further control keratinocyte proliferation by influencing the ubiquitination and subcellular distribution of PTEN. By influencing the recruitment and/or function of NEDD4-1, XIAP or CHIP ΔNp63α could indirectly control the ubiquitination of PTEN and thereby regulate the stability and subcellular localization of PTEN. The ability of ΔNp63α to modulate the subcellular localization of PTEN may also affect mitotic progression. ΔNp63α and Akt are essential for proper progression through mitosis and this may be accomplished via regulation of PTEN (Hau et al., 2011; Liu et al., 2008).

The work detailed in this dissertation will test the hypothesis that ΔNp63α controls keratinocyte proliferation by inhibiting the expression, ubiquitination and subcellular translocation of PTEN. This study will determine if ΔNp63α is capable of controlling global PTEN expression, thereby forming a critical regulatory loop with Akt to maintain normal cellular proliferation. Secondly, it will also establish whether the ΔNp63α/PTEN axis is clinically relevant by studying the expression of ΔNp63α and PTEN in human NMSC. Finally, the work presented in this dissertation will also demonstrate an essential and novel role for PTEN and Akt in regulating the mitotic machinery. Together this dissertation will increase the understanding of how ΔNp63α contributes to increased cell proliferation and how loss of PTEN plays a multi-faceted role in tumor progression.
II. Methods

A. Cell Lines, Reagents and Plasmids.

H1299, PC3 and A431 were purchased from American Type Culture Collection (ATCC) while HaCaT cells were obtained from Dr. Dori Germolec (National Institute of Environmental Health Sciences). Dr. Elsa Flores (University of Texas M.D. Anderson Cancer Center, Houston, TX) generously gifted primary and E1A transformed wildtype and p63 null mouse embryonic fibroblasts (MEF). All cell lines except primary keratinocytes were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 8% fetal bovine serum and 100 U/mL of penicillin/streptomycin and maintained at 37° C in 5% CO₂. Adult primary human keratinocytes (HEK) were maintained in Epilife media supplemented with human keratinocyte growth serum and 100 U/mL of penicillin and streptomycin and 0.25 μg/mL amphotericin B (Cascade Biologics, Portland, OR). Neonatal human primary keratinocytes (NHEK) were maintained in KGM-Gold media supplemented with BPE, 2 mL bovine pituitary extract, 0.5 mL human epidermal growth factor, 0.5 mL insulin, 0.5 mL hydrocortisone, 0.5 mL transferrin, 0.25 mL epinephrine and 0.5 mL gentamycin according to manufacturer’s protocol (Lonza Walkersville, Walkersville, MD). The Akt specific inhibitor MK2206 was dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 10 μM for all experiments (Selleckchem, Houston, TX).

The expression plasmid encoding murine ΔNp63α in the pcDNA3.1A backbone was kindly provided by Dr. Frank McKeon (Harvard Medical School, Boston, MA).
Human hemagglutinin (HA) tagged ΔNp63α was donated by Dr. Subham Basu (Barts and The London School of Medicine and Dentistry, London, UK). Constitutively active myristol-Akt (CA-Akt) and kinase dead Akt were donated by Dr. Lindsey D. Mayo (Indiana University, Indianapolis, IN). Dr. Steven Berberich (Wright State University, Dayton, OH) kindly donated the 6x-His tagged ubiquitin (His-Ub) expression plasmid while the HA-ubiquitin and human wild-type NEDD4-1 expression plasmids were graciously provided by Dr. Miguel Martins (University of Leicester, Leicester, UK).

The full length PTEN-luc reporter used was described previously (Virolle et al., 2001). Four minimal PTEN luciferase reporters (designated A-C, E) were created by annealing primers to the regions of interest followed by ligation into the pGL3 Basic promoter-less luciferase reporter (Promega, Madison, WI) at the KPNI and HINDIII sites with T4 ligase (New England Bioscience, Ipswich, MA) for 2 hours at room temperature. The sequence of primers used can be found in Table 1 located in the methods appendix.

B. Transient Transfections.

For plasmid transfections cells were transfected at 80% confluence using Lipofectamine 2000 in antibiotic free DMEM (Life Technologies, Grand Island, NY). Cells were processed for downstream analysis 24 hours post transfection. Knockdown studies were conducted by two rounds of Oligofectamine or Lipofectamine RNAiMax based transfections carried out 24 hours apart (Life Technologies). All siRNAs and the Allstars Non-Silencing Control (NSC) were purchased from Qiagen (Valencia, CA). The target sequences for the siRNAs can be found in Table 2 of the methods appendix.

C. Real Time PCR.
Total RNA from cells was extracted using the E.Z.N.A RNA isolation kit (Omega Bio-Tek, Norcross, GA) as per manufacturer’s protocol. A total of 1 μg RNA was used to synthesize cDNA by using TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA). TaqMan based real-time PCR analysis was performed on ABI Prism7900HT sequence detection system using TaqMan Gene Expression Master mix and gene-specific assays on demand (AOD) for genes of interest and normalized to endogenous GAPDH for human genes or to β-actin for murine genes of interest (PE Applied Biosystems, Foster City, CA). Human AODs used were GAPDH (4325792), VDR (Hs_0017213_m1), p63 (Hs_00978340_m1), ΔNp63 (Hs_00978339_m1), TAp63 (Hs_00186613_m1), TAp73 (Hs_01056228_m1), ΔNp73 (Hs_1065727) IGFBP-3 (Hs_00426287_m1), PTEN (Hs_00829813_s1), and NEDD4-1 (Hs_0040645_m1). Murine AODs were p63 (Mm00495788_m1), PTEN (Mm00477210_m1) and β-actin (Mm00607939_s1). Fold change in genes of interest, relative to endogenous loading controls, was calculated using the comparative ΔΔCT method on SDS2.2.2 software (Pfaffl, 2001).

D. Immunoblot analysis.

Whole cell lysates were prepared by lysing the cells in phosphatase inhibitor containing buffer (50 mM Tris-HCl pH 8, 120 mM NaCl, 5 mM NaPPi, 10 mM NaF, 30 mM paranitrophenylphosphate, 1 mM benzamidine, 0.1% NP-40, 1% Triton X-100 and 0.2 mM PMSF, 100 nM sodium orthovanadate) supplemented with protease inhibitors. Subcellular fractionations were carried out using the NE-PER kit as per manufacturer’s instructions (Pierce, Rockford, IL). Total protein amounts were measured using BCA (Pierce, Rockford, IL) protein detection method and equivalent amounts of protein extracts were mixed with an appropriate volume of 10x loading dye (0.5 M dithiothreitol,
0.3 M Tris pH 6.8, 10% SDS, 50% glycerol and 0.05% bromophenol blue) and separated by 10% SDS-PAGE, or 7.5% SDS-PAGE for ubiquitinated proteins. Following separation by PAGE, proteins were transferred onto polyvinylidene difluoride membranes and blocked with 5% milk in Tris buffered saline with 0.5% Tween (TBST) or 1% BSA in TBST for detection of phosphorylated proteins. Membranes were subsequently immunoblotted for specific proteins as follows: anti-p63 (4A4), anti-p63α (H-129), anti-β-actin (AC-15), anti-α-tubulin (B-7), normal mouse IgG (sc-2025), normal rabbit IgG (sc-2027) (Santa Cruz Biotechnologies, Santa Cruz, CA); anti-pAkt (S473) #9271, anti-Akt #9272, anti-PTEN #9559, anti-NEDD4 #3607, anti-p-S6 #4838, anti-S6 #2217, anti-PARP #9542 (Cell Signaling, Boston, MA). Anti-Lamin A/C (BD Biosciences #612162) was donated by Dr. Steven Berberich (Wright State University) while the ΔNp63 specific RR14 antibody was gifted by Dr. Satrijit Sinha (SUNY-Buffalo). Antibodies specific to total p73 (IMG-259A) and ΔNp73 (IMG-313A) were purchased from Imgenex (Imgenex, San Diego, CA). Appropriate horseradish peroxidase-conjugated secondary antibodies (Promega) were used for chemiluminescence detection with Western Lightening Pro Chemiluminescent Substrate kit (Perkin-Elmer, Waltham, MA) and imaged with a Fujifilm Las3000 and Multi-Gauge Software (GE Healthcare-Fujifilm, Pittsburgh, PA).

E. Immunofluorescence analysis.

Cells were plated onto sterile, acid washed glass coverslips at least 24 hours prior to treatment. Cells were fixed with 2% paraformaldehyde for 15 minutes at room temperature. Coverslips were washed thrice to remove excess paraformaldehyde prior to permeabilization with 0.2% Triton X-100 diluted in PBS for 5 minutes. Cells were simultaneously washed and blocked by three 5 minute washes with 0.5% normal goat
serum in PBS (NGS-PBS). Proteins of interest were detected by incubating coverslips with the designated antibody diluted in 0.5% NGS-PBS for 1 hour at room temperature in a humid chamber. Excess primary antibody was removed by three consecutive washes with NGS-PBS followed by incubation with isotype appropriate AlexaFluor-488 or AlexaFluor-568 conjugated secondary antibodies for 1 hour. Coverslips were mounted onto glass slides and nuclei counterstained using VectaShield + DAPI (4',6-diamidino-2-phenylindole) Mounting Medium (Vector Laboratories, Burlingame, CA). p63 was visualized with rabbit anti-p63α H-129 (diluted 1:50 in NGS-PBS, Santa Cruz). PTEN was detected with mouse monoclonal anti-PTEN 6H2.1 antibody in NGS-PBS (1:50 dilution from Cascade Bioscience, Winchester, MA). Confirmation of PTEN subcellular localization by immunofluorescence was also made using rabbit monoclonal anti-PTEN (#9559) at a dilution of 1:25 (Cell Signaling, Boston, MA). Gamma tubulin was detected with rabbit polyclonal anti-γ-tubulin at dilution of 1:2000 (DQ-19, Sigma-Aldrich). Rabbit polyclonal PLK-1 and Pericentrin were detected at 1 μg/mL, while ninein (ab4447) was detected at 20 μg/mL (AbCam, Cambridge, MA).

Formalin fixed, paraffin-embedded embryonic mouse sections used for immunofluorescence in Figures 3, 25B and 36 were a kind gift from Dr. Satrajit Sinha (SUNY Buffalo). All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo. Human tissue samples were obtained and handled in full approval and accordance with Wright State University Institutional Review Board protocols. Human skin microarray (SK805, Biomax, Rockville, MD) and paraffin embedded embryonic mouse sections were first treated to four, 10 minute incubations at room temperature in
HistoClear (National Diagnostics, Atlanta, GA) with gentle agitation to remove the paraffin. The tissues were re-hydrated by 5 minute washes in gradient ethanol (95%, 70%, 50%) followed by distilled water for 5 minutes. A heat based antigen retrieval technique was used to unmask p63 and PTEN epitopes by immersing slides in Tris/EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0) and heating in a pressure cooker at 90°C for 12 minutes. Slides were cooled to room temperature before blocking for 3 hours in 5% normal goat serum in PBS plus 0.5% Tween 20. Tissues were incubated in primary antibodies, mouse p63 4A4 (Santa Cruz) and rabbit PTEN #9559 (Cell Signaling) diluted 1:25 in blocking buffer, overnight at 4°C. Excess primary antibody was removed by three consecutive washes with NGS-PBS followed by incubation with isotype appropriate AlexaFluor-488 or AlexaFluor-568 conjugated secondary antibodies for 1 hour. Coverslips were mounted onto tissues slides and nuclei counterstained using VectaShield + DAPI Mounting Medium (Vector Laboratories).

Images were acquired using a Leica DMI6000 B (Leica Microsystems, Wetzlar, Germany) inverted epifluorescent microscope and ImagePro 6.2 Software (Media Cybernetics, Bethesda, MD). Analysis of mean fluorescent intensity (MFI) was done as previously described (Lee and Rhee, 2011). Briefly, the fluorescence intensity within the region of interest was defined by drawing a circle around the centrosome using ImagePro 6.2 (Media Cybernetics). The background intensity from an identically sized circle adjacent to the area of interest was subtracted from the MFI of the area of interest. At least 5 measurements of each protein were taken of each sample for studies of paraffin embedded tissues while centrosome studies measured at least 25 centrosomes per
condition for each experiment with an n = 3 independent experiments. Statistical significance of differences was determined by Student’s t-test for all centrosome related experiments. Significance for MFI in human epidermal samples was determined by one-way ANOVA with a Bonferroni ad-hoc test and the Pearson Product Moment analysis was used to determine correlations between p63 and PTEN. A probability value of < 0.05 was considered statistically significant.

**F. Chromatin Immunoprecipitation.**

Chromatin Immunoprecipitation (ChIP) was done using the ChIP-IT Express Kit as per manufacturer’s protocol (Active Motif, Carlsbad, CA) and as described previously (Kommagani et al., 2009). Briefly, asynchronously growing HaCaT and A431 cells were fixed with 2.7% formaldehyde in minimal media for 10 minutes with gentle agitation. Fixation was stopped with ice cold 1x Glycine Stop solution for 5 minutes at room temperature with gentle agitation. Cells were scrapped off culture dishes using cold PBS supplemented with PMSF and pelleted by centrifugation for 10 minutes at 1257x g at 4°C. Pellets were resuspended in 1 mL of ice cold lysis buffer supplemented with 5 μL protease inhibitor cocktail (PIC) and 5 μL PMSF. Cells were lysed by 10 strokes in an ice cold dounce homogenizer and nuclei were subsequently pelleted by centrifugation for 10 minutes at 2350x g at 4C. Nuclei were resuspended in the provided shearing buffer supplemented with PIC and PMSF. A431 chromatin was sonicated on ice using a Fisher Scientific model 500 sonic dismembrator for pulses lasting for 59 sec with 10 sec resting in between pulses. HaCaT chromatin was sonicated for 3 total cycles of 30 sec pulses with 10 sec resting in between. Fragmentation of chromatin to sizes of 300-1,000 base pairs, the majority which being 300-500 base pairs, was confirmed by electrophoresis on
a 1% agarose gel after de-proteinization with proteinase K for 2 hours at 42°C. A total of 16 μg of sheared chromatin was pre-cleared with Protein G magnetic beads for 2 hours at 4°C before immunoprecipitation overnight at 4°C with 2 μg of IgG control, anti-p63 4A4, anti-63α, or anti-p53 AB-6 diluted in 25 μL of Protein G magnetic beads, 20 μL of ChIP buffer 1, and 2 μL PIC. A portion (10%) of sheared chromatin that was not subjected to immunoprecipitation was used as input DNA. Chromatin was eluted from antibody-Protein G complex by three 15 minute incubations in 50 μL ChIP elution buffer (1M NaHCO₃, 1% SDS) at room temperature.

Input and immunoprecipitated chromatin were reverse cross linked by incubating chromatin in 5 M NaCl with RNase A overnight at 65°C. Any remaining protein was digested by proteinase K treatment for 2 hours at 42°C. Final DNA was cleaned using the QiaQuick PCR Purification Kit (Qiagen) before PCR amplification with GoTaq Green Master Mix (Promega, Madison, WI). Sequences for primers can be found in Table 3 of the methods appendix. Primers for the p21 promoter were as described previously (Kommagani et al., 2009). PCR conditions used are as follows, a total of 40 cycles were performed each consisting 30 sec at 94°C, 30 sec at 55°C and 30 sec at 68°C.

**G. Cell Proliferation.**

Cells harvested after the second round of siRNA transfection were re-seeded at 2 x 10⁴ cells/well in a 6 well plate for colony formation assays. At 72 hours post re-seeding, cells were stained with 1% crystal violet. Twenty random images were taken per condition and colony number and area from each frame was measured using ImagePro 6.2 Software (Media Cybernetics). For cell growth assays, cells were re-seeded at 5,000 cells/well in 96 well plates MTS assays or at 10,000 cells/well in 12 well plates for trypan
blue exclusion assays after 24 hours from the second round of siRNA transfection. The MTS assay was performed as per manufacturer’s instructions at days indicated (Promega). Cell proliferation as measured by trypan blue exclusion was done in triplicate per condition per day post siRNA transfection.

H. Dual Luciferase Assay.

Cells were transfected with 100 ng of either a luciferase reporter with the full length pten promoter (PTEN-luc) or the designated minimal pten promoter reporter along with the ΔNp63α or empty vector. To control for transfection efficiency cells were also transected with 5 ng Renilla luciferase expression vector. Dual-luciferase assays were performed using Dual-Luciferase Reporter 1,000 Assay System as per manufacturer’s protocol (Promega, Madison, WI). The luciferase activity of the experimental reporter was normalized to Renilla luciferase activity as measured in relative light units (RLU). The effect of ΔNp63α overexpression on each experimental reporter is further reported as change RLU values as compared to vector transfected cells.

I. Ubiquitination Assays.

H1299 cells were transfected with the indicated expression plasmids and either HA-ubiquitin or His-Ub. For experiments using HA-Ub, H1299 cells were lysed in a high salt buffer (300 mM NaCl, 100 mM Tris pH 8, 0.2 mM EDTA, 0.1% NP40, 10% glycerol). A total of 1 mg protein was diluted to a final concentration of 100 mM NaCl with a salt free buffer (100 mM Tris pH 8, 0.2 mM EDTA, 0.1% NP40, 10% glycerol); 10% of the total protein was saved for input control. Protein lysates were pre-cleared with 20 μl of magnetic Protein G Dynabeads (Life Technologies) for 1 hour followed by overnight incubation at 4°C with 1 μg anti-hemagglutinin (clone 12CA5) donated by Dr.
Patrick Dennis. Antibody-ubiquitinated protein complexes were pulled out of solution with Protein G Dynabeads (Life Technologies) by an additional 2 hours incubation at 4°C. The isolated beads were resuspended in 70 μL 100 mM NaCl buffer (100 mM NaCl, 100 mM Tris pH 8, 0.2 mM EDTA, 0.1% NP40, 10% glycerol) and resolved by immunoblot analysis as described above.

Ubiquitination assays with the His-Ub expression plasmid were completed as described previously (Jaffray and Hay, 2006). Briefly, 24 hours post transfection with His-Ub expression plasmid, cells were pelleted and 20% of the pelleted cells were lysed in phosphatase inhibitor containing buffer (PhIB) for determination of protein concentration and saved for the input control. The remaining 80% of the cells were lysed in guanidinium-HCl buffer (6 M guanidinium-HCl, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 8, 0.01 M Tris HCl, 10 mM β-mercaptoethanol, 5 mM imidazole). Equivalent amounts of total protein in 6 M guanidinium HCl buffer (1 mg), as determined by BCA analysis of the fraction lysed in PhIB, were rotated overnight with 75 μl Nickle-NTA beads (Qiagen) at 4°C. The beads were pelleted by centrifugation at 2350x g for 5 minutes at 4°C. Beads were successively washed for 15 minutes each at room temperature in the following buffers: Wash Buffer 1 (6 M guanidinium-HCl, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 8, 0.01 M Tris HCl, 10 mM β-mercaptoethanol), wash buffer 2 (8 M urea, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, 0.01 M Tris HCl, 10 mM β-mercaptoethanol), wash buffer 3 (8 M urea, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 6.3, 0.01 M Tris HCl, 10 mM β-mercaptoethanol, 0.2% Triton X-100), wash buffer 4 (8 M urea, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 6.3, 0.01 M Tris HCl, 10 mM β-mercaptoethanol, 0.1% Triton X-100), and wash buffer 5 (8 M urea, 0.1 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 6.3, 0.01 M Tris HCl, 10 mM β-mercaptoethanol). His-
ubiquitinated proteins were eluted off of the washed beads by 30 minute incubation at room temperature in 100 μL elution buffer (200 mM imidazole, 0.15 M Tris-HCl, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol, 5% SDS). Input control and eluted His-Ub proteins were processed for immunoblot analysis as described above.

I. Methods Appendix.

### Table 1: Primers used for generation of minimal luciferase reporters

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### Table 3: Primers used for chromatin immunoprecipitation

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41
III. Results

A. p63 negatively regulates PTEN expression.

To examine how p63 controls cell proliferation in vitro, several keratinocyte-derived cell lines were employed. Primary Human Epidermal Keratinocytes (HEKs) were used to whenever possible. Because of the difficulty growing, limited lifespan and culture costs associated with HEKs, transformed keratinocyte cell lines were used for the majority of the experiments within this dissertation. A431 cells, originating from a cutaneous squamous cell carcinoma of the vulva, were used as a model for atypical cancerous keratinocytes while the spontaneously transformed, non-tumorigenic HaCaT cell line was used to model relatively normal human keratinocytes.

Due to the often opposing functions of the TAp63 and ΔNp63 isoforms, the predominant p63 isoforms in any cell line must first be identified in order to determine how p63 influences keratinocyte proliferation. Consistent with earlier reports, ΔNp63α was found to be the predominant p63 isoform in both these cell lines (Di Como et al., 2002; Mangiulli et al., 2009; Megyeri et al., 2009). Quantitative real time PCR (qRT-PCR) analysis with TAp63 and ΔNp63 specific primers demonstrated that ΔNp63 mRNA levels were several orders of magnitude higher than TAp63 mRNA levels in A431, HaCaT, and HEK cells when compared to the p63 null, non-small cell lung carcinoma H1299 cell line (Figure 6A). Endogenous TAp63 transcript levels were frequently near the limit of detection, indicating very low expression of the TAp63 isoforms during normal cell growth.
Figure 6: ΔNp63α is the predominant isoform in keratinocyte cell lines. (A) RT-PCR analysis of total cDNA from the indicated cell lines using primers specific to TA or ΔNp63 isoforms. H1299 cells lacking p63 were used as a negative control. (B) Immunoblot analysis using pan-p63 (4A4), ΔNp63 specific (RR14), and p63α isoform specific antibodies (H-129) and compared to p63 isoforms overexpressed in H1299 cells.
In order to determine the predominant C-terminal splice variants of p63 present in A431 and HaCaT cells, whole cell lysates were subjected to Western blot analysis alongside extracts from H1299 cells overexpressing each of the six main isoforms of p63 as positive controls. A distinct band of ~70 kDa was observed in both A431 and HaCaT lysates when probed with an antibody detecting all isoforms of p63 (4A4) (Figure 6B). This band migrated identically to that of the ΔNp63α control and showed similar intensities when probed with the pan-p63 and ΔNp63 specific antibodies. Occasionally, bands smaller than the main 70 kDa band were observed in lysates from either A431 or HaCaT cells. Because of the unreliable presence of these bands as well as their detection in H1299 cells with forced expression of a single p63 isoform, they were determined to be degradation products of ΔNp63α rather than bands corresponding to ΔNp63β or ΔNp63γ. No higher molecular weight bands in A431 or HaCaT lysates were observed with either the pan-p63 or TAp63 specific antibodies, demonstrating the lack of endogenous TAp63 protein in actively growing keratinocyte cultures. The inability to detect endogenous TAp63 protein along with the molecular weight and use of isoform specific antibodies confirms ΔNp63α as the predominant p63 isoform in both A431 and HaCaT cells.

Having determined that ΔNp63α is the predominant isoform, and only detectable p63 protein in keratinocyte cell lines, the effects of ΔNp63α on PTEN expression were next determined. Using multiple pan-p63 siRNAs, p63 expression was knocked down in A431 (Figure 7A), HaCaT cells (Figure 7B), and adult HEKs (Figure 7C). Silencing p63 led to a modest, but consistent increase in PTEN transcript levels in all cell lines with all siRNAs tested. Increased IGFBP-3 levels, previously shown to be negatively regulated
by ΔNp63α served as a positive control (Barbieri et al., 2005). The increase in PTEN transcript levels upon silencing of p63 correlated with increased protein levels compared to non-silencing control (NSC) transfected cells (Figure 7A-C, lower panels). We also confirmed that the pan-p63 siRNAs equally knockdown TAp63 and ΔNp63 (Figure 8A). To demonstrate that the increased PTEN expression observed upon p63 silencing is due to ΔNp63α we knocked down ΔNp63α expression using a ΔNp63 specific siRNA and showed that loss of only ΔNp63α, the predominant isoform, still led to an increase in PTEN mRNA (Figure 8B). Although the corresponding increase in PTEN protein levels in Figure 7 seems modest, minute changes in PTEN protein levels have been shown to have profound biological impact and thus the modest increases in PTEN observed upon p63 knockdown were considered biologically significant (Alimonti et al., 2010).

Mouse embryonic fibroblasts (MEFs) from p63−/− and wild type mice were utilized to confirm that increased PTEN expression upon loss of p63 was not an artifact. PTEN transcript and protein levels were found to be elevated in both E1A transformed MEFs (Figure 9A) as well primary MEFs obtained from p63−/− mice when compared to those of wild type mice (Figure 9B). A minor amount of residual p63 mRNA was detectable in the p63−/− MEFs due to the location of primers used for qRT-PCR spanning between exons 4 and 5 while the p63 protein was rendered nonfunctional due to the deletion of exons 6-8 in the Trp63 gene (Yang et al., 1999). The residual p63 mRNA fails to generate any p63 proteins and is barely within the limit of detection by qRT-PCR. The increase in PTEN mRNA and protein in p63−/− MEFs was very similar to the increases observed from p63 knockdown in keratinocyte cell lines (Figure 7), consistent with PTEN being negatively regulated by ΔNp63α.
Figure 7: p63 negatively regulates PTEN expression in keratinocyte cell lines. (A) A431, (B) HaCaT cells and (C) Primary adult human epidermal keratinocytes (HEKa) were transfected with non-silencing control (NSC) siRNA or siRNAs targeting all isoforms of p63. Total RNA was extracted and transcript levels of p63, PTEN and IGFBP-3 were analyzed by qRT-PCR. Y-axis represents the change in PTEN and p63 transcript levels relative to NSC transfected cells. Representative immunoblots of p63 and PTEN are shown in the bottom panels. Fold change in protein, after normalization to β-actin and relative to non-silencing control, is listed above each band.
It has been demonstrated that PTEN is regulated at the transcriptional level by the p63 family members p53 and p73 (Stambolic et al., 2001; Vella et al., 2009). Because ΔNp63α can act in a dominant negative manner to prevent the pro-apoptotic transcriptional activities of other p53 family members (Yang et al., 1998), the involvement of the various p53 family members on regulation of PTEN by ΔNp63α was investigated. Both A431 and HaCaT cells harbor mutations in p53 that disrupt the normal binding pattern of p53 and abrogate the tumor suppressive functions of p53 (Park et al., 1994; Rolley et al., 1995). Loss of ΔNp63α resulted in increased PTEN expression in cells harboring mutant p53 (Figure 7A-B) as well as in primary keratinocytes (Figure 7C) and MEFs (Figure 9B) expressing wild type p53, demonstrating that repression of PTEN expression by p63 is independent of p53.

In order to rule out the possibility that the induction of PTEN observed after silencing ΔNp63α was not simply caused by alleviation of the dominant negative effects of ΔNp63α on either the TAp73 or TAp63 isoforms, the expression of p73 in keratinocytes first had to be examined. The levels of both TA and ΔN isoforms of p73 were examined by qRT-PCR and immunoblot analyses (Figure 10). As shown in Figure 10A, ΔNp73 was the predominant isoform of p73 observed in both A431 and HaCaT cell lines, suggesting that these cells may have a deficient p73-dependant pro-apoptotic program since the ΔNp73 isoforms can act in a dominant negative manner toward the TAp73 isoforms similar to the relationship of ΔNp63/TAp63 proteins. TAp73α and TAp73β were, however, still detectable at the protein level and could therefore influence the expression of PTEN (Figure 10B).
Figure 8: Selective knockdown of ΔNp63 increases PTEN expression. (A) HaCaT cells were transfected with NSC or siRNA against all isoforms of p63 or siRNA specific to either the ΔNp63 isoforms or TAp63 isoforms. Total RNA was extracted and analyzed by qRT-PCR. Y-axis represents the change in transcript levels relative to NSC transfected cells after normalization to GAPDH. (B) HaCaT cells were transfected with NSC or siRNA specific to the ΔNp63 isoforms. Total RNA and protein were extracted and analyzed as in (A) for ΔNp63α and PTEN levels. Y-axis represents the fold change in PTEN and ΔNp63 transcript levels relative to NSC transfected cells. Representative immunoblots of ΔNp63α and PTEN are shown in the bottom panels. Fold change in protein, after normalization to β-actin and relative to non-silencing control, is listed above each band.
Figure 9: Elevated PTEN expression in p63 null MEFs. Total RNA extracted from (A) E1A transformed and (B) primary mouse embryonic fibroblasts (MEF) obtained from wild-type and p63 null mice were subjected to qRT-PCR to detect transcript levels in the top panel. Bottom panel represents immunoblot analysis of whole cell extracts from wild-type and p63 null MEFs; change in PTEN protein levels compared to wildtype MEFs is listed above each band. Note: p63 was undetectable at the protein level and near the limit of detection for qRT-PCR in p63 null MEFs. Error bars represent standard deviation.
Figure 10: ΔNp73β is the predominant p73 isoform in A431 and HaCaT cells. (A) qRT-PCR analysis of total cDNA from indicated cell lines using primers specific for the TA or ΔNp73 isoforms. (B) Immunoblot analysis using pan-p73 or ΔNp73 specific antibodies and compared to p73 isoforms overexpressed in H1299 cells.
In order to rule out the possibility that silencing ΔNp63α was inducing PTEN expression by simply reversing its dominant negative effects on either the TAp73 or TAp63 to allow transcriptional up-regulation by TAp63 and/or TAp73, cells were transfected with non-silencing control siRNA or siRNA to ΔNp63α and/or siRNA specific to either TAp63 (Figure 11A) or TAp73 (Figure 11B). In A431 cells, which showed higher levels of TAp63 mRNA (Figure 6A), silencing ΔNp63α significantly inhibited ΔNp63α expression and increased PTEN mRNA similar to the use of pan-p63 siRNAs in Figure 7A (Figure 11A). Interestingly, silencing TAp63 also led to an increase in PTEN transcript levels, however this may be the result of the minor reduction in ΔNp63 mRNA caused by silencing TAp63 (Figure 11A). Silencing both ΔNp63α as well as TAp63 isoforms also led to significant PTEN induction similar to silencing ΔNp63 alone, therefore suggesting that PTEN induction observed upon silencing ΔNp63α occurs primarily independent of any effects on TAp63 activity. Immunoblot analysis results were consistent with the qRT-PCR results in that silencing ΔNp63 had more profound effects on PTEN protein than silencing TAp63 (Figure 11A, lower panel). Endogenous TAp63 protein could not be detected by immunoblot analysis (Figure 6B).

Similarly to the effects on TAp63, silencing TAp73 alone in HaCaT cells did not significantly affect the PTEN expression when compared to non-silencing control (Figure 11B). HaCaT cells were used since they expressed the highest amount of endogenous p73 (Figure 10A). When both ΔNp63α and TAp73 were silenced together, the increase in PTEN expression was similar to the condition in which only ΔNp63α was silenced. Together our results demonstrate that up-regulation of PTEN upon ΔNp63α knockdown is not due to repression of p53 or the TA isoforms of p63 and p73.
**Figure 11: ΔNp63α is the predominant isoform responsible for changes in PTEN.**

(A) A431 cells were transfected with non-silencing control (NSC) siRNA, siRNA specific to the ΔNp63 or TAp63 isoforms, or both. Total RNA was extracted and changes transcript levels were analyzed by qRT-PCR. Y-axis represents the change in transcript levels relative to NSC transfected cells after normalization to the endogenous loading control. Representative immunoblots for the indicated proteins are shown in the bottom panel. (B) HaCaT cells were transfected with non-silencing control (NSC) siRNA, siRNA specific to ΔNp63 or TAp73, or both. Total RNA was extracted and changes transcript levels were analyzed by qRT-PCR. Y-axis represents the change transcript levels relative to NSC transfected cells. Representative immunoblots for the indicated proteins are shown in the bottom panel. Fold change in protein, after normalization to β-actin and relative to non-silencing control, is listed above each band.
B. PTEN is a direct target of ΔNp63α.

Loss of ΔNp63α led to increases in both PTEN transcript and protein levels, suggesting that PTEN may be a direct transcriptional target of p63. Examination of the pten promoter revealed the presence of several putative p63 responsive elements (REs) in addition to the p53 RE described previously (Figure 12A) (Stambolic et al., 2001). To define the region of the pten promoter to which ΔNp63α binds, chromatin immunoprecipitation (ChIP) assays were performed in A431 and HaCaT cells. ChIP analysis was performed using antibodies that recognize all p63 isoforms (4A4 antibody) or a p63 antibody that specifically recognizes only the p63α isoforms (H-129 antibody). Not surprisingly, p63 bound to the same locations of the pten promoter with similar affinities in both A431 and HaCaT cells. p63 bound to regions of the pten promoter with multiple p63 REs as seen when immunoprecipitated with either pan-p63 or alpha isoform specific antibodies (Regions A and B, Figure 12B). p63 also bound the p53 responsive element, which is consistent with previous reports demonstrating that p63 can to bind to promoters of other p53 targets (Region C, Figure 12B) (Westfall et al., 2003). Not all putative p63 responsive elements, however, showed binding of p63 at the pten promoter. Region D contained two half sites that matched the criteria for a p63 specific RE, yet failed to demonstrate binding. Interestingly, Region D corresponds to the region reported to be bound by p73 in thymic cells further confirming the results from Figure 11 that p63 does not interfere with p73-mediated regulation of PTEN (Vella et al., 2009). Region E with a canonical p63 RE only showed strong binding when immunoprecipitated with the pan-p63 antibody, indicating weak binding to this RE.
Figure 12: ΔNp63α binds the pten promoter. (A) Schematic of putative p63 binding sites and documented p53 binding sites in the pten promoter (Accession #AF067844). Each gray oval represents a single putative p63 half-site, while each black oval represents a single putative p53 half-site. (B) Chromatin immunoprecipitation assay was performed on A431 and HaCaT cells wherein chromatin was immunoprecipitated with normal IgG control antibody or antibodies that recognize all p63 isoforms or p63α isoforms as indicated. Eluted DNA was PCR-amplified with primers specific for multiple regions of the pten promoter as shown in (A). The 5’ locations of primers used are indicated in parentheses for each region. The p21 promoter was used as a positive control for p63 binding.
Because mutant p53 has previously been demonstrated to complex with p63 and affect the binding of p63 to its transcriptional targets, the binding of p53 to the \textit{pten} promoter was also evaluated in these cells (Gaiddon et al., 2001; Strano et al., 2002). p53 was found to bind nearly all of the same regions of the \textit{pten} promoter as p63 (Figure 13). Region E, which showed binding by p63 failed to show binding by p53 in either A431 or HaCaT cells, while regions A and B showed strong binding of both p63 and p53. Interestingly, the mutant p53 in A431 cells failed to bind the p21 promoter, a known target of wild type p53, suggesting that this mutation severely disrupts the normal binding pattern of p53 and is consistent with previous reports (Park et al., 1994; Rolley et al., 1995). Contrary to prior studies, the results shown in Figure 13 suggest that while mutant p53 may interact with p63 it does not prevent p63 from binding to the promoter of \textit{pten} (Gaiddon et al., 2001; Strano et al., 2002). However, we cannot discern from these experiments if mutant p53 physically interacts with \Delta Np63\alpha or whether mutant p53 influences where p63 binds the \textit{pten} promoter or vice versa. These results show that endogenous \Delta Np63\alpha is capable of binding to the \textit{pten} promoter in A431 and HaCaT cells in multiple locations unique to p63 in addition to binding the p53 RE.

To confirm that p63 binding of the \textit{pten} promoter correlated with a decrease in the transcriptional activity of \textit{pten}, the effect of \Delta Np63\alpha on the transcriptional activity of a luciferase reporter containing the full-length \textit{pten} promoter (PTEN-Luc) was analyzed. The p63 null cell line H1299 was co-transfected with the PTEN-Luc reporter and either \Delta Np63\alpha or empty vector control. Consistent with the ChIP results, \Delta Np63\alpha overexpression led to a statistically significant reduction (p<0.05) in PTEN-Luc reporter activity as well as protein levels when compared to vector transfected control cells.
Conversely, when ΔNp63α was silenced in HaCaT cells the PTEN-Luc reporter activity increased, albeit to a more modest degree, as did the protein levels of endogenous PTEN (Figure 14B).

In order to define which regions within the pten promoter were critical for ΔNp63α mediated repression of pten transcriptional activity, a series of minimal luciferase reporters were created based on the areas of the pten promoter to which p63 bound. These minimal PTEN-luc reporters contained smaller fragments of the pten promoter than the corresponding regions pulled down in the ChIP assays (Figure 12B), encompassing only the putative p63 REs. When co-transfected into H1299 cells with ΔNp63α only the minimal PTEN-Luc reporter corresponding to Region B of the pten promoter showed a significant change in luciferase activity, suggesting that this region is critical for ΔNp63α-mediated repression of PTEN (Figure 15). Region B contains five p63 half sites (sequence shown in Figure 13C, lower panel), and the density of the p63 REs within this region may explain why it is so potent at repressing PTEN expression. However, because the minimal PTEN-Luc reporters lack most of the PTEN promoter, we cannot determine if the lack of change in luciferase activity in response ΔNp63α by the other minimal reporters is due to the possible deletion of co-factor binding sites or simply that these regions are unnecessary for ΔNp63α mediated repression of PTEN. Nonetheless, these results demonstrate that ΔNp63α binds to the pten promoter, in a region distinct from other p53 family members, and negatively regulates PTEN expression.
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Figure 13: p53 occupies many of the same locations of the pten promoter as p63. Chromatin immunoprecipitation assays were performed on A431 and HaCaT cells wherein chromatin was immunoprecipitated with normal IgG control antibody or an antibody specific to p53 as indicated. Eluted DNA was PCR-amplified with primers specific for multiple regions of the pten promoter. Specific nucleotide locations of the regions amplified are shown in parentheses. The p21 promoter was used as a positive control, however A431 cells harbor a mutation in p53 that affects DNA contact and may explain why p53 fails to bind to the p21 promoter in these cells.
Figure 14: ΔNp63α reduces transcriptional activity of the *pten* promoter. (A) Dual luciferase assays performed on H1299 cells co-transfected with the full-length PTEN-Luc reporter and empty vector or ΔNp63α. (B) Dual luciferase assays performed on HaCaT cells after knockdown of p63 by siRNA. Y-axis represents change in relative luciferase units compared to control transfected cells. Representative immunoblots are shown below to demonstrate adequate overexpression or knockdown.
Figure 15: Definition of the ΔNp63α-responsive elements in the pten promoter. (A) Dual luciferase assays in H1299 cells after co-transfection with the Full-length (FL) PTEN-luc reporter or the designated minimal PTEN-luc reporter and either empty vector or ΔNp63α expression plasmids. Minimal reporter plasmids correspond to the regions of the pten promoter pulled down by ChIP assay from Figure 12B. The sequence corresponding to region B is shown below, with the core sequence of the putative p63 response elements bolded and underlined. Lined up underneath each p63 half site is the canonical p63 response element where R = purines, W = A or T and Y = pyrimidines. Error bars represent standard deviation. * =p<0.05 compared to control cells.
C. Activation of the Akt pathway via ΔNp63α-mediated PTEN repression.

The most well-known function of PTEN is to dephosphorylate PIP$_3$ and thereby inhibit the Akt survival pathway. To determine if the regulation of PTEN by ΔNp63α affects the Akt pathway, increasing concentrations of ΔNp63α were transfected into p63 null, PTEN positive H1299 cells. Forced expression of ΔNp63α led to a dose-dependent reduction in endogenous PTEN protein levels with a concomitant increase in active Akt as measured by phospho-serine 473 levels, hereafter referred to as p-Akt (Figure 16A). Induction of p-Akt by ΔNp63α is dependent on ΔNp63α-mediated repression of PTEN as overexpression of ΔNp63α in PTEN null PC3 cells failed to increase p-Akt levels (Figure 16B).

Since activated Akt has been show to increase the levels of ΔNp63α and ΔNp63α can repress the expression of PTEN, it was next determined if ΔNp63α and constitutively active Akt (CA-Akt) could synergistically decrease PTEN levels (Segrelles et al., 2006). ΔNp63α alone led to a dramatic reduction in endogenous PTEN when overexpressed in H1299 cells, while CA-Akt alone led to a more modest decrease in PTEN (Figure 17). Consistent with previous reports, an enhancement of ΔNp63α protein was observed when ΔNp63α and CA-Akt were co-expressed (Barbieri et al., 2003; Segrelles et al., 2006). Co-expression of ΔNp63α and CA-Akt, however, did not synergistically reduce PTEN expression as compared to overexpression of only ΔNp63α, but was capable of further reducing PTEN as compared to CA-Akt or vector alone conditions (Figure 17). The inability of ΔNp63α and CA-Akt to act synergistically in reducing PTEN levels is consistent with ΔNp63α and Akt functioning in the same pathway rather, but may also be the result reaching saturating levels of ΔNp63α with the transfection protocol used.
### A) H1299; PTEN positive

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### B) PC3; PTEN null

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Figure 16: Exogenous ΔNp63α induces Akt activation via down regulating PTEN.

(A) H1299 cells were transfected with decreasing amounts of ΔNp63α expression plasmid as indicated. (B) PTEN null PC3 cells were transfected with empty vector or ΔNp63α and subjected to immunoblot analysis for the indicated proteins. The level of PTEN and the ratio of p-Akt/Akt protein levels, normalized to β-actin, is listed below each band.
Figure 17: ΔNp63α and constitutively active Akt do not synergistically decrease PTEN. H1299 cells were transfected with ΔNp63α alone or together with constitutively active Akt (CA-Akt) and whole cell lysates were subject to immunoblot analysis as indicated. The change in ΔNp63α and PTEN protein levels normalized to β-actin is listed above each band.
Forced expression of ΔNp63α in p63 null cells increased p-Akt levels by reducing the expression of PTEN. To determine if the same pathway was functioning in p63 positive keratinocytes, ΔNp63α levels were knocked down with three different siRNA (Figure 18A). Silencing ΔNp63α led to an increase in endogenous PTEN corresponding with decreased p-Akt levels (Figure 18A). Knockdown of PTEN in HaCaT cells resulted in increased p-Akt levels, as expected, but also caused significant increases in ΔNp63α expression (Figure 18B). Interestingly, silencing both ΔNp63α and PTEN resulted in p-Akt levels similar to that of control transfected cells (Figure 18A). To confirm that the increases in ΔNp63α observed upon loss of PTEN were indeed caused by activation of the Akt pathway, primary neonatal HEKs (NHEKs) were treated with the Akt specific inhibitor MK2206 after knockdown of PTEN. Incubation with MK2206 prevented the increase in ΔNp63α levels caused loss of PTEN (Figure 19). Altogether, this confirms that ΔNp63α and PTEN function within a regulatory loop to control p-Akt levels, which in turn also mediates the expression of ΔNp63α and PTEN.
D. The ΔNp63α/PTEN/pAkt Regulatory loop controls keratinocyte proliferation.

The Akt pathway is well known for promoting cell survival and proliferation. In order to examine whether the control of p-Akt by the ΔNp63α-PTEN regulatory loop maintains cell proliferation rates, colony formation assays were performed after silencing ΔNp63α and/or PTEN. Silencing ΔNp63α reduced colony formation (Figure 20A-B), while silencing PTEN not only led to an increase in total colony number (Figure 20B), but also in colony size (Figure 20C) in both A431 and HaCaT cells. Simultaneous knockdown of ΔNp63α and PTEN did not statistically alter colony number (Figure 20B) or size (Figure 20C) as compared to non-silencing control transfected cells. Silencing of ΔNp63α and PTEN was confirmed by immunoblot analysis as shown in Figure 20B.

Consistent with the colony formation assays, cell proliferation determined by MTS assays also demonstrated that silencing both ΔNp63α and PTEN resulted in proliferation rates similar to control transfected cells (Figure 21A). Loss of only ΔNp63α or PTEN resulted in decreased or increased proliferation rates, respectively. Cell proliferation was also measured by trypan blue exclusion in A431 and HaCaT cells after transfection with p63 and/or PTEN specific siRNA (Figure 21B). When measured by MTS assays A431 cells did not show much reduction in proliferation in response to silencing ΔNp63α alone as compared to the reduced rates measured by colony forming and trypan blue assays (Figure 21A vs Figures 20B and 21B). This may be due to the indirect measurement of metabolizing cells in MTS assays, rather than directly measuring the increase in cell number over time as with colony formation and trypan blue assay. Despite the discrepancies between methodologies, the results clearly show that a tight
balance between ΔNp63α and PTEN expression must be met in order to maintain normal proliferation rates in keratinocytes.
Figure 18: ΔNp63α affects Akt activation by regulating PTEN expression. (A) HaCaT cells were transfected with three different siRNAs targeting p63 or NSC as indicated and whole cell extracts were subjected to immunoblot analysis as indicated. (B) HaCaT cells were transfected with NSC or siRNA against p63 and/or PTEN and whole cell lysates were subject to immunoblot analysis as indicated. The change in protein level, normalized β-actin or to Total Akt and β-actin, is listed above each band.
Figure 19: PTEN influences ΔNp63α levels via inhibition of Akt activity. Primary neonatal human epidermal keratinocytes (NHEKs) were transfected with NSC or PTEN specific siRNA followed by DMSO control or 10 μM MK2206 to specifically inhibit Akt activity. Immunoblot analyses for the indicated proteins were performed on whole cell lysates; the changes in protein levels, relative to NSC DMSO treated cells, are listed below each band.
Figure 20: A balance between ΔNp63α and PTEN controls cell proliferation. (A) Representative images of crystal violet stained colonies 3 days post re-seeding of transfected cells with NSC siRNA, p63 siRNA and/or PTEN siRNA as indicated. (B) Analysis of total colony number after knockdown of p63 and/or PTEN. Y-axis represents total number of colonies from (A) as counted with ImagePro 6.2. Error bars represent standard deviation. *= p<0.05. Knockdown was confirmed by immunoblot analysis and a representative experiment is shown. (C) Analysis of colony size after knockdown of p63 and/or PTEN.
Figure 21: Concomitant silencing of ΔNp63α and PTEN return growth rate to normal. A431 and HaCaT cells were transfected with NSC siRNA, p63 siRNA and/or PTEN siRNA as indicated and re-plated 24hr post transfection. The change in proliferation rates were measure by (A) MTS assays or (B) trypan blue staining for viability. (A) The Y–axis represents the change in absorbance relative to day 1. (B) The Y–axis represents total cell number. Error bars representing standard deviation within a single representative experiment.
E. ΔNp63α and PTEN expression and localization in non-melanoma skin cancers.

The balance between ΔNp63α and PTEN was shown to be critical for regulating proliferation rates in cell culture (Figure 20-21), while in the clinical setting elevated levels of ΔNp63α have been reported in a variety of squamous and basal cell carcinomas (SCC and BCC, respectively) (Di Como et al., 2002; Nylander et al., 2000; Parsa et al., 1999). To determine if the amplification of ΔNp63α seen in human non-melanoma cancers coincided with reduced PTEN, the expression and localization of both p63 and PTEN were examined by indirect immunofluorescence using a tissue microarray consisting of normal skin cores (n=9), hyperplastic epidermal tissue (n=7), benign basal cell papillomas (n=6), cores of human papilloma virus (HPV) induced condyloma acuminatum (n=9), BCC cores (n=11), and SCC cores (n=11). The influence of the ΔNp63α-PTEN axis on tumor development could also be evaluated because the tissue microarray contained benign overgrowths (hyperplasia, papillomas and condylomas) in addition to cancerous tissue (BCC and SCC). Representative images of PTEN and ΔNp63α expression in each of the tissue types is shown in Figure 22.

A statistically significant increase in p63 staining (ANOVA F$_{5,54}$=12.700; p<0.001), as measured by the mean fluorescence intensity (MFI), was observed in hyperplastic skin, condylomas and BCC as compared to normal epidermal tissue (Figure 23A). p63 expression was highest in HPV induced condyloma acuminatum samples, most likely due to the presence of HPV and its ability of inhibit mir-203 mediated suppression of p63 (Melar-New and Laimins, 2010). While there was a trend towards increased p63 levels in benign papillomas and SCC as compared to normal tissue, it was not statistically significant. Except in papillomas, p63 expression was highest in benign
or locally invasive neoplasms, suggesting that p63 expression is important in tumor
development but not necessarily for invasion. Increasing the sample size for each type of
diseased tissue would clarify whether these trends are truly representative of p63
expression in the early stages of tumor development.

A significant correlation was observed between the MFI values of p63 and PTEN
in normal tissue (r=0.693, p=0.042), as well as in BCC (r=0.6687, p=0.00268) and SCC
(r=0.7356, p=0.012). While there was no decrease in PTEN staining accompanying the
increased p63 in diseased skin, it is the overall ratio of p63 to PTEN that governs
proliferation as demonstrated in Figures 20-21. A statistically significant difference
(ANOVA F_{5,53}=12.37; p<0.001) in the ratio between p63 and PTEN was observed in
condylomas and cancerous tissues (Figure 23B), highlighting the importance of balance
between ΔNp63α and PTEN.

In all tissue types p63 was only observed in nuclei. In normal tissue PTEN
staining was most abundant in the cytoplasm of epidermal keratinocytes (Figure 24, top
left panel). A few cells with intense PTEN staining were seen in the dermis and in
melanocytes, consistent with earlier reports (Luukko et al., 1999). Within the dermis of
most cancerous tissue samples, intense staining for PTEN was observed in areas of solar
elastosis, partially due to some non-specific uptake of secondary antibodies by these
damaged fibers (Figure 24, bottom left panel). It is important to note, however, that these
cells are of mesenchymal origin, do not express any p63 and are not the sites of origin for
non-melanoma skin cancers. Interestingly, in both BCC and SCC very little nuclear
PTEN was observed in the cancerous tissue, with a robust lack of nuclear PTEN observed
in BCC as compared to normal epidermal tissue (Figure 24, middle column).
Figure 22: ΔNp63α and PTEN expression in normal and diseased skin.

Representative images from indirect immunofluorescence performed on a tissue microarray containing multiple cores of normal human skin, hyperplastic skin, precancerous lesions (benign papillomas and HPV induced condyloma acuminatum) and cancerous skin (basal cell carcinoma and squamous cell carcinoma).
Figure 23: The balance between ΔNp63α and PTEN expression is upset in NMSC.

(A) Quantification of the mean fluorescence intensity for p63 and PTEN staining, in arbitrary units, from normal epidermal tissue, hyperplastic epidermis (Hyper.), benign papillomas (Pap.), HPV induced condyloma acuminatum (Cond.), basal cell carcinoma (BCC), and squamous cell carcinomas (SCC). Error bars represent standard error of the mean. (B) The average ratio of p63 fluorescence intensity to PTEN fluorescence intensity is plotted for each tissue type. Error bars represent standard error of the mean; *=p<0.05.
Figure 24: Absence of nuclear PTEN in non-melanoma skin cancers. Representative images of PTEN staining from normal human skin, basal cell carcinoma (BCC), and squamous cell carcinoma (SCC) are shown. The right two columns are magnified images from the areas surrounded by a box in the left column to better illustrate the subcellular distribution of PTEN. The absence of nuclear PTEN in normal and cancerous epidermal tissue is shown from the lack of co-localization with DNA (DAPI, blue) or p63 (green) in the left most column.
F. ΔNp63α suppresses nuclear PTEN expression.

To better understand how ΔNp63α affects the nuclear levels of PTEN, the subcellular distribution of PTEN in the epidermis of adult human (Figure 25A) and E18.5 mouse (Figure 25B) skin was examined. In both adult and embryonic skin, keratinocytes of the basal layer and hair follicles expressed the highest levels of p63 with nearly complete nuclear exclusion of PTEN (Figure 25A-B). Moreover, PTEN staining was only observed in the nucleus of supra-basal layer cells that no longer expressed ΔNp63α. ΔNp63α expression is normally down-regulated as keratinocytes differentiate and move upward into the superficial layers of the epidermis. However, in keratinocytes of the supra-basal layer which have yet to degrade ΔNp63α no nuclear PTEN is observed (Figure 25A-B, marked by asterisk). This is consistent with reports demonstrating that nuclear PTEN is most abundant in differentiated cells, while ΔNp63α is associated with proliferating cells (Gimm et al., 2000).

In order to determine if ΔNp63α suppresses nuclear PTEN, the effects of silencing ΔNp63α on nuclear and cytoplasmic PTEN levels were measured. Loss of ΔNp63α increased both nuclear and cytoplasmic levels of PTEN (Figure 26A). Conversely, overexpression of ΔNp63α led to a marked reduction of cytoplasmic PTEN with a corresponding increase in cytoplasmic p-Akt levels. A small, but reproducible, decrease in nuclear PTEN expression was observed after overexpression of ΔNp63α that correlated with a robust increase in nuclear p-Akt (Figure 26B). Furthermore, nuclear PTEN levels were also increased after knockdown of ΔNp63α in intact HaCaT cells as determined by immunofluorescence (Figure 27). Together, these studies demonstrate that ΔNp63α inhibits localization of PTEN to the nucleus in proliferating keratinocytes.
Figure 25: PTEN is not present in the nucleus of healthy basal layer keratinocytes. Indirect immunofluorescence was performed on (A) normal adult human skin and (B) E18.5 mouse skin. Representative images for nuclei (DAPI, blue), p63 (green), and PTEN (red) are shown for each skin type. Basal layer cells that lack nuclear PTEN are shown by arrows while an asterisk shows supra-basal layer cells lacking nuclear PTEN.
Figure 26: ΔNp63α suppresses nuclear PTEN. (A) HaCaT cells were transfected with NSC or p63 specific siRNA and subjected to sub-cellular fractionation. Immunoblot analysis of the indicated proteins is shown in the left panel. Nuclear protein levels were normalized to the intensity of PARP, while α-tubulin served as a loading control for normalization of cytoplasmic proteins. The change in protein level, normalized to the respective loading control and relative to NSC transfected cells, is listed above each band. (B) H1299 cells were transfected with empty vector or ΔNp63α prior to sub-cellular fraction and immunoblot analysis as in (A).
Figure 27: Knockdown of ΔNp63α increases nuclear PTEN. HaCaT cells were transfected with NSC or p63 specific siRNA and subjected to indirect immunofluorescence for p63 (red) and PTEN (green). Nuclei are shown with DAPI staining (blue). White arrows designate cells in which p63 was silenced.
G. ΔNp63α regulates PTEN ubiquitination via the E3 ligase NEDD4-1.

Retention of PTEN within nucleus is maintained by a balance between nuclear import and export. Previous studies have implicated multiple ways by which PTEN is translocated into the nucleus (Chung et al., 2005; Liu et al., 2005a; Trotman et al., 2007). Examination of a gene expression microarray conducted on the HaCaT and A431 keratinocyte lines after knockdown of p63 revealed the E3 ligase NEDD4-1, the first ligase shown to control the subcellular distribution of PTEN (Trotman et al., 2007), to be a potential transcriptional target of ΔNp63α (data not shown). To test the hypothesis that ΔNp63α regulates PTEN ubiquitination via the E3 ligase NEDD4-1, the ubiquitination of endogenous PTEN was first measured after transfection of p63 null cells with hemagglutinin tagged ubiquitin (HA-Ub) together with either empty vector or ΔNp63α expression plasmids. Overexpression of ΔNp63α reduced the levels of ubiquitinated endogenous PTEN, detected as higher migrating smears by Western blot with an anti-PTEN antibody following immunoprecipitation of HA-tagged ubiquitin (Figure 28A). The reduction in ubiquitinated PTEN in response to overexpression of ΔNp63α correlated with a decrease in NEDD4-1 expression (Figure 28A). To ensure that the reduction in ubiquitination of PTEN was not simply a by-product of increased transcriptional repression of PTEN, the effects of ΔNp63α overexpression on ubiquitination of exogenous PTEN was also measured. Similar to the effects on endogenous PTEN, ΔNp63α clearly reduced the levels of exogenous PTEN (Figure 28B). Forced expression of PTEN, consistent with previous reports (Ahn et al., 2008), reduced the levels of endogenous NEDD4-1 impeding interpretation of the effects of ΔNp63α on NEDD4-1
mediated ubiquitination of PTEN from this experiment (Figure 28B). Nonetheless, these data demonstrate that ΔNp63α is able to decrease the amount of ubiquitinated PTEN.
**Figure 28: ΔNp63α decreases ubiquitination of PTEN.** (A) H1299 cells were transfected with HA tagged ubiquitin (HA-Ub) and either empty vector or ΔNp63α expression plasmids. Endogenous ubiquitinated PTEN was immunoprecipitated with an anti-HA antibody and detected by anti-PTEN antibodies by Western blot. The change in protein levels, relative to vector transfected cells, in the input lysate is listed below each band. (B) H1299 cells were transfected with the 6xhistidine tagged ubiquitin (His-Ub) and the indicated expression plasmids. Ubiquitinated proteins were isolated using Ni-NTA beads and ubiquitination of exogenous PTEN was detected as higher migrating smears by Western blot with an anti-PTEN antibody.
Figure 29: Exogenous ΔNp63α decreases NEDD4-1 in a dose-dependent manner.

H1299 cells were transfected with decreasing concentrations of ΔNp63α as indicated and whole cell extracts were subjected to immunoblot analysis for the indicated proteins. The change in NEDD4-1 protein levels, relative to vector transfected cells after normalization to β-actin, is listed above each band.
A) Primary MEFs

NEDD4-1

PTEN

β-actin

WT  p63-/-

1.0  7.45

1.0  1.63

B) Primary Keratinocytes

Change in Transcript levels

p63

NEDD4-1

ΔNp63α

NEDD4

β-actin

NSC  sip63-1  sip63-2

1.0  2.06  2.05
Figure 30: ΔNp63α represses NEDD4-1 in primary cells. (A) Whole cell extracts from wild-type and p63 null MEFs were subjected to immunoblot analysis for the indicated proteins. The changes in proteins levels, relative to wildtype, are plotted above each band. Note: p63 was undetectable at the protein level. (B) Primary adult human epidermal keratinocytes were transfected with NSC siRNA or siRNAs targeting all isoforms of p63. Total RNA was extracted and transcript levels of p63 and NEDD4-1 were analyzed by qRT-PCR. Y-axis represents the change in PTEN and p63 transcript levels relative to NSC transfected cells. Representative immunoblots of p63 and PTEN are shown in the bottom panels. The change in protein levels, relative to NSC transfected cells after normalization to β-actin, is listed above each band.
Figure 31: ΔNp63α represses NEDD4-1 in transformed cells. (A) A431 and (B) HaCaT cells were transfected with non-silencing control (NSC) siRNA or siRNAs targeting all isoforms of p63. Total RNA was extracted and transcript levels of p63 and NEDD4-1 were analyzed by qRT-PCR. Y-axis represents the change in transcript levels relative to NSC transfected cells. Representative immunoblots of p63 and NEDD4-1 are shown in the bottom panels. The change in protein levels relative to NSC transfected cells is listed above each band.
**Figure 32: Selective knockdown of ΔNp63 increases NEDD4-1 in transformed cells.**

(A) A431 and (B) HaCaT cells were transfected with non-silencing control (NSC) siRNA or siRNA directed against only the ΔNp63 isoforms. Total RNA was extracted and transcript levels of p63 and NEDD4-1 were analyzed by qRT-PCR. Y-axis represents the change in transcript levels relative to NSC transfected cells. Representative immunoblots of p63 and NEDD4-1 are shown in the bottom panels. The change in protein levels relative to NSC transfected cells is listed above each band.
To better ascertain if ΔNp63α is mediating ubiquitination of PTEN by regulating NEDD4-1, the expression of NEDD4-1 was measured after forced expression of ΔNp63α. Overexpression of ΔNp63α led to a dose-dependent decrease in NEDD4-1 expression (Figure 29). Moreover, NEDD4-1 protein levels were 7.45 times higher in p63−/− MEFs compared to wild type cells (Figure 30A). Because p63 null mice do not produce keratinocytes, the change in NEDD4-1 levels was measured after knockdown of p63 in primary adult human keratinocytes (Figure 30B). Knockdown of p63 with two different pan-p63 siRNAs lead to increased levels of NEDD4-1 transcript and protein levels as compared to control transfected cells (Figure 30B). The induction of NEDD4-1 after silencing p63 was shown not be dependent on transformation status or cell line, as knockdown of p63 also increased NEDD4-1 transcript and protein levels in HaCaT (Figure 31A) and A431 (Figure 31B) cells. The use of ΔNp63 specific siRNA further confirmed that ΔNp63α negatively regulates NEDD4-1 in both HaCaT (Figure 32A) and A431 cells (Figure 32B).

In addition to mediating the subcellular localization of PTEN, NEDD4-1 can also lead to poly-ubiquitin mediated proteosomal degradation of PTEN (Wang et al., 2007). To test whether knockdown of NEDD4-1 could synergize with silencing of ΔNp63α to further enhance PTEN expression, ΔNp63α and NEDD4-1 were simultaneously knocked down in HaCaT cells. Consistent with the ability to transcriptionally repress PTEN, knockdown of ΔNp63α led to increased levels of PTEN (Figure 33). Silencing of NEDD4-1, which can down regulate PTEN expression by poly-ubiquitin mediated proteosomal degradation, also led to increased levels of PTEN (Wang et al., 2007).
Concomitant knockdown of both ΔNp63α and NEDD4-1, however, did not further increase whole cell levels PTEN levels (Figure 33).

Since loss of NEDD4-1 did not synergize with knockdown of ΔNp63α to increase whole cell levels of PTEN, it was speculated that the relationship between ΔNp63α and NEDD4-1 was controlling the subcellular distribution of PTEN within keratinocytes instead. To test this possibility, the ability of NEDD4-1 to restore ubiquitination of PTEN after forced expression of ΔNp63α was next measured. Similar to the results in Figure 28B, forced expression of ΔNp63α dramatically reduced the levels of ubiquitinated PTEN (Figure 34). Co-expression of both NEDD4-1 and ΔNp63α attenuated the effects of ΔNp63α on ubiquitination of PTEN (Figure 34).

Although ΔNp63α appeared to reduce both mono- and poly-ubiquitinated PTEN as evidenced by the decrease in all molecular weights of ubiquitinated PTEN in Figures 28 and 34, poly-ubiquitination of PTEN by NEDD4-1 has been shown to be predominantly comprised of lysine 63 linked ubiquitin chains (Guo et al., 2012). Lysine 63 linked ubiquitin chains are correlated with both signal transduction and proteasome mediated degradation, unlike lysine 48 ubiquitin chains which are predominantly degradation signals (Pickart and Fushman, 2004; Thrower et al., 2000). To determine if ΔNp63α mediated repression of NEDD4-1, and the subsequent reduction in PTEN ubiquitination, could act as a signaling event to mediate nuclear levels of PTEN in keratinocytes, the levels of nuclear PTEN were measured after silencing both ΔNp63α and NEDD4-1 in HaCaT cells. Knockdown of p63 in HaCaT cells increased NEDD4-1 levels and also increased both nuclear and cytoplasmic PTEN (Figure 35). Conversely, loss of NEDD4-1 resulted in reduced nuclear PTEN levels, but increased cytoplasmic
levels of PTEN (Figure 35). Concomitant knockdown of ΔNp63α and NEDD4-1 attenuated the increase in nuclear PTEN as compared to knockdown of only ΔNp63α, suggesting that inhibition of NEDD4-1 by ΔNp63α is responsible for the suppression of nuclear PTEN (Figure 35). In epidermal tissue both ΔNp63α and NEDD4-1 are highly expressed in the basal layer (Figure 36). The localization of ΔNp63α and NEDD4-1 to the basal layer also correlates with the exclusion of PTEN from the nuclei of basal layer keratinocytes (Figure 25), demonstrating that the ability of ΔNp63α to inhibit NEDD4-1 mediated ubiquitination of PTEN inhibits the nuclear translocation of PTEN in vivo as well as in vitro. Altogether, these data strongly suggest that ΔNp63α is able to inhibit nuclear levels of PTEN by inhibiting the ubiquitination of PTEN by NEDD4-1 (Figure 37).
Figure 33: ΔNp63α and NEDD4-1 negatively regulate endogenous PTEN expression.

HaCaT cells were transfected with NSC or siRNA against p63 and/or NEDD4-1 and whole cell lysates were subject to immunoblot analysis for the indicated proteins. The change in protein levels, relative to NSC transfected cells after normalization to normalized β-actin, is listed below each band.
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**His Pulldown**

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**Input: PTEN**

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Figure 34: NEDD4-1 rescues ΔNp63α-mediated reductions in PTEN ubiquitination. H1299 cells were transfected with 6xhistidine tagged ubiquitin (His-Ub) and the indicated expression plasmids. Ubiquitinated proteins were isolated using Ni-NTA beads and ubiquitination of exogenous PTEN and ΔNp63α were detected as higher migrating smears by Western blot. The change in total protein levels as detected from the input lysate are listed below each band.
Figure 35: ΔNp63α and NEDD4-1 antagonistically regulate nuclear PTEN levels.

(A) HaCaT cells were transfected with NSC or siRNA against p63 and/or NEDD4-1 and subjected to subcellular fractionation one day post transfection. Immunoblot analysis of the indicated proteins is shown in the left panel. Nuclear protein levels were normalized to the intensity of PARP, while α-tubulin served as a loading control for normalization of cytoplasmic proteins. (B) The change in protein level from three independent experiments is plotted. Y-axis represents the change PTEN levels relative to NSC transfected cells after normalization to the loading control.
Figure 36: ΔNp63α and NEDD4-1 expression is high in the basal layer of epithelial tissues. Representative images showing the localization of p63 (green) and NEDD4-1 (red) in epidermal tissues from wild type E18.5 mouse embryos are shown in the top row. Representative images for nuclei (DAPI, blue), p63 (green), and PTEN (red) in epidermal tissues from wild type E18.5 mouse embryos are shown in the bottom row. Formalin fixed paraffin embedded whole-embryo mounts were donated by Dr. Satrijit Sinha, SUNY Buffalo.
**Figure 37: Model of ΔNp63α mediated regulation of nuclear PTEN.** ΔNp63α is able to suppress nuclear levels of PTEN by inhibiting the expression of E3 ligase NEDD4-1. The repression of NEDD4-1 by ΔNp63α subsequently decreases the mono-ubiquitination of PTEN, thereby preventing nuclear import of PTEN.
H. ΔNp63α influences the localization of PTEN to novel subcellular compartments.

PTEN has many functions dependent on its location within cells. Cytoplasmic PTEN is most notably associated with Akt inhibition, but is also important in suppressing invasion and migration as well as contributing to proper cell polarization through dephosphorylation of PIP₃ (Davidson et al., 2010; Langlois et al., 2010). Nuclear PTEN is important for inducing cell cycle arrest and maintaining chromosome stability; these processes may, at least in part, be independent of Akt inhibition (Liu et al., 2005b; Shen et al., 2007). Interestingly, PTEN -/- mouse embryonic fibroblasts (MEFs) escape taxol-mediated mitotic arrest more quickly than wild type cells, suggesting that PTEN is important for a mitotic spindle checkpoint (Gupta et al., 2009). PTEN has also been implicated in the induction of genes associated with microtubule formation and/or stabilization (Gupta et al., 2009; Tibarewal et al., 2012). To this end, the potential of a mitosis-specific role for PTEN was investigated.

Examination of mitotic cells after knockdown of p63 in HaCaT cells demonstrated an increase in PTEN fluorescence at discrete foci that were reminiscent of centrosomes (Figure 38A, quantitated in 38B). These PTEN “foci” were confirmed to be mitotic centrosomes by co-localization the centrosome marker γ-tubulin (Figure 39). Moreover, PTEN was only observed to localize at centrosomes during mitosis; there was little to no co-localization of PTEN with γ-tubulin during G1 or G2 in either HaCaT cells (Figure 39) or H1299 cells (Figure 40). Immunofluorescence labeling of PTEN at the centrosomes is specific, as no signal was detected in PTEN-null PC3 cells (Figure 41, top row). Co-localization of PTEN and γ-tubulin was detected in multiple cell lines, further demonstrating that localization of PTEN to mitotic centrosomes is not cell line specific.
Figure 38: Loss of ΔNp63α increases centrosomal PTEN. (A) HaCaT cells were transfected with non-silencing control (NSC) or p63 specific siRNA then stained for PTEN (green), p63α (red) and DNA (blue) 48 hr later. Arrowheads point to the centrosome enlarged within the inset box. Bars = 5 μm. (B) The mean fluorescent intensities of p63 and centrosomal PTEN from (A) are plotted. Error bars represent s.e.m. from the mean, >25 centrosomes measured. *=p values ≤0.004.
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Figure 39: PTEN localizes to centrosomes during all stages of mitosis in HaCaT cells. Actively growing asynchronous HaCaT cells were fixed and stained for PTEN (green), γ-tubulin (red) and DNA (DAPI, blue). Representative cells from each stage of the cell cycle are shown in each row. Arrowheads point to the centrosome enlarged within the inset box while full arrows point to the remaining centrosome. Bars = 5 μm.
Figure 40: PTEN localizes to centrosomes during all stages of mitosis in H1299 cells. Actively growing asynchronous H1299 cells were fixed and stained for PTEN (green), γ-tubulin (red) and DNA (DAPI, blue). Representative cells from each stage of the cell cycle are shown in each row. Arrowheads point to the centrosome enlarged within the inset box while full arrows point to the remaining centrosome. Bars = 5 μm.
Figure 41: Centrosomal PTEN is not cell line specific. PTEN null PC3 cells and PTEN positive HaCaT, A431 and H1299 cells were stained for PTEN (green) and γ-tubulin (red). The PTEN +/+ cell lines show varying amounts of co-localization (observed as yellow in the last column) with γ-tubulin. Arrowheads indicate the centrosome enlarged within the inset box while full arrows indicate the remaining centrosome. Bars = 5 μm.
The localization of PTEN to mitotic centrosomes was also detected with a second PTEN-specific antibody (Figure 42), excluding the possibility that staining of PTEN at mitotic centrosomes was an artifact.

Centrosomes are critical to proper spindle assembly and chromosome separation during mitosis. To determine if PTEN was involved in this process, the expression of PTEN at mitotic centrosomes in primary keratinocytes was first examined. Primary neonatal human epidermal keratinocytes (NHEKs) where chosen for investigation into the function of centrosomal PTEN as transformed cancer cell lines can be prone to genomic instability and would thus confound the interpretation of how PTEN influences centrosome and/or chromosome integrity. Similar to HaCaT and H1299 cells, little to no centrosomal labeling of PTEN was observed during interphase in NHEKs (Figure 43). Clear localization of PTEN to centrosomes during mitosis was observed by co-staining with centrosome markers γ-tubulin, pericentrin (PCNT) and polo-like kinase 1 (PLK-1) (Figure 43). PTEN is located in the pericentrosomal matrix (PCM), as shown by co-localization with PLK-1, PCNT, and γ-tubulin (Figure 43). Ninein, a protein associated with centrosomes predominantly during interphase (Chen et al., 2003), showed no co-localization with PTEN during interphase and minimal co-localization in the few mitotic cells with detectable centrosomal ninein (Figure 43, last column). The intensity of PTEN at the centrosomes was most evident during prophase and metaphase (Figure 44), which coincides with the time frame for centrosome maturation (Palazzo et al., 2000). The relative intensity of PTEN at the PCM during mitosis was more similar to PLK-1, another transiently associated PCM protein, confirming the mitosis specific centrosomal localization of PTEN (Figure 45).
Figure 42: Centrosome PTEN is not a staining artifact. H1299 cells were stained for PTEN with either Cascade Bioscience PTEN 6H2.1 (cb PTEN) or Cell Signaling rabbit monoclonal anti-PTEN (#9559, cs PTEN). Both PTEN images were taken at the same exposure time (500 ms). Nuclei were counter stained with DAPI. Arrows point to centrosomes. Bars = 5 μm.
Figure 43: PTEN co-localizes with numerous pericentrosomal matrix proteins 
during mitosis. Actively growing, asynchronous, neonatal primary epidermal 
kernatinocytes (NHEK) were fixed and stained for the indicated proteins. Representative 
cells from each stage of the cell cycle as determined by chromatin condensation and 
centrosome number are shown in each row. Arrowheads point to the centrosome enlarged 
within the inset box while full arrows point to the remaining centrosome. Bars = 5 μm.
Figure 44: Quantitation of centrosomal PTEN during mitosis. Mean fluorescence intensity (arbitrary units) of PTEN, PLK-1, γ-Tubulin and pericentrin (PCNT) during each stage of mitosis from NHEK. Error bars represent s.e.m from three independent experiments. At least 25 centrosomes per experiment were measured.
I. PTEN regulates centrosome composition in both an Akt-dependent and independent manner.

To determine the role of PTEN at mitotic centrosomes, the effects of PTEN knockdown on PCM associated proteins were examined. Immmoblot analyses of whole cell extracts after knockdown of PTEN in NHEKs revealed modest, but statistically significant increases in both PLK-1 and γ-tubulin (Figure 45). Pericentrin could not be consistently detected by immunoblot analysis, in part due to its large size (~330 kDa) affecting protein transfer, and was thus only measured during mitosis by immunofluorescence. Contrary to the effects on whole cell levels, knockdown of PTEN did not alter PLK-1 or γ-tubulin levels at centrosomes (Figure 46A, quantitated in 46B), but lead to a 29% reduction in centrosomal PCNT levels (Figure 46B). The difference between immunoblot and immunofluorescence results may be due to the fact that the immunofluorescence measurements were taken of only mitotic centrosomes without considering changes in whole cell immunofluorescence, while the Western blot analyses in Figures 45A measure total protein levels (at all subcellular compartments) and at all stages of the cell cycle. Furthermore, global changes may not always mirror the changes occurring at a specific cellular compartment. This is often important for driving processes forward while also limiting the biochemical changes of the process to a particular subcellular location.

Induction of PLK-1 upon PTEN loss may occur via a phosphatase independent mechanism as has been reported previously in prostate cancer cells (Song et al., 2011). To determine if PLK-1 and γ-tubulin were similarly regulated in normal keratinocytes by an Akt-independent mechanism, PTEN was silenced followed by treatment with the Akt
inhibitor MK2206. Although MK2206 alone had little effect on whole cell levels of either PLK-1 or γ-tubulin (Figure 47A), inhibition of Akt activity by MK2206 completely blocked the increases in both PLK-1 and γ-tubulin caused by silencing PTEN (quantitated in Figure 47B).

Intriguingly, inhibition of Akt with MK2206 reduced levels of PTEN, PLK-1 and γ-tubulin at mitotic centrosomes (representative images in Figure 48, quantitated in Figure 49). MK2206 had little effect on levels of PCNT at mitotic centrosomes (Figure 50). Furthermore, inhibition of Akt with MK2206 could not rescue centrosomal levels of PCNT after knockdown of PTEN, indicating that PTEN is regulates PCNT through an Akt-independent manner (Figure 49). Altogether, these studies indicate that both PTEN and Akt are critical for proper association of PCNT, PLK-1 and γ-tubulin with mitotic centrosomes. Furthermore, the respective roles of PTEN and Akt on regulating centrosome composition are not entirely redundant.
Figure 45: PTEN loss increases whole cell levels of γ-tubulin and PLK-1. PTEN was silenced in NHEKs followed by immunoblot analysis for the indicated proteins. Quantification of the change in proteins levels is graphed in the right panel. Error bars represent standard deviation from three separate experiments.
A) DNA/PTEN/γTub.  DNA/PTEN/PLK-1  DNA/PTEN/PCNT

NSC  siPTEN  NSC  siPTEN  NSC  siPTEN

B) Percent of NSC Intensity at Centrosomes

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**Figure 46: PTEN differentially affects centrosome proteins.** (A) PTEN was silenced in NHEKs then stained for the indicate proteins by immunofluorescence. (B) The mean fluorescent intensities of each protein at mitotic centrosomes, relative to control transfected cells, are plotted. Error bars represent standard error of the mean from three separate experiments, at least 25 centrosomes measured for each protein per condition per experiment. *=p values ≤ 0.05; # = p values ≤ 0.009. Arrowheads point to the centrosome enlarged bottom panels; Bars = 5 μm.
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Percent of NSC Levels

- * indicates statistically significant difference.
Figure 47: PTEN influences whole cell levels of γ-tubulin and PLK-1 in an Akt dependent manner. (A) NHEKs were transfected with non-silencing control (NSC) or PTEN specific siRNA followed by 10 μM MK2206 or DMSO control. Immunoblot analysis for the indicated proteins was performed. (B) The change in total protein levels is plotted. Error bars represent standard deviation from at least three separate experiments.
Figure 48: Akt activity regulates centrosome composition. (A) Immunofluorescence analysis for the indicated proteins was performed on NHEKs transfected with non-silencing control (NSC) or PTEN specific siRNA followed by 10 μM MK2206 or DMSO control. Arrowheads point to the centrosome enlarged bottom panels; Bars = 5 μm.
Figure 49: Quantitation of centrosome proteins after inhibition of Akt activity. Immunofluorescence analysis for the indicated proteins was performed on NHEKs transfected with non-silencing control (NSC) or PTEN specific siRNA followed by 10 μM MK2206 or DMSO treatment. The mean fluorescent intensities of each protein at mitotic centrosomes, relative to NSC/DMSO treated cells, are plotted on the right. Error bars represent standard error of the mean from three separate experiments, at least 25 centrosomes measured for each protein per condition per experiment. * = p values ≤ 0.05; # = p values ≤ 0.009.
J. Both PTEN expression and Akt activity are required to prevent centrosome defects.

In addition to reducing the recruitment of PCM proteins to mitotic centrosomes, inhibition of Akt signaling with MK2206 drastically increased the frequency of centrosome defects during metaphase (Figure 50). Concomitant knockdown of PTEN and MK2206 treatment reduced the frequency of metaphase cells with centrosome defects as compared to MK2206 treatment alone, but was still significantly higher than in cells treated with non-silencing control and DMSO (Figure 50). MK2206 treatment and PTEN knockdown did not drastically alter the frequency of prophase cells with centrosome defects compared to control treated cells (Figure 50). This may be the result of combined PTEN knockdown and MK2206 treatment allowing cells to progress through prophase without resolving defects, as it has been reported earlier that PTEN null have a deficient mitotic checkpoint (Gupta et al., 2009). No clear effects on centrosome number or structure were observed in anaphase cells (data not shown). The most common defects observed were fragmented centrosomes and centrosome amplification, as determined by staining for constitutive centrosome components PCNT and γ-tubulin (Figure 51). The combination of PTEN knockdown and MK2206 treatment increased the frequency of fragmented centrosomes (Figure 51A). Consistent with previous reports, loss of PTEN increased the frequency of centrosome amplification (Li et al., 2009; Nam et al., 2010). Inhibition of Akt with MK2206 after knockdown of PTEN reduced the number of cells with supernumerary centrosomes, confirming that centrosome amplification is caused by increased Akt activity rather than PTEN loss (Figure 51B).
It is important to note that even though loss of PTEN and MK2206 treatment increased the number of centrosome defects, no overt chromosomal abnormalities were observed in NHEK cells during our short term study. Because these studies were conducted in primary cells it indicates that disruption of the PTEN/Akt pathway is sufficient to cause centrosome defects but that additional mutations may be required before the effects on chromosome stability are detectable. Consistent with this idea, knockdown of PTEN alone increased the prevalence of centrosomal and chromosomal defects in H1299 cells, a non-small cell lung carcinoma cell line (Figure 52). The most common of these defects were centrosome amplification, broken chromosomes, failure of chromosomes to align at the metaphase plate, and anaphase bridges (examples depicted in Figure 52B). These defects likely contribute to the chromosome instability and aneuploidy that has been previously associated with PTEN loss (Gupta et al., 2009; Levine et al., 1998; Puc et al., 2005). Furthermore, the induction of mitotic defects in the absence of PTEN highlights the need for continued PTEN expression even after transformation in order to conduct mitosis in an organized fashion.
Figure 50: Concomitant loss of Akt and PTEN reduces the frequency of centrosomal defects observed when only Akt is inhibited. Immunofluorescence analysis for the indicated proteins was performed on NHEKs after treatment NSC or PTEN specific siRNA followed by 10 μM MK2206 or DMSO control. The number of centrosomal defects in each stage of mitosis was scored. Error bars represent standard deviation from at least five separate experiments: *p values ≤ 0.05. No defects were observed NSC, DMSO treated cells in metaphase.
**Figure 51: Akt activity and PTEN are necessary to prevent centrosome defects.**

Representative images of (A) fragmented and (B) supernumerary centrosome defects. Arrowheads point to the centrosome enlarged within the inset box while full arrows point to the remaining centrosome. Bars = 5 μm. The frequency of each type of defect is graphed in the lower panels. Error bars represent standard deviation from at least five separate experiments: *=p values ≤ 0.05.
Figure 52: Silencing only PTEN is sufficient to induce mitotic defects in H1299 cells.

(A) Asynchronously growing H1299 cells were stained for PTEN, γ-tubulin and DNA. The number of chromosomal and centrosomal defects scored per 100 mitotic cells per experiment is graphed. Error bars represent s.e.m from three separate experiments. *=p values ≤0.05. (B) Representative images from cells scored in (A) showing supernumerary centrosomes and broken chromosomes; arrows point to centrosomes or misaligned chromosomes, respectively. Arrowheads point to the centrosome enlarged within the inset box while full arrows point to the remaining centrosome. Bars = 5 µm.
IV: Discussion

A. Significance of transcriptional regulation of PTEN by ΔNp63α.

At an estimated 3.5 million new cases each year, non-melanoma skin cancers (NMSCs) are the most common type of cancer in the United States, comprising more new cases each year than all other cancers combined (Rogers et al., 2010). The increase in NMSC frequency has reached epidemic proportions with some states showing a nearly 350% increase in the occurrence of SCC from 1979 to 1999 (Karagas et al., 1999). By understanding the molecular and cellular etiology of NMSC, better treatment and prevention modalities can be designed. Elucidating the critical genetic paradigms associated with keratinocyte proliferation, and the de-regulation of these processes, is essential to understanding of NMSC development. Normal epidermal development is dependent upon the expression of the transcription factor p63 (Mills et al., 1999; Yang et al., 1998; Yang et al., 1999). In particular, the ΔNp63α isoform helps maintain the proliferative potential of keratinocytes. Amplification of ΔNp63α is also frequently observed in SCC, making it an excellent candidate for understanding the etiology of NMSC (Bircan et al., 2006; Di Como et al., 2002; Sakiz et al., 2009).

Due to the difficulty in dissecting the molecular events involved in epidermal proliferation in whole tissues, many studies often employ keratinocytes culture systems. ΔNp63α was shown to be the predominant p63 isoform in cultured primary keratinocytes, the spontaneously transformed HaCaT cell line and the atypical squamous cell carcinoma
A431 cell line (Figure 6). In all three cell lines, knockdown of ΔNp63α led to increased transcript and proteins levels of PTEN (Figures 7-8), indicating that PTEN is negatively regulated by p63 in keratinocytes.

ΔNp63α can function in a dominant negative manner towards p53 and the pro-apoptotic TAp63 and TAp73 isoforms, further demonstrating the pro-proliferative nature of ΔNp63α and implicating it as a possible oncogene (Yang et al., 1998). The exponentially higher transcript levels of ΔNp63 compared to TAp63 in normal and cancerous keratinocytes (Figure 6), as well as the inability to detect any protein levels of TAp63 make it unlikely that the TAp63 isoforms exert much control over PTEN in keratinocytes. Furthermore, siRNAs targeting all p63 isoforms (Figure 7) resulted in similar inductions of PTEN to those observed with ΔNp63 specific siRNA (Figure 8), indicating that TAp63 is not a positive regulator of PTEN in adult keratinocytes. This is consistent with previous studies demonstrating that overexpression of TAp63α in keratinocytes led to activation of Akt, a common functional readout of decreased PTEN levels (Ogawa et al., 2007). Together, this suggests that p63 inhibits PTEN independently of any repressive effects of the ΔNp63 isoforms toward the TAp63 isoforms.

Head and neck squamous cell carcinomas are reliant on high levels of ΔNp63α for survival through repression of p73-mediated apoptosis (Rocco et al., 2006). PTEN has previously been shown to be a direct transcriptional target of both p53 and TAp73 (Stambolic et al., 2001; Vella et al., 2009), making it possible that ΔNp63α inhibited PTEN expression by impairing the ability of p53 and/or p73 to induce PTEN. In the HaCaT and A431 keratinocytes lines, however, the anti-apoptotic ΔNp73 isoforms are the predominant p73 species (Figure 10). Isoform specific knockdown of TAp73 did not
significantly alter PTEN levels (Figure 11B), suggesting that regulation of PTEN by the different p53 family members may be tissue specific. Furthermore, p63 did not bind to Region D of the *pten* promoter (Figure 12B), which corresponds to the region of the *pten* promoter previously shown to be bound by p73 in thyroid cells, suggesting that p63 and p73 do not compete for same responsive elements (Vella et al., 2009). The studies in Figures 10 to 12 suggest that, in at least HaCaT and A431 cells, TAp73 plays little role in the transcriptional regulation of PTEN in the absence of genotoxic stress. More studies, however, would need to be conducted to determine if the transcriptional repression of PTEN by ΔNp63α contributes to the ΔNp63α-mediated resistance of SCC cells to genotoxic agents like cisplatin (Rocco et al., 2006; Sen et al., 2011).

Arguing against the possibility that ΔNp63α induced PTEN by acting in a dominant negative manner toward p53 was the observation of similar increases in PTEN after silencing ΔNp63α in A431, HaCaT and primary human epidermal keratinocytes (HEKs), since these cell lines all harbor different forms of p53 (Figure 7). HEKs have wild type p53, while A431 and HaCaT cells harbor mutant p53, R273H and H179Y/R282W, respectively. Interestingly, it has been shown that mutant p53 can co-immunoprecipitate with ΔNp63α from HaCaT cells (Gaiddon et al., 2001; Strano et al., 2002). The interaction between mutant p53 and ΔNp63α may explain why many of the same regions of the *pten* promoter to which p63 bound were also occupied by p53 (Figure 13). However, luciferase assays showed repression of PTEN-Luc activity upon overexpression of ΔNp63α in the absence of any p53, mutant or wild type, demonstrating that p53 is not required for ΔNp63α mediated repression of PTEN transcription (Figure 14A). Knockdown of ΔNp63α in p53 mutant HaCaT cells also alleviated the repression
on the activity of the PTEN-Luc construct, suggesting that even if mutant p53 bound to ΔNp63α it does not impede the ability of ΔNp63α to transcriptionally repress PTEN (Figure 14B). This may be due to fact that keratinocytes express very high levels of ΔNp63α and studies have shown that mutant p53 only bound ~10% of the total p63 expressed, leaving a significant portion of p63 to control transcription of its target genes unimpeded (Gaiddon et al., 2001). Altogether, these studies suggest that ΔNp63α acts as a master regulator, independent of p53 status, to repress PTEN in keratinocytes. To truly confirm the ability of ΔNp63α to transcriptionally repress PTEN independently of p53, one would need to examine the occupancy of ΔNp63α at the pten promoter by ChIP analysis after knockdown of p53 in cell lines with wildtype and mutant p53 in addition to measuring the effect of concomitant loss of p53 and ΔNp63α on transcription rates of PTEN.

Interestingly, some studies have shown a paradoxical tumor-promoting function for PTEN in cells expressing gain-of-function p53 mutations (Li et al., 2008). PTEN stabilizes p53 in phosphatase-independent and phosphatase-dependent manners; protecting p53 from Mdm2-mediated ubiquitination through inhibition of Akt signaling and through direct physical interaction (Freeman et al., 2003; Mayo et al., 2002). By binding to mutant p53, PTEN protects mutant p53 from Mdm2-mediated destruction thereby enhancing tumor growth in xenograft studies (Li et al., 2008). Since mutation of p53 is frequently observed in NMSC (Benjamin et al., 2008), it would be interesting to examine if the protection of mutant p53 by PTEN could synergize with ΔNp63α-mediated transcriptional repression of PTEN to enhance the development of NMSC.
While mutations in p53 are found in 50% of all human cancers, p63 is rarely mutated in cancers (Hagiwara et al., 1999). Rather, ΔNp63α is overexpressed in multiple types of SCC and BCC, typically through amplification of the p63 locus (Bircan et al., 2006; Choi et al., 2002; Di Como et al., 2002; Hibi et al., 2000; Hu et al., 2002; Park et al., 2000; Parsa et al., 1999; Sakiz et al., 2009). Overexpression of ΔNp63α in H1299 cells, mimicking the amplification of ΔNp63α observed in NMSC, led to phosphorylation and activation of Akt by repressing PTEN (Figure 16). The repression of PTEN by ΔNp63α was found to be critical for ΔNp63α-mediated induction of Akt phosphorylation since overexpression of ΔNp63α was unable to induce Akt phosphorylation in PTEN null cells (Figure 16B). This is in contrast to a recent study by Sen et al. suggesting that Akt1 is a direct transcriptional target of ΔNp63α (Sen et al., 2011). However, no changes in total Akt levels were observed upon overexpression of ΔNp63α (Figure 16) or knockdown of p63 (Figures 18), arguing against a direct transcriptional effect of ΔNp63α on Akt. Any changes that may have occurred in Akt1 in response to modulation of ΔNp63α levels in Figures 16-18 may have been eclipsed by using a pan-Akt antibody (capable of detecting Akt1, Akt2 and Akt3) rather than the Akt1 specific antibody used by Sen et al. (Sen et al., 2011). The dissimilarity between these two studies could further be due to the difference in cell lines used; the studies shown in this dissertation primarily utilized cell lines from a cutaneous origin while Sen et al. used ovarian cancer and head and neck SCC cell lines (Sen et al., 2011). While the studies presented in this dissertation support the indirect activation of Akt by ΔNp63α through transcriptional repression of PTEN, they do not entirely exclude the possibility that ΔNp63α may also induce Akt through direct transcriptional means.
In addition to ΔNp63α mediated activation of Akt, Akt can also increase ΔNp63α, potentially accentuating ΔNp63α-mediated repression of PTEN (Barbieri et al., 2003; Segrelles et al., 2006). However, while artificial proliferative signals in the form of ΔNp63α and CA-Akt over expression did not lead to a synergistic or additive decrease in PTEN expression, it did result in a larger decrease in PTEN expression compared to CA-Akt alone (Figure 17). Activation of Akt can initiate the ARF/Egr-1 axis that leads to induction of PTEN (Yu et al., 2009), potentially explaining why CA-Akt was not as potent at repressing PTEN as ΔNp63α. The potent ability of ΔNp63α to reduce PTEN expression suggests that ΔNp63α amplification, even in the absence of constitutive activation of the PI3K/Akt pathway maybe be enough to tip the balance toward hyperplasia and cancer development. This is supported by studies demonstrating that cutaneous SCC is dissimilar from other forms of SCC in that cutaneous SCC rarely exhibits constitutively activating mutations in PI3K or Akt (Hafner et al., 2010).

By inhibiting the activation of Akt, PTEN can also antagonize ΔNp63α levels as shown in Figure 19. Altogether, this creates a feedback loop between ΔNp63α/PTEN/Akt that must be finely balanced to prevent an overabundance of proliferative signals as diagramed in Figure 53. Loss of PTEN significantly increases the normal proliferation rates of keratinocyte cell lines which can be restored by concomitant knockdown of ΔNp63α (Figure 20-21). This is consistent with previous reports demonstrating that minute reductions in PTEN expression are sufficient to drive tumorigenesis and further highlight the delicate balance between ΔNp63α/PTEN/Akt in controlling cell proliferation (Alimonti et al., 2010; Trotman et al., 2003). Because of the myriad of genes and pathways regulated by ΔNp63α and Akt, the simplified model depicted in Figure 53
represents only a fraction of events required for keratinocyte proliferation. More studies will need to be conducted in order to determine what other pathways may contribute to how PTEN balances the activation of Akt and the subsequent stabilization of ΔNp63α. A potential pathway for future studies on keratinocytes proliferation would be the induction of PTEN by the Egr-1/Arf axis, considering that this axis can be triggered by Akt (Yu et al., 2009).

Interestingly, the atypical squamous cell line A431 displayed the same changes in growth rates in response to silencing ΔNp63α and/or PTEN as HaCaT cells, suggesting that the ΔNp63α/PTEN axis is conserved in both atypical squamous cell carcinomas and non-tumorigenic cells (Figure 20). A431 cells, however, were less susceptible to changes in growth rates after silencing ΔNp63α and/or PTEN, when measured by MTS assay (Figure 21A). This may be due to the high degree of epidermal growth factor receptor amplification in the cells which could impact the metabolism. The indirect measurement of metabolizing cells in MTS assays, rather than directly measuring the increase in cell number over time as with colony formation and trypan blue assay, could then explain the inconsistencies between HaCaT and A431 cells in Figure 21A. Despite the discrepancies between methodologies, the results clearly show that a tight balance between ΔNp63α and PTEN expression must be met in order to maintain normal proliferation rates in keratinocytes.

The importance of the balance between ΔNp63α and PTEN is further highlighted in squamous and basal cell carcinomas, the two most common cancers, where the ratio of p63 to PTEN is significantly perturbed (Figure 23). The amplification of ΔNp63α and the subsequent imbalance between ΔNp63α and PTEN in both basal cell carcinoma and
squamous cell carcinoma suggests that regulation of PTEN in keratinocytes relies primarily on ΔNp63α to control proliferation. A similar statistically significant increase in the ratio of p63 to PTEN was observed in samples of HPV induced condyloma acuminata (Figure 23B). There is strong evidence showing that HPV is involved in the development of some forms of skin cancer, in addition to its known role in cervical cancer (Faridi et al., 2011; Harwood et al., 2004; Nindl et al., 2007). Together, this study demonstrates that ΔNp63α maintains normal cell proliferation by negatively regulating basal PTEN levels to cause Akt activation and that disruption of this balance is an early insult that contributes to non-melanoma skin cancer.

The studies presented in this dissertation suggest that the repression of PTEN by ΔNp63α may be involved in the development of NMSC, however, tumor formation studies in animal models and would need to be conducted to confirm this possibility. For instance, one could generate a mouse model wherein ΔNp63α was overexpressed in the epidermis while PTEN levels were reduced and measure the development of NMSC by two-step chemical carcinogenesis or chronic UVB irradiation. This could be accomplished by crossing the K5-tTA/pTRE-ΔNp63α (K5-ΔNp63α) mouse model developed by Satrijit Sinha (Romano et al., 2010), wherein ΔNp63α is overexpressed in the skin via a keratin 5 promoter, with the skin-specific PTEN knockout mouse (k5-PTEN+/floox) created in the laboratory of Tak Mak (Suzuki et al., 2003). If the model proposed in Figure 53 is correct, the overexpression of ΔNp63α would allow mice heterozygous for PTEN to initially develop more tumors than PTEN+/+ mice, but overtime the enhanced repression of PTEN by ΔNp63α would cause K5-ΔNp63α; PTEN+/+ mice to phenocopy k5-PTEN+/floox mice. Furthermore, in the absence of
doxycycline K5-tTA/pTRE-ΔNp63α bi-transgenic animals express ΔNp63α, allowing for temporal control the ΔNp63α transgene to better address the role of ΔNp63α-mediated repression of PTEN during tumor development and progression as alluded to in Figure 23.
Figure 53: The ΔNp63α, PTEN and Akt feedback loop controls keratinocyte proliferation. ΔNp63α transcriptionally represses PTEN, leading to increased Akt phosphorylation and activation. Active Akt also increases the levels of ΔNp63α; both proteins enhance cell survival and proliferation. PTEN inhibits activation of Akt, and thereby ΔNp63α, aiding in growth suppression.
B. Significance of ΔNp63α-mediated regulation of PTEN subcellular distribution.

In addition to regulation at the transcriptional level, PTEN is also subject to post-translational modifications and changes in subcellular distribution. In the nucleus PTEN has many functions that may be independent of those associated with its cytoplasmic localization such as cell cycle arrest (Chung and Eng, 2005; Ginn-Pease and Eng, 2003), protection of p53 and p73 from degradation (Lehman et al., 2011; Mayo et al., 2002) and maintaining chromosome stability (Gupta et al., 2009; Shen et al., 2007). Since nuclear PTEN is most frequently observed in G1 arrested or quiescent cells, ΔNp63α may be able to stimulate keratinocyte proliferation through transcriptional repression of PTEN (discussed above) as well as by inhibiting the nuclear translocation of PTEN (Figure 26) (Chung and Eng, 2005; Ginn-Pease and Eng, 2003; Jacob et al., 2009; Whiteman et al., 2002). The ability of ΔNp63α to support keratinocyte proliferation by controlling PTEN localization is supported by the absence of PTEN from the nuclei of basal layer keratinocytes, which are the proliferative cells of the epidermis, while PTEN is abundant in the nuclei of non-proliferating, differentiated cells of the upper epidermis (Figure 25). The dramatic lack of nuclear PTEN in both BCC and SCC (Figure 27), presumably due in part from amplification of ΔNp63α, might prevent cells from undergoing a proper cell cycle arrest or differentiation program and thus allow for cancer development. In melanoma the loss of nuclear PTEN is associated with cancer progression and could be used as a prognostic marker (Whiteman et al., 2002). Given that little nuclear PTEN is observed in basal layer keratinocytes of normal skin (Figure 25), it is unlikely that the lack of nuclear PTEN seen in BCC and SCC could be used as a prognostic marker as with melanoma (Figure 24).
In order to determine if the repression of nuclear PTEN by ΔNp63α was contributing to enhanced proliferation more experiments would need to be conducted. For example, if the lack of nuclear was allowing keratinocytes to proliferate one would expect to observe decreased proliferation, potentially measured by MTS assays or colony forming ability, after transfection of PTEN harboring a nuclear localization signal to force expression of PTEN in the nucleus. Furthermore, expression of a PTEN construct that was locked in the cytoplasm, either through introduction of a nuclear exclusion signal or mutation of lysine 289 to prevent import of PTEN into the nucleus, should rescue the effects on keratinocyte proliferation after silencing ΔNp63α.

Modulation of ΔNp63α expression was able to affect nuclear PTEN in both HaCaT and H1299 cells, albeit to varying degrees, suggesting that the ability of ΔNp63α to repress nuclear PTEN is not restricted to keratinocytes (Figure 26). Knockdown of ΔNp63α in HaCaT cells was clearly able to increase nuclear levels of PTEN (Figure 26A). H1299 cells already had a low nuclear to cytoplasmic ratio of PTEN, and thus the reduction in nuclear PTEN caused by over expression of ΔNp63α was less pronounced than the changes observed in HaCaT cells (Figure 26B). Despite the discrepancies in the magnitude of change between the cell lines, high levels of ΔNp63α in either cell line correlated with low levels of nuclear PTEN and subsequent increases in nuclear phospho-Akt levels. The inverse relationship between nuclear PTEN and nuclear phosphorylated Akt supports the recent study by Dr. Charis Eng’s laboratory showing that nuclear PTEN is necessary and sufficient for inhibiting nuclear Akt (He et al., 2012). More studies, however, should also be conducted to determine if the Akt-independent functions of PTEN, such the binding of CENP-C or the APC/Cdh1 mediated degradation of PLK-1
are also affected by modulation of ΔNp63α. This could be accomplished by studying the effects of concomitant overexpression of ΔNp63α and a phosphatase–dead PTEN mutant, such as C124S PTEN, on chromosome stability and cell cycle arrest.

ΔNp63α was found to inhibit nuclear levels of PTEN by inhibiting the ubiquitination of PTEN by NEDD4-1 as diagramed in Figure 37. By negatively regulating the expression of NEDD4-1 (Figures 29-32), p63 was able to indirectly control the ubiquitination (Figure 27 and 34), and thus the localization, of PTEN (Figures 35). While exogenous NEDD4-1 did not increase ubiquitination of PTEN over baseline levels in H1299 cells, it sufficiently blocked ΔNp63α-mediated reductions in PTEN ubiquitination (Figure 34). The increases in whole cell levels of PTEN (Figure 33) and the reduction in nuclear PTEN (Figure 36) caused by knockdown of NEDD4-1 in HaCaT cells would suggest that NEDD4-1 is in fact ubiquitinating PTEN. The inability to detect the ubiquitination of PTEN by exogenous NEDD4-1 could be the result of the overexpression system used, perhaps though insufficient supply of NEDD4-1 co-factors, and further ubiquitination studies should be conducted in keratinocytes after knockdown of endogenous NEDD4-1 to confirm this possibility. ΔNp63α could also regulate the ubiquitination of PTEN by competing for binding to NEDD4-1. This is supported by previous studies showing that ΔNp63α is also ubiquitinated by NEDD4-1 leading to its destabilization (Bakkers et al., 2005).

Despite the strong circumstantial evidence pointing to the regulation of PTEN ubiquitination by ΔNp63α to be via inhibition of NEDD4-1, ΔNp63α may also inhibit the ubiquitination of PTEN through additional mechanisms. Microarray analyses conducted
previously in the laboratory identified several genes potentially regulated by ΔNp63α that could theoretically alter the ubiquitination of PTEN. The microarray studies, performed after knockdown of p63, demonstrated that p63 may positively regulate the expression of ubiquitin specific protease 31 (USP31). USP31 catalyzes the removal of ubiquitin from proteins (Lockhart et al., 2004), suggesting that ΔNp63α could theoretically induce the de-ubiquitination of PTEN via USP31 rather than inhibit the addition of ubiquitin to PTEN. ΔNp63α also potentially regulates the expression of NEDD4 binding partner 1 (N4BP1). As the name suggests N4BP1 physically interacts with NEDD4-1 thereby preventing the binding and ubiquitination of other target proteins. N4BP1 has also been shown to bind to other members of the NEDD4 family of ubiquitin ligases, protecting p63 and p73 from ITCH mediated degradation (Oberst et al., 2007). While only a mild decrease in N4BP1 abundance was observed in one of the four p63-knockdown conditions used in the microarray study, the potential positive regulation of N4BP1 by ΔNp63α could indirectly inhibit ubiquitination of PTEN by competing with PTEN for a number of ubiquitin ligases. The ability of ΔNp63α to regulate N4BP1 or USP31 would need to be confirmed first, before attempting to determine if either protein was responsible for the reduction in ubiquitination of PTEN caused by ΔNp63α.

While ubiquitination of PTEN could not be confirmed with exogenous NEDD4-1 in H1299 cells, in HaCaT cells endogenous NEDD4-1 clearly inhibited PTEN expression (Figure 33). Moreover, ΔNp63α was shown to negatively regulate the expression of NEDD4-1 in multiple keratinocyte cell lines (Figures 30-31). The inhibition of NEDD4-1 by ΔNp63α likely serves more as a signaling mechanism than as a means to stabilize PTEN protein. This is supported by several pieces of data. First, overexpression of
ΔNp63α led to dose-dependent decreases in PTEN protein (Figure 16), which would not be expected if ΔNp63α was also indirectly stabilizing PTEN via repression of NEDD4-1. Secondly, poly-ubiquitination of PTEN by NEDD4-1 has been shown to be predominantly comprised of lysine 63 linked ubiquitin chains (Guo et al., 2012). Lysine 63 linkages are correlated with signal transduction and proteasome mediated degradation, unlike lysine 48 ubiquitin chains which are predominantly degradation signals (Pickart and Fushman, 2004; Thrower et al., 2000).

The ability of NEDD4-1 to ubiquitinate PTEN and ΔNp63α (Bakkers et al., 2005; Trotman et al., 2007), combined with the results from this study demonstrating that ΔNp63α inhibits NEDD4-1 (Figures 30-31), suggests that ΔNp63α/NEDD4-1/PTEN form a natural negative feedback mechanism. This feedback loop serves to keep ΔNp63α and NEDD4-1 levels in check so that nuclear PTEN levels are kept low, thereby protecting the proliferative capacity of keratinocytes within the basal layer of the epidermis. Restriction of NEDD4-1 expression to the basal layer of the epidermis and the exclusion of PTEN from the nuclei of basal layer keratinocytes (Figure 37), further demonstrates that the inhibition of NEDD4-1 by ΔNp63α is physiologically relevant in maintaining homeostasis in normal epidermal tissue. The indirect regulation of PTEN ubiquitination via NEDD4-1 and the direct transcriptional repression of PTEN by ΔNp63α may also be separated from each other temporally. The experiments performed in this dissertation do not address the kinetics of the direct and indirect regulation of PTEN by ΔNp63α, making it possible that they do not occur simultaneously as implied in the simplified model shown in Figure 37. It will be intriguing to understand how the feedback loop between ΔNp63α/NEDD4-1/PTEN is altered in response to differentiation.
signals, such as high calcium, since both ΔNp63α and NEDD4-1 expression was mainly restricted to proliferative basal layer of the epidermis (Figure 36). High calcium levels have been demonstrated to activate NEDD4-1 as well as lead to the degradation of ΔNp63α, and may represent a key step in inducing keratinocyte differentiation and the development of a stratified epidermis (De Laurenzi et al., 2000; Wang et al., 2010).

The development of a healthy epidermis also relies on the expression of Akt as demonstrated by the hypoplastic skin of Akt1/Akt2 double knockout mice (Peng et al., 2003). The thin, translucent skin of Akt1/Akt2 knockout mice is caused by reduced proliferation of basal layer keratinocytes (Peng et al., 2003). Interestingly, activation of Akt has also been shown to lead to nuclear export of PTEN and may also contribute to the absence of PTEN from the nuclei of basal layer keratinocytes (Liu et al., 2007). As ΔNp63α also regulates the activation of Akt via PTEN (Figure 16), there is likely cross talk between the Akt-mediated nuclear export of PTEN and ΔNp63α-mediated inhibition of nuclear import of PTEN via NEDD4-1. This is supported by studies show that inhibition of Akt signaling reduces NEDD4-1 expression (Ahn et al., 2008). Hyperactivation of Akt caused by NEDD4-1 mediated degradation of PTEN has also been implicated in keloid formation; an abnormal, and often painful, overgrowth of scar tissue (Chung et al., 2011). Additionally, forced expression of Akt in basal layer keratinocytes sensitizes mice to two-stage chemical carcinogenesis (Segrelles et al., 2007). Repression of NEDD4-1 by ΔNp63α may thus serve to limit the ubiquitin mediated degradation of PTEN, and subsequent hyperactivation of Akt in skin. Further studies will be required to understand the complex interplay between ΔNp63α and
NEDD4-1 in regulating the PTEN/Akt axis to control proliferation in normal and pathological skin.

To form a proper epidermis during development, basal progenitor cells undergo both symmetric cell divisions parallel to the basement membrane to increase surface area as well as asymmetric cell divisions perpendicular to the basement membrane to increase thickness of the tissue (Lechler and Fuchs, 2005; Smart, 1970). However, embryos from p63 deficient mice exhibit random division orientation (Poulson and Lechler, 2010). It has also been demonstrated that PTEN is critical in maintaining spindle orientation during metaphase by ensuring appropriate levels and localization of PIP3 to the mid-cortex (Toyoshima et al., 2007), suggesting that the regulation of PTEN by ΔNp63α is essential for proper mitotic spindle orientation in addition to controlling the proliferation of keratinocytes. Supporting this hypothesis was the identification of a novel pool of PTEN localized to mitotic centrosomes and the ability of ΔNp63α to influence the abundance of centrosomal PTEN (Figure 38). The ability of ΔNp63α to control both the nuclear and centrosomal levels of PTEN is diagramed in Figure 54.

Aberrant PTEN expression has also previously been correlated with poor prognosis in breast cancers with concomitant up-regulation of many centrosome associated proteins (Matsushima-Nishiu et al., 2001; Saal et al., 2007). The studies in Figures 45 and 47 demonstrate that both PTEN and Akt coordinate to properly regulate centrosome composition during mitosis without significantly affecting steady state levels of centrosome associated proteins. In primary keratinocytes knockdown of PTEN increased whole cell levels of γ-tubulin and PLK-1 in an Akt-dependent manner and had little effect on recruitment of either protein to mitotic centrosomes (Figures 46 and 47).
Conversely, knockdown of PTEN reduced centrosomal levels of pericentrin in an Akt-independent manner (Figures 48 and 49).

Inhibition of Akt activation with MK2206 reduced the whole cell and centrosome levels of PLK-1 and γ-tubulin and also prevented the recruitment of PTEN to mitotic centrosomes (Figures 48 and 49). Previous studies have implicated a role for Akt in removing the G2/M block caused by DNA damage by preventing the ubiquitination and degradation of PLK-1 (Shtivelman, 2003). However, no study to date has specifically demonstrated that Akt activity is critical for protein recruitment to centrosomes. It is currently unclear how Akt activity is able to influence the localization of PTEN to mitotic centrosomes, but one could speculate that Akt-mediated control of either GSK3-β or S6K2 could influence recruitment of PTEN since both proteins are known to physically interact with PTEN as well as localize to mitotic centrosomes (Al-Khoury et al., 2005; Liu et al., 2007; Rossi et al., 2007; Wakefield et al., 2003).

The ability of Akt and PTEN to regulate centrosome composition during mitosis likely contributes to proper spindle formation as demonstrated by the increase in centrosome defects caused by the knockdown of PTEN and/or inhibition of Akt with MK2206 (Figures 50 and 51). The recruitment of γ-tubulin to the proximal ends of centrioles is critical for both centrosome maturation and microtubule nucleation (Fuller et al., 1995; O'Toole et al., 2012). However, the ability of centrosomes to nucleate microtubules after depletion of PTEN and/or Akt was not evaluated in these experiments and would vital for future studies to help identify the exact roles of PTEN and Akt in controlling centrosome function during mitosis. This could easily be explored by measuring microtubule regrowth after nocodazole treatment in the presence and absence of
MK2206 and siRNA against PTEN. It is possible that PTEN may directly influence centrosome function by modulating PI3K and Akt activity at the centrosomes in addition to the plasma membrane, as both PI3K and Akt have been reported to localize to centrosomes in other cell lines (Kalous et al., 2006; Kapeller et al., 1995; Lelievre et al., 2008).

Akt inhibitors in addition to MK2206 have been shown to cause spindle abnormalities during mitosis (Liu et al., 2008), but the studies in Figures 46 and 48 provide the first evidence that PTEN and Akt play a direct role in controlling centrosome composition during mitosis. Because of the direct effects on recruiting PTEN and PLK-1 to mitotic centrosomes in addition to the traditional growth suppressive effects of Akt inhibition, combinatorial therapies of MK2206 with other mitotic inhibitors may prove extremely beneficial in cancer treatment. The potential therapeutic value of centrosomal PTEN and its regulation by Akt activity warrants future studies.

C. Conclusions.

ΔNp63α has been called the master regulator of epithelial stem cells, controlling keratinocyte growth and the proper development of a stratified epidermis. The studies presented in this dissertation demonstrate that ΔNp63α is able to maintain the proliferative potential of keratinocytes by activating Akt through transcriptional repression of PTEN. ΔNp63α was also shown to inhibit the nuclear localization of PTEN, which may further the increase the proliferative potential of basal layer keratinocytes since nuclear PTEN is associated with G1 arrest (Chung and Eng, 2005; Ginn-Pease and Eng, 2003). Inhibition of NEDD4-1 by ΔNp63α was implicated in reducing the ubiquitination of PTEN, thereby preventing its import into the nucleus. The studies
presented here also identify a novel pool of PTEN that is localized to centrosomes only during mitosis. Coordinated control of both Akt and PTEN regulate centrosome composition and integrity during mitosis and provides insight into how PTEN functions as a multifaceted tumor suppressor. The importance of the ΔNp63α/PTEN/Akt signaling loop in epidermal biology was highlighted by the significant disruption of ΔNp63α and PTEN levels in non-melanoma skin cancers. Altogether, these studies provide important molecular insight into the control of keratinocyte proliferation by the ΔNp63α-PTEN-Akt signaling loop.

**D. Future Directions.**

The studies presented in this dissertation provide the framework for understanding the development of NMSC by elucidating how ΔNp63α controls keratinocyte proliferation through regulating the expression and localization of PTEN. There is however, much more work to be done before this information can be applied to in a clinical setting. For instance, the balance between ΔNp63α and PTEN was shown to be critical for controlling Akt activation (Figure 18) and that this balance is disrupted in NMSC (Figure 23); could the ΔNp63α/PTEN/Akt axis be a potential target for pharmacological intervention in the treatment of NMSC? The Akt inhibitor MK2206, which was demonstrated here to have novel effects on the composition (Figure 48) and quality of centrosomes (Figure 50), is already in phase II clinical trials for other solid cancers (Institute, 2012a; Institute, 2012b). Since MK2206 is currently delivered intravenously, would topical inhibition of Akt with MK2206 be sufficient to treat NMSC? Could MK2206 be used in combination with microtubule destabilizing agents to selectively target cancer cells, which typically display centrosome abnormalities and
hyperactivation of Akt? All of these questions would need to first be addressed in cell
culture and animal models before even beginning to design human intervention strategies.

Additionally, there is still very little known about how the ΔNp63α/PTEN/Akt axis is turned off in normal keratinocytes. Identification of the inhibitors of the ΔNp63α/PTEN/Akt axis could provide another possible therapeutic option. The induction of PTEN by the Egr-1/Arf axis could be another potential pathway for future studies, considering that this axis is also regulated Akt (Yu et al., 2009).

This dissertation has focused on the involvement of the ΔNp63α/PTEN/Akt in NMSC, but this axis may be critical for other epithelial cancers as well. It will be important to investigate whether the ΔNp63α/PTEN/Akt axis functions similarly in prostate and breast cancers as it does in keratinocytes since the former cancer types have considerably higher mortality rates than NMSC.
Figure 5: Schematic of ΔNp63α-mediated control of PTEN subcellular localization. 
ΔNp63α transcriptionally represses NEDD4-1, inhibiting the ubiquitination and subsequent nuclear translocation of PTEN. ΔNp63α also transcriptionally represses PTEN, which can inhibit the amount of PTEN available for localization to mitotic centrosomes. PTEN can indirectly affect the phosphorylation and activation of Akt. Both PTEN and active Akt contribute to the composition of centrosomes during mitosis.
References


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