Molecular Mechanisms of p63-Derived Ectodermal Dysplasia

by

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Abstract

Molecular defects in the \( p63 \) gene give rise to severe physiological abnormalities in patients with ectodermal dysplasia, however the mechanisms by which \( p63 \) mutations disrupt \( p63 \) function are unknown. In this study we examined four \( \Delta Np63\alpha \) mutants; Ectrodactyly-Ectodermal Dysplasia with Clefting (EEC) R204W, R304W and Ankyloblepharon-Ectodermal Dysplasia with Clefting (AEC) mutants, L514F and G530V, and characterized DNA binding, transcription factor activity, oligomerization with wild-type \( p63 \) and changes in protein stability/nuclear localization. We also investigated the putative OD-SAM interaction in \( p63 \) and \( p73 \). We demonstrated that both the EEC and AEC mutants cannot transcriptionally activate the \( PERP \) promoter and can hetero-oligomerize forming dominant negative complexes with wild-type \( p63 \). We show that both EEC mutants and AEC L514F mutants are more stable which is not due to aberrant degradation by the E3 ligase Itch. Finally, we discovered that a novel interaction between the \( p73 \) OD and SAM domain is absent in \( p63 \).
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# Table of Contents

Acknowledgments ......................................................................................................................... iii

List of Figures ................................................................................................................................. vii

Chapter 1 Review of Literature ................................................................................................. 1

1 INTRODUCTION ......................................................................................................................... 1

1.1 *Introduction to the p53 Family and Their Structure* ....................................................... 1

   1.1.1 Overview of the p53 Family ......................................................................................... 1

   1.1.2 Structure of the p53 Family Proteins ........................................................................ 2

1.2 *p63: Role in Development and Skin Maintenance* ...................................................... 4

   1.2.1 p63 Knockout Mice ..................................................................................................... 4

   1.2.2 p63 Is Essential for Skin Formation and Maintenance ........................................... 6

   1.2.3 p63 Regulates Genes Involved in Cell-Cell Adhesion and Maintenance of the
        Structural Integrity of the Skin ....................................................................................... 10

1.3 *p63 Derived Ectodermal Dysplasia: Ectrodactyly and Ankyloblepharon* ................. 12

   1.3.1 Overview of Ectodermal Dysplasia ............................................................................ 12

   1.3.2 *p63* Gene Mutations as a Cause of Ectodermal Dysplasia ....................................... 12

   1.3.3 Differential Regulation of Target Genes by EEC and AEC p63 Mutants ................. 15

   1.3.4 Mouse Model of Akyloblepharon Ectodermal Dysplasia ......................................... 17

1.4 *Ubiquitin E3 Ligase Itch Negatively Regulates p63* ....................................................... 18

   1.4.1 Overview ...................................................................................................................... 18

   1.4.2 Itch and p63 ................................................................................................................. 18

   1.4.3 Modular Structure of Itch ........................................................................................... 20

   1.4.4 Proteins Implicated in p63 Degradation ..................................................................... 21

   1.4.5 MDM2 and p63 .......................................................................................................... 22

   1.4.6 Potential Autofeedback Loop Mediates p63 Degradation ......................................... 24
2 AIMS, HYPOTHESIS AND RATIONALE ................................................................. 25

3 MATERIALS AND METHODS ............................................................................. 27

3.1 Cell Lines and Transfections ........................................................................ 27
3.2 Plasmids ........................................................................................................... 27
3.3 Antibodies ........................................................................................................ 28
3.4 Immunoprecipitation and Immunoblotting ..................................................... 28
3.5 Ubiquitinylation Assays ................................................................................... 29
3.6 Protein Degradation: Cycloheximide Chase Assay ........................................ 29
3.7 In Vitro Translation .......................................................................................... 30
3.8 Electrophoretic Mobility Shift Assay .............................................................. 30
3.9 Dual Luciferase Assay ...................................................................................... 31
3.10 Immunofluorescence Microscopy .................................................................. 31
3.11 NMR Spectroscopy and Protein Purification ................................................ 32

4 RESULTS ............................................................................................................ 34

4.1 AEC but not EEC Mutants Can Bind to a p63 Response Element ..................... 34
4.2 EEC and AEC Mutants are Transcriptionally Inactive and Form Dominant Negative Complexes with Wild Type p63 ......................................................... 39
4.3 Nuclear Localization is Unaffected by EEC and AEC Mutations ..................... 42
4.4 EEC and AEC mutations increase p63 stability .............................................. 45
4.5 Oligomerization domain and SAM domain of p73 but not p63 interact and residues involved are not conserved in p63 .................................................. 51

5 DISCUSSION ...................................................................................................... 53

5.1 p63 Mutants Exhibit Differential Binding of a p63 Response Element ............. 53
5.2 EEC and AEC Mutants Are Transcriptionally Inactive and Can Form Dominant Negative Hetero-oligomeric Complexes with Wild Type p63 ......................... 56
5.3 EEC and AEC mutations have a stabilizing effect on p63 ............................... 58
5.4 Oligomerization and SAM domains of p73 but not p63 interact and residues involved are not conserved in p63 ............................................................... 62
6 CONCLUSIONS AND FUTURE DIRECTIONS

7 REFERENCES
List of Figures

Figure 1: Structure of the p63 isoforms. ............................................................................................................ 3

Figure 2: p63′ exhibit severe developmental defects such as limb, craniofacial and skin abnormalities. ................................................................................................................................. 4

Figure 3: Location of p63 mutations correlate to a genotype-phenotype relationship suggesting that DNA binding domain mutations cause EEC syndrome and SAM domain mutations cause AEC syndrome. ................................................................................................................................. 14

Figure 4: Modular Structure of the E3 Ubiquitin Ligase Itch and Expression Pattern in the Epidermis. ........................................................................................................................................ 20

Figure 5: AEC (SAM Domain) mutants but not EEC (DNA-Binding Domain) mutants bind to a p63 binding-site. ................................................................................................................................. 36

Figure 6: AEC mutants produce multiple protein-DNA complexes that correlate to molecular weights of tetrameric, dimeric and monomeric ΔNp63α. .............................................................................................. 37

Figure 7: Deletion of the SAM domain causes differential banding reminiscent of AEC mutants and oligomeric defective mutant p63 (I378P) is unable to bind DNA .................................................................................................. 38

Figure 8: EEC and AEC mutants are transcriptionally inactive and exert a dominant negative affect over wild type p63. ................................................................................................................................. 40

Figure 9: EEC and AEC Mutant p63 is able to form hetero-oligomers with wild type p63. ....... 41

Figure 10: Nuclear Localization Is Unaffected by EEC and AEC Mutations. ............................... 44

Figure 11: Protein stability of the EEC and AEC mutants in the presence of Itch. ....................... 48

Figure 12: Itch binds to both EEC and AEC mutants. ................................................................. 49

Figure 13: Itch ubiquitinylates EEC and AEC mutants

Figure 14: p73 residues involved with OD-SAM interaction are not conserved in p63......... 52
Figure 15: Mutations in the SAM domain may alter the oligomeric equilibrium of p63, shifting the tetrameric archetype in favour of lesser oligomeric states. ................................................................. 55

Figure 16: Proposed p63-negative regulator autoinhibitory loop. ................................................................. 61

Figure 17: Putative masking of residues by the SAM-OD interaction in p73 may be necessary for gene modulation and function on various p73 target promoters................................................................. 63
Abbreviations

AEC: Ankyloblepharon Ectodermal Dysplasia with/without Cleft Lip
AER: Apical Ectodermal Ridge
ATP: Adenosine-5’-triphosphate
BSA: Bovine Serum Albumin
C2: Ca\(^{2+}\)-Dependent Phospholipid Binding Domain
CEP-1: *Caenorhabditis elegans* p53/p63 Homologue
CHX: Cycloheximide
DBD: DNA Binding Domain
E1: Ubiquitin Activating Enzyme
E2: Ubiquitin Conjugating Enzyme
E3: Ubiquitin Ligase Enzyme
EEC: Ectrodactyly Ectodermal Dysplasia with/without Cleft Lip
EMSA: Electromobility Shift Assay
HA: Hemagglutinin
HECT: Homologous to the E6 Carboxy Terminus
HSQC: Heteronuclear Single Quantum Coherence
IKK\(\alpha\): IκB Kinase-Alpha
IVT: *In Vitro* Translation
K1: Keratin 1; Keratinocyte Terminal Differentiation Marker
MDM2: Murine Double Minute 2
NMR: Nuclear Magnetic Resonance
OD: Oligomerization Domain
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
PEI: Polyethylenimine
PERP: p53 Apoptosis Effector Related PMP-22
PY: Proline-Rich Consensus Sequence
SAM: Sterile Alpha Motif
SDS PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SKP: Skin Derived Precursor
shRNA: Small Hairpin Ribonucleic Acid
SUMO-1: Small Ubiquitin-Like Modifier
TA: Transient Amplifying Cells
TA: Transactivation Domain
TID: Trans-inhibitory Domain
Ub: Ubiquitin
WW: Protein Interaction Module Containing Two Conserved Tryptophan Residues (W)
3F: Triple Flag Tag
Chapter 1

Review of Literature

1 INTRODUCTION

1.1 Introduction to the p53 Family and Their Structure

1.1.1 Overview of the p53 Family

p63 and p73 are homologues of the tumour suppressor p53 and together these three transcription factors comprise the p53 family. Although p63 and p73 are able to bind to canonical p53 DNA promoter sequences and induce apoptosis and cell cycle arrest, it has become clear that these proteins exhibit distinct biological functions. Unlike p53, p63 and p73 exhibit a tissue-specific expression pattern predominantly in ectodermal-derived structures and the nervous system, respectively (1-3). During development, p63 promotes the formation of the epidermis and the complex epithelial-mesenchymal interactions that give rise to ectodermal structures such as the limbs, hair, teeth and several glands in the body including mammary, lachrymal and salivary glands (1, 4). In the mature skin, p63 maintains the proliferative potential of epidermal and dermal stem cells ensuring tissue regeneration/homeostasis and regulates the commitment of epidermal lineages such as keratinocytes towards stratification and terminal differentiation (1, 5, 6). p73 on the other hand has important roles in the development and maintenance of the vertebrae nervous system. During embryogenesis, p73 promotes neuronal survival through the expression of the antiapoptotic ΔNp73 isoform throughout development (3). In the mature nervous system, p73 helps to maintain adult neurons and neural stem cells throughout adulthood (2, 3, 7).
1.1.2 Structure of the p53 Family Proteins

There is a high degree of sequence and structural homology between p63, p73 and the tumour suppressor protein, p53. Several p53 functional protein domains are conserved in p63 and p73, including an acidic N-terminal transactivation domain (TA) for the recruitment of additional protein cofactors involved in transcription, a DNA binding domain (DBD) that binds to promoter sequences of target genes and an oligomerization domain (OD) that is essential for the tetramerization of the active transcription factor (8-10). The p63 gene contains two transcriptional initiation start sites that give rise to two fundamentally distinct proteins termed TA and ΔN p63. The full length TA transcript encodes a protein isoform with a N-terminal TA domain that shares ~22% sequence identity with p53. In contrast, the ΔN p63 mRNA is transcribed from a distal cryptic promoter on intron 3, resulting in a N-terminally truncated (ΔN) protein that lacks the TA domain. The ΔN isoforms can act as dominant negative proteins towards TAp63 proteins, as well as other full length p53 family proteins (10-12). This occurs through two mechanisms: (1) ΔN isoforms bind to target gene promoter sites and effectively outcompete the TA proteins for binding sites on DNA consensus sequences and (2) through protein-protein interactions between TA and ΔN proteins which form hetero-oligomeric complexes that are transcriptionally inert (10-12). Initially, the ΔN forms were believed to be transcriptionally inactive due to the absence of a classical TA domain. However, in recent years a subset of p63 target genes have been identified that can be induced by both TA and ΔN p63. Some genes are selectively activated by ΔNp63 (12-20). The ability of ΔNp63 to transactivate genes has been attributed to the presence of a second intrinsic TA domain located within the first 26 amino acids of the N-terminus of the ΔN p63 isoforms (12-20).

The 3’ end of the p63 (as well as p73) transcript also undergoes complex alternative splicing leading to three possible C-terminal variants α, β, or γ, resulting in a total of six individual proteins that arise from the p63 gene. Of the six proteins, TAp63α is the longest transcript of the p63 gene. The α-isoforms of p63 contain a SAM (sterile alpha motif), which is also present in p73, but not p53 (9, 21, 22, 22). SAM domains are highly conserved protein motifs in eukaryotes. They are comprised of 65-70 amino acids that form a compact globular bundle of five α-helices consisting of four α-helices and a small 3_10-helix (21, 22). The SAM domain likely mediates protein-protein interactions with binding partners that are specific only to the p63α isoforms. The biological significance of the SAM domain is suggested by the finding that
SAM point mutations are detected in the clinical disease known as Ankyloblepharon Ectodermal Dysplasia with or without cleft lip/cleft palate (AEC) syndrome. Downstream of the SAM domain p63 contains a C-terminal trans-inhibitory domain (TID) which binds to the N-terminal transactivation domain of TAp63α thereby inactivating its transcriptional potential. Lastly, upstream of the SAM domain is a proline-rich region that contains a PY motif that is essential for p63α degradation (23, 24).

Figure 1: Structure of the p63 isoforms. (A) Schematic overview of the p53 family and the sequence identity among their domains. TA, transactivation domain, DBD, DNA binding domain, OD, oligomerization domain, proline-rich (PY) motif, SAM, sterile α-motif domain and TID, trans-inhibitory domain or PS, post-SAM domain. (B) Exon-intron and splicing pattern diagram of p63 (Adapted from (10)). The P1 promoter gives rise to the TA isoforms where as the P2 promoter results in the generation of N-terminally truncated ΔN isoforms. The carboxy termini is subject to complex alternative splicing which gives rise to α, β, or γ proteins in p63 (C) Diagram highlighting the high sequence identity between the functional domains of p63 and p73.
1.2  **p63: Role in Development and Skin Maintenance**

1.2.1  **p63 Knockout Mice**

p63 orchestrates the formation of the epidermis and the complex epithelial-mesenchymal interactions that give rise to ectodermal structures such as the limbs, hair, teeth and several glands in the body (1, 4, 25). Initial studies with p63 knockout mice provided the first insight into the role of p63 in development. Two independent research groups created p63 knockout mice by disrupting the DBD that is common to both the TA and ΔN variants (1, 25). p63\(^{+/−}\) mice have severe developmental defects including craniofacial malformations such as cleft-palate, soft-tissue and bone abnormalities characterized by the absence or truncation of hind and forelimbs, in addition to the complete loss of the epidermis, stratified epithelia and its derivatives (hair, teeth, and glands such as mammary, lachrymal and salivary glands).

![Figure 2: p63\(^{+/−}\) exhibit severe developmental defects such as limb, craniofacial and skin abnormalities](image)

*Top Left and Right Panels: p63 null mice fail to develop hair, teeth, limbs, eyelids and exhibit cleft lip; Blue Stain: Cartilage, Magenta Stain: Bone. Lower Panel: Stratified epithelium of the epidermis and tongue are not present compared to wild type mice. Images taken from Yang et al. 1999 (1).*
Furthermore, $p63^{-/+}$ mice die shortly after birth due to dehydration resulting from a loss of barrier function that is normally provided by the skin. $p63$ exhibits a tissue-specific expression pattern that predominantly includes the ectodermal layers of the developing epidermis, limb buds, brachial arches (involved in soft-tissue and craniofacial skeletal formation) and epithelial appendages. Loss of $p63$ also disrupts key ectodermal-mesenchymal tissue interactions during development that are essential for the correct formation of limb and epithelial structures. The apical ectodermal ridge (AER) is a key structure responsible for reciprocal ectodermal-mesenchymal signaling. In $p63^{-/-}$ mice, the AER is absent and is unable to properly direct the patterning of the underlying mesenchyme for the outgrowth and formation of limbs and several other ectodermal organs that rely on ectodermal-mesenchymal cross-talk. For example, hair, whiskers, teeth, mammary, salivary and lachrymal glands are all absent in $p63$ deficient mice. In contrast to their wild type littermates, $p63$ knockout mice lack an epidermis and instead of skin, are covered by a primitive single-layered epithelium deficient of stratified epithelia and its appendages. The two groups who generated $p63^{-/-}$ provided different explanations for this skin phenotype. Yang et al. (1999) detected small patches of skin that expressed terminal differentiation markers (filaggrin and involucrin), and suggested that $p63$ resides in the regenerative stem cell population (1). Thus, they concluded that the epithelial abnormalities could be explained due to the depletion of stem cells necessary for the regenerative capacity of the skin. In contrast, Mills et al. (1999) did not detect early or late markers of terminal differentiation (K10, filaggrin and loricin) and as such they concluded that $p63$ is critical for committing keratinocytes towards terminal differentiation (25). Taken together, it is clear that $p63$ is essential for epidermal morphogenesis and is important for the development of ectodermal structures. These studies suggest that $p63$ is important for maintaining the progenitor cells that give rise to the skin and is also crucial for initiating stratification and terminal differentiation programs that direct the formation of the upper epidermis. It should be noted that in these studies, the murine phenotypes observed could not be attributed to specific isoforms since the strategies used resulted in the deletion of all $p63$ isoforms.
1.2.2 p63 Is Essential for Skin Formation and Maintenance

In recent years, studies identifying specific p63 (non p53 and non p73) targets have provided clues how p63 signalling directs the formation and maintenance of the epidermis and ectodermal structures. The epidermis results from the proliferation of cells in the basal layer of the skin as well as the progression of keratinocytes through the terminal differentiation program within the upper stratum of the skin (26). Together, these two processes maintain a constant state of tissue homeostasis by replenishing keratinocytes that have slough off the skin surface with new cells that terminally differentiate and migrate outwards towards the surface of the epidermis (26, 81).

The skin is organized into several layers, beginning with the basal layer which is anchored to the basement membrane through hemidesmosome adhesion complexes, integrins and a network of keratin intermediate filaments such as keratins 14 and 5 that are exclusively expressed in basal cells (82). Moreover, the basal regenerative layer is comprised of a heterogeneous population of cells consisting of stem cells and transient amplifying cells (TAC) which serve to maintain and replenish the skin. Asymmetrical replication of epidermal stem cells typically gives rise to two progeny: a stem cell that remains in the stem cell niche and a transient amplifying cell which is destined to terminally differentiate (26-28). Young TACs retain a high proliferative capacity and rapidly progress through a few rounds of mitotic division before exiting the cell cycle and committing to stratification and terminal differentiation in the first suprabasal level of the skin known as the spinous layer (26-28).

The transition of basal cells into the spinous layer is marked by a switch in expression of the basal cell markers K14 and K5 to the spinous cell markers K10 and K1 (82). In the spinous layer, keratinocytes begin to synthesize large amounts of keratin intermediate filaments which form a web-like pattern that radiates outwards from the perinuclear ring throughout the cytoplasm (81, 82). The terminal ends of the keratins are connected to cell-cell adhesion complexes known as desmosomes. These structural connections have important role that forms a mechanical network of cross connected cells. As keratinocytes migrate further up into the granular layer, keratin production is reduced and the cells begin express and synthesize specialized cornified envelope proteins such as loricin, filaggrin, involucrin which are deposited beneath the plasma membranes (81, 82). Granular cells also begin to secrete highly specialized hydrophobic lipids into the extracellular matrix forming a lipid rich layer that helps to create the water-impermeable barrier of the skin. The final stages of keratinocyte terminal differentiation
occur in cornified layer of the skin where cornified envelop proteins that were synthesized in the granular layer are cross-linked by transglutaminase which transforms the cell envelope into a rigid structure composed of integrated proteins and covalently bound lipids (26, 28, 81, 82). Cells also change in morphology due to filaggrin which organizes keratin filaments into tight macrofibril bundles that cause the cells to flatten out. In the final stages, the cell undergoes programmed cell death were organelles are destroyed including the nucleus thus producing a layer of dead squame cells that form the impermeable barrier function of the skin (81, 82).

Both the TA and ΔN isoforms are essential for maintaining the high proliferative potential as well as the controlled rate of self-renewal of the underlying stem and TAC cells. In addition both p63 isoforms regulate crucial genes required for the formation of the upper stratified layers of the skin. ΔNp63α is the predominant isoform expressed in the basal regenerative layer of the epidermis where it serves in part to maintain the proliferative capacity of epidermal stem/progenitor cells and prevent premature depletion. Several studies have shown that ΔNp63α is essential for maintaining the self-renewal, clonogenicity and high proliferative capacity of epithelial stem cells (1, 29, 30). A recent study by Senoo et al. (2007) demonstrated with clonogenic assays that shRNA knockdown of ΔNp63 resulted in a significant loss in proliferative potential of epidermal and thymic epithelial stem cells (29). p63 knockdown resulted in fewer and smaller colonies compared to wild type controls, which were larger and expressed higher levels of the Ki67 proliferation marker, suggesting that loss of ΔNp63 results in decreased renewal and depletion of the stem cell population.

Interestingly, Su et al. (2009) recently demonstrated that specifically the TAp63 isoform is essential for the maintenance of adult stem cell populations. For these studies, they generated two TAp63−/− murine models; one which ablated TAp63 in the germline and the other which knocked out TAp63 in K14 expressing cells of the basal layer of the epidermis. Germline TAp63−/− mice displayed a premature aging phenotype characterized by ulcerated skin with impaired wound healing, kyphosis and a shortened life span compared to their wild type littermates. Furthermore, TAp63 was expressed in skin-derived precursors (SKPs) located in the dermal sheath and papilla. In cultured SKPs, TAp63−/− cells exhibited hyper-proliferation and increased self-renewal as detected by denser clonal spheres compared to SKPs cultured from wild type mice. Furthermore, colony formation assays demonstrated that TAp63 was also essential for
epidermal precursor proliferation, however, TAp63 specific deletion in the epidermis, did not recapitulate the germline TAp63\textsuperscript{\textminus/\textminus} mouse skin abnormalities. Taken together, they concluded that loss of TAp63 affects self-renewal of dermal and epidermal stem cells, leading to hyperproliferation and premature senescence. The loss of precursor cells likely explains the premature aging phenotype of the TAp63\textsuperscript{\textminus/\textminus} mice. The ability of TA and ΔN p63 to maintain the proliferative capacity of epidermal progenitor cells is due, in part, to the induction of genes involved in cell cycle progression as well as repression of genes that control cell cycle withdrawal (12, 13, 15, 17, 31-33). For example, Westfall \textit{et al.} (2003) demonstrated that ΔNp63α functions as a transcriptional repressor for two cyclin-dependant kinase inhibitors, p21 and 14-3-3-σ, leading to proliferation of keratinocytes \textit{in vivo} (31). During terminal differentiation, ΔNp63α levels are down-regulated resulting in the induction of p21 and 14-3-3-σ, which promote cell cycle withdrawal and initiation of terminal differentiation within the suprabasal levels of the epidermis (30, 31). Recent data has shown that ΔNp63α can also repress \textit{Notch1}, a key inducer of keratinocyte differentiation, by binding to a p53-response element located within the \textit{Notch1} promoter (34). In cultured human cervical keratinocytes, ΔNp63α knockdown effectively increased \textit{Notch1} expression causing hypo-proliferation and a reduction in clonogenicity.

In addition to maintaining the proliferative potential of basal epithelial cells, p63 is also responsible for regulating crucial aspects of keratinocyte terminal differentiation and cell fate. As keratinocyte stem cells divide, they give rise to TACs, which progress through a few rounds of mitotic division before exiting the cell cycle and committing to stratification and terminal differentiation programs in the outer layers of the epidermis (28). In p63\textsuperscript{\textminus/\textminus} mice that have been selectively re-complemented to express either TA (p63\textsuperscript{\textplus/TA}) or ΔN (p63\textsuperscript{\textplus/ΔN}) (18), regenerative epidermal organotypic\textsuperscript{1} culture model (6), or mice model experiments (35) have all suggested that ΔNp63 is required for epidermal formation and keratinocyte terminal differentiation. TAp63 acts synergistically with ΔNp63 to upregulate genes involved in the later stages of keratinocyte maturation. For example, Truong \textit{et al.} (2006) used a regenerative organotypic tissue model to demonstrate that siRNA knockdown of ΔNp63 resulted in severe tissue hypoplasia, characterized

\textsuperscript{1} Organotypic Culture: Tissue removed from an organ that continues to develop in culture as it would normally in the organ where it was derived from.
by the complete absence of the differentiation markers K1, K10, and transglutaminase and also resulted in the formation failure of the spinous and suprabasal layers. In contrast, epidermal tissue generated from cells in which TAp63 expression was decreased by siRNA had only minimal abnormalities. Although the stratum corneum, the outer-most layer consisting of fully mature cornified keratinocytes, was absent in cells with decreased TAp63, the granular and spinous layers were properly formed and expressed K1, K10, trantraglutaminase and loricrin terminal differentiation markers (6). Koster et al. (2009) also reported that downregulation of ΔNp63 in an inducible mouse model resulted in developmental abnormalities of the spinous layer.

Proliferating cells were detected in the suprabasal level and keratinocytes exhibited a delay in the expression the K1 terminal differentiation marker. These results suggest that the ΔNp63 isoforms ensure the proper formation of the basal layer and initiate early genes in differentiation, providing the framework and architecture for the spinous layer to properly develop. In contrast, TAp63 isoforms act synergistically with ΔN to contribute to the formation of the upper stratified layers of the skin. Indeed, this view is supported by two groups who independently concluded that the TA isoforms drive the expression of proteins that are crucial to the formation of the upper granular and cornium stratum layers (6, 18). These include keratins 1 and 10, as well as transglutase 3 and 5, which cross-link structural proteins loricrin, filaggrin and involucrin. These proteins comprise the cornified envelope and scaffolding necessary for the barrier function of the upper stratum (6, 18).

p63 also regulates genes that ensure keratinocytes properly exit from the cell cycle before their commitment to stratification. For instance, p57kip2, a cyclin-dependent kinase inhibitor is induced by ΔNp63 when keratinocytes begin to terminally differentiate allowing them to withdraw from the cell cycle (15). p63 may also prevent hyperproliferation of stem cells, allowing them to divide at a controlled rate and prevent premature senescence through induction of p57kip2 (5). The IKKa gene is also a downstream target of p63 and is involved in epidermal keratinocyte differentiation and cell cycle exit (17, 32, 36). Interestingly, IKKa−/− mice exhibit similar phenotypes to p63 null mice including craniofacial, skin and limb defects (1, 25, 37, 38). Several groups have shown that TA and ΔN isoforms directly induce IKKa expression. Furthermore, p63 can indirectly upregulate IKKa through GATA-3 and by the TAp63-specific transactivation of Ets-1 (17, 32, 36). Loss of IKKa leads to decreased expression of early (K1) and late terminal differentiation markers including loricrin and filaggrin. In skin, p63 is crucial for the initial
induction of IKKα during the first stages of keratinocyte differentiation, but is not necessary to maintain them in the suprabasal levels suggesting that terminal differentiation can proceed through additional mechanisms independent of p63 (36). Furthermore, downregulation of ΔNp63α results in lower levels of IKKα and the early differentiation marker K1. IKKα is also a key regulator of the cell cycle. siRNA knockdown of ΔNp63α results in hyperproliferation of differentiating keratinocytes, however reintroduction of IKKα was shown to restore the low proliferation rate of terminally differentiating cells, suggesting that p63 induction of IKKα is necessary for keratinocytes to exit the cell cycle and initiate terminal differentiation (17, 32, 36).

Taken together, p63 is essential for the formation and maintenance of the epidermis. p63 is required for ensuring the high proliferative potential of the underlying stem cells and/or TACs as well as regulating crucial genes required for keratinocyte differentiation in the upper stratified layers of the skin. However, there are conflicting explanations as to the mechanisms of how p63 exerts its dual functions. For example, Senoo et al. (2007) and others have concluded that p63 is essential to maintain the proliferative capacity of stem cells through its repression of genes that promote cell cycle exit. In contrast, p63 promotes terminal differentiation by also activating genes such as IKKα and p57kip2 required for cell cycle withdrawal. One possible explanation is that in different contexts temporal and spatial expression of cofactors ensure that p63 maintains the proliferative potential of cells in the basal regenerative layer, as well as promoting keratinocytes to cease mitotic division upon exposure to differentiation stimuli in order to form the stratified epithelia layers.

1.2.3 p63 Regulates Genes Involved in Cell-Cell Adhesion and Maintenance of the Structural Integrity of the Skin

The skin is a versatile organ that provides an essential anatomical barrier from external pathogens and other environmental insults. Maintaining the structural integrity and barrier function of the skin relies on several structural proteins that participate in epithelial cell-cell adhesion which provides the epidermis with tensile strength against mechanical stress through the formation of a network of cross-linked cells. PERP is a p63 target that is important for maintaining the structural integrity of the skin (19, 20). PERP is a tetraspan membrane protein
that integrates into desmosomal adhesion complexes that promote cell-to-cell adhesion in stratified epithelium. Initially, PERP was identified as a p53 response target gene that is upregulated to induce apoptosis in response to DNA damage (80). However, PERP null mice are not predisposed to spontaneous tumors but rather exhibit severe defects in epithelial structures such as the skin (69). Specifically, PERP−/− null mice die postnatally due to severe blisters of the skin and oral muscosa which are attributed to defects in the proper assembly of desmosome complexes necessary for cell-cell adhesive contacts. Other epithelial defects include abnormal skin thickness due to hyperproliferation of keratinocytes in the skin, dystrophic nails and greasy disorganized (69). Interestingly, PERP null mice display phenotypes similar to patients with a clinical disease known as ectodermal dysplasia caused by mutations in p63 (19, 20, 42, 43, 69). In these patients, severe skin erosions are often observed in the disease which has been linked to aberrant PERP expression in the skin (19). In addition to PERP, p63 regulates genes other proteins that are crucial to maintain the structural integrity and barrier function of the skin such as Claudin-1 (14), which participates in the formation of tight junctions between epithelial cells. Claudin-1 knock out mice exhibit severe skin abnormalities and die postnatally from dehydration due to a lack of barrier function (14). These phenotypes are reminiscent of the p63 knock out mice suggesting that these genes are linked within the same signaling pathway (1, 14). Lopardo et al. (2008) demonstrated that ΔNp63α specifically binds to the Claudin-1 promoter in vivo and activates endogenous expression of Claudin-1 in cells. Furthermore, p63 null mice and AEC patient skin biopsies express reduced levels of Claudin-1 compared to controls suggesting that aberrant expression of Claudin-1 may contribute in part to the skin phenotype observed in AEC patients.

Recent studies with TAp63−/− mice have shown that TAp63α plays a crucial role in maintaining the genomic stability of the female germline (39, 40). At birth, TAp63 is constitutively expressed in mouse oocytes. Genotoxic insults (e.g. irradiation or chemotherapy drugs) that damage oocyte genomic material activate c-Abl, which promotes TAp63 phosphorylation on specific tyrosine residues. As a consequence, TAp63 is stabilized and in turn, activates proapoptotic genes that lead to cell death of these damaged oocytes (39, 40). The McKeon group also demonstrated that upregulation of the pro-apoptotic genes PUMA and NOXA was independent of p53 function (39, 40). In summary, p63 plays critical roles in development by orchestrating a complex cascade of
signaling pathways that modulate the formation of several structures such as limbs, bones, skin and various ectodermal-derived appendages. In terms of the mature epidermis and epithelial tissue, p63 is essential for maintaining the proper morphogenesis and homeostasis of these tissues by preserving the self-renewal properties of stem cells and modulating epithelial commitment and differentiation programs within epithelialized tissue. Thus it is not surprising that mutations which affect p63 result in significant developmental defects.

1.3 **p63 Derived Ectodermal Dysplasia: Ectrodactyly and Ankyloblephron**

1.3.1 **Overview of Ectodermal Dysplasia**

Ectodermal dyplasia represents a broad spectrum of rare inherited diseases that affect the proper development of ectodermal-derived tissues and their structures such as the epidermis, hair, nails, glands and teeth, which can also be accompanied by craniofacial and limb malformations (41-44). Ectodermal dysplastic syndromes include different modes of Mendelian inheritance (X-linked; dominant or recessive, autosomal dominant or recessive) and have an incidence of approximately 7 cases in 10,000 births, although the penetrance and expression of this disease is variable. To date, there are over 170 genes implemented in this disease. Clinical phenotypes vary but all are characterized by ectodermal malformations and hypoplasias (41). Although there are some specific characteristics associated with given gene defects there are no clear genotype-phenotype correlations.

1.3.2 **p63 Gene Mutations as a Cause of Ectodermal Dysplasia**

Two subsets of ectodermal dysplasia syndromes have been linked to mutations in the *p63* gene: ectrodactyly-ectodermal dysplasia clefting syndrome (EEC), and ankyloblepharon ectodermal dysplasia clefting syndrome (AEC). Both of these syndromes are characterized by developmental defects that affect the skin, hair, limb, craniofacial regions and other organs reminiscent of the phenotype observed in *p63*+/− knockout mice. Patients with both EEC and AEC exhibit heterozygous autosomal dominant inheritance and share similar characteristics, including
abnormal skin and hair, developmental delay, hypo- or oligodontia, craniofacial defects such as cleft lip with or without cleft palate, absent or dystrophic nails, conductive hearing loss, as well as nasal-lacrimal duct and gland dysplasias. Although EEC and AEC syndromes both arise from mutations in p63, these diseases also have distinguishing features (41-45). For instance, EEC patients typically have severe limb defects such as ectrodactyly (“lobster claw”) or syndactyly digits and have sparse or coarse/wirey hair. In contrast, AEC syndrome is less frequently associated with limb abnormalities and instead patients exhibit unique characteristics such as ankyloblepharon (partial fusion of eyelids), skin fragility, alopecia and erosive dermatitis, particularly on the scalp (42, 45). There is a partial genotype-phenotype correlation linking the two clinical syndromes to specific functional domains (42-44). EEC syndrome is usually associated with missense mutations clustered in the core DBD, which generate amino acid substitutions that are predicted to interfere with direct contact of DNA, or cause extensive structural rearrangements that affect its binding (43, 44). These alterations affect all p63 proteins since the DNA binding domain is conserved in all isoforms. In contrast to EEC, AEC mutations are centralized within exon 13 which encodes the C-terminal SAM domain, thus exclusively affects two p63 isoforms; TAp63α and ΔNp63α (42).
Figure 3: Location of p63 mutations correlate to a genotype-phenotype relationship suggesting that DNA binding domain mutations cause EEC syndrome and SAM domain mutations cause AEC syndrome. Modular structure of p63 highlighting the domains in which EEC (Green) and AEC (Blue) are localized within the $p63$ gene. EEC syndrome, associated with mutations in the DNA binding domain, is characterized by severe limb defects such as ectrodactyly or syndactyly digits, as well as cleft lip with or without cleft palate. AEC syndrome is characterized by ankyloblepharon, severe skin fragility which often cause robust skin erosions, as well as alopecia and cleft lip with or without cleft palate. Images adapted from references (42, 43).
As described earlier, the p63 SAM domain is a putative protein-protein interaction module. For p63, the SAM domain likely serves as a docking site for co-activators or co-repressors that comprise the transcriptional complex required for activation or repression of target gene promoters. In addition, the SAM domain may also be important for directly binding DNA based on indirect evidence from reporter gene assays where point mutations in the SAM domain abolish p63 transcriptional activation (19). Nevertheless, the SAM domain is comprised of five α-helices that pack into a tight globular conformation (21, 22). Based on 3D-modeling studies of the SAM domain, AEC mutations can be divided into two subclasses based on their solvent accessible surface area (42). The residues L514, C522 and I538 are buried deep within the hydrophobic core of the SAM domain and are exposed to a small, solvent accessible surface (1.3%, 6.2% and 1%, respectively) (42). These mutations are thought to result in major structural changes that destabilize the protein by disrupting key hydrophobic interactions that pack the α-helices tightly within the SAM domain. The other subclass is comprised of mutations that are exposed to a large solvent accessible area: G530 (26.2%), T533 (47.1%), and Q540 (37.4%). These mutations do not alter the SAM domain conformation but are predicted to abolish interactions with p63 binding proteins. Interestingly, structural investigations of the p53-like protein in the model organism Caenorhabditis elegans (CEP-1) by nuclear magnetic resonance (NMR) have shown that the CEP-1 SAM domain helps to stabilize the protein through key contacts with its OD domain (9). In light of the sequence similarity and overall structural topology between p63, p73 and CEP-1, these structural elements may also serve a stabilizing role for human p63 and p73.

1.3.3 Differential Regulation of Target Genes by EEC and AEC p63 Mutants

The mechanisms by which the various p63 mutations result in different ectodermal phenotypes largely remain an enigma. As alluded to earlier, both EEC and AEC syndromes exhibit autosomal dominant modes of inheritance, however, loss or mutation in one p63 allele does not always recapitulate characteristics of ectodermal dysplasia (25, 43, 44). This evidence suggests that the phenotypic changes associated with these syndromes is not solely due to haploinsufficiency but instead, is likely due to differential regulation of downstream target genes and pathways by the various p63 EEC and AEC mutant proteins. The ability of p63 to activate or
repress downstream target genes depends on the specific p63 mutation; its effect on p63 binding to a particular promoter and its ability to recruit and interact with the necessary cofactors on the promoter site. For example, a recent study by Lo Iacono et al. (2008) investigated the distal-less-related homeodomain transcription factors Dlx5 and Dlx6, which are p63 target genes that are involved in the development of skeletal bones of the face and limbs. The AEC mutant L514F was able to bind to and transactivate both Dlx5 and Dlx6 promoters. In contrast, the EEC-derived mutant C306R was unable to activate these promoters, suggesting one possible explanation why limb defects are absent in AEC patients. Moreover, other studies have shown that additional EEC mutants are transcriptionally defective. This is attributed to the nature of the mutation, which causes severe changes to the DBD and effectively abolishes their ability to physically bind DNA. Indeed, this was confirmed by in vitro EMSA data for C306R, R204W and R304W using a probe containing a p53 consensus sequence (46). In contrast to EEC, AEC syndrome mutants differentially regulate various target genes which is dependent on both the location of the mutation within the SAM domain and is also promoter dependent (14, 15, 19, 36, 47, 48). p63 mutant proteins that are defective for activating transcription from one promoter may have activity similar to wild type for another promoter. Beaudry et al. (2009) showed that the p63-derived AEC mutants, L514F, G530V and Q536L possess variable ability to activate the PERP promoter. Interestingly, the L514F variant, previously described to activate the Dlx5 and Dlx6 promoters, was unable to induce PERP activation as measured by PERP promoter luciferase-reporter. In contrast, the Q536L mutant retained the ability to activate the PERP promoter at levels similar to wild type. Moreover, Testoni and Mantoani (2006) demonstrated that the SAM domain is important for the recruitment of p63 to various G2/M cell-cycle target genes (33). Specifically, ΔNp63α binds to the NF-Y transcription factor, which is required within the transcription complex to repress the promoters of various cell cycle target genes including cyclin B2 (33). AEC mutants fail to associate with the NF-Y cofactor, which prevents p63 from binding and repressing transcription of some cell-cycle genes. Interestingly, EEC C306R, which has an intact SAM domain and retains its ability to bind to NF-Y, was successfully recruited to the Cyclin B2 promoter and surprisingly, exhibited a “gain-of-function” activity, increasing transcription of Cyclin B2 opposed to repressing its expression. This goes to show that even though some mutations may have a diminished capacity to directly bind DNA such as the majority of the EEC mutants, interactions with regulatory partners can still manifest p63 to bind indirectly to target gene promoters and exhibit differential effects. Taken together, differential
expression of various downstream target genes can in part be explained by differences in DNA binding capacity of the various p63 mutations. Furthermore, this capacity may also be mediated by altered or abolished cofactor interactions that are necessary to properly recruit the transcription machinery and modulate important p63 target genes.

1.3.4 Mouse Model of Akyloblepharon Ectodermal Dysplasia

Koster et al. (2009) recently generated an inducible ΔNp63 mouse model for AEC termed, ΔNp63 i-kd by selectively downregulating the ΔNp63 isoform upon topical treatment with RU486 (a progesterone receptor agonist). This mouse model successfully recapitulated the skin fragility and erosion phenotype observed in the epidermis of AEC patients (35). Immunochemistry staining of skin biopsies from ΔNp63 i-kd mice and AEC patients revealed marked similarities compared to control groups. First, the spinous layer failed to properly form in both the AEC mice and human patients. Notably, proliferating keratinocytes were observed in the suprabasal levels of the skin of ΔNp63 i-kd mice and AEC patients, as indicted by BrdU incorporation and Ki67 staining, respectively. Cells in this layer have normally exited the cell cycle and initiated terminal differentiation. Interestingly, earlier studies have documented strong p63 staining in AEC patient skin biopsies within the upper layers of the epidermis where p63 is normally absent (42). Thus, it is interesting to speculate that the presence of proliferating cells in the spinous layer in the AEC patient’s skin biopsy may be due to the aberrant degradation of p63 (either at the transcriptional or protein level). As a consequence, accumulated levels of mutant p63 in the suprabasal levels may prevent cell cycle withdrawal and delay terminal differentiation. Although Koster et al. did not perform immunohistochemical staining for p63; the researchers did observe a delay in the expression of the keratinocyte terminal differentiation marker, K1. In the non-lesional skin of AEC patients and ΔNp63 i-kd mice, keratinocytes exhibited postponed initiation of terminal differentiation, whereas in lesional skin from patients, the K1 marker was essentially absent. In addition to abnormalities documented for terminal differentiation, Koster et al. observed that the integrity of the basement membrane was also compromised by downregulating ΔNp63. Specifically, collagen IV, which is normally found in the basement membrane exclusively, exhibited discontinuous staining in both mice and AEC patients. This may be a consequence of improper induction of the ΔNp63α target...
gene *Fras1*, a component of the basement membrane that promotes integrity of the epidermal-dermal interface via the extracellular matrix (33). At the biochemical level, AEC-derived ΔNp63α mutants (L514F and Q536L) can act in a dominant negative manner towards wild type p63 on the IKKα promoter; whether these AEC mutants have a dominant effect on other target genes has not been determined (35). Together, additional investigations to determine how different p63 mutations cause pleiotropic effects on downstream target genes and pathways are essential to dissect the mechanisms that result in EEC and AEC.

### 1.4 Ubiquitin E3 Ligase Itch Negatively Regulates p63

#### 1.4.1 Overview

Ubiquitin mediated protein degradation is a highly complex process involving ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes that sequentially coordinate the covalent transfer of ubiquitin molecule(s) to specific lysine residue(s) of a particular protein substrate, which flags it for 26S proteasome degradation (49). All members of the p53 family are subject to degradation via the ubiquitin-proteasome pathway, allowing the cell to regulate and maintain these proteins at specific levels. In recent years, E3 ubiquitin ligases that regulate p63 and the other p53 family members have received considerable attention due to their involvement in regulating signaling pathways that mediate development and tumourigenesis and it is becoming clear that p53, p63 and p73 are differentially regulated by specific E3 ligases (50).

#### 1.4.2 Itch and p63

Itch (also known as atrophin-1 interacting protein 4; AIP-4) is a E3 ubiquitin ligase that was first identified during genetic studies investigating the *agouti* locus responsible for coat colouration in mice (51). Radiation-induced chromosome inversions produced the non-agouti-lethal 18H mice (“Itchy” mice), which inactivated both the *agouti* gene and a previously uncharacterized gene
locus that encoded the E3 ubiquitin ligase Itch. 18H mice (lacking both *itch* and *agouti*) developed profound immune and inflammatory diseases that were previously unseen in other *agouti* mutant animals. 18H mice were characterized by severe inflammation of the large intestine, stomach and skin, resulting in constant itching, and hyperplasia of hematopoietic lymphoid cells and cells of the epithelium. Thus, implicating Itch as having an important role in immunological and inflammatory responses, as well as epithelial and hematopoietic cell growth (51). Since these initial studies, Itch has been shown to be pivotal in regulating signaling cascades that modulate immuno-inflammatory response, epidermal stem cell regeneration and keratinocyte differentiation specifically through p63 as well as other proteins such as notch (52, 53).

Rossi et al. (2006) were the first to identify a role for Itch in maintaining steady-state levels of p63 in the epidermis. Interestingly, p73 is also susceptible to Itch degradation, although the physiological relevance is clearer with regards to p63 involvement in skin morphology (23, 54). Nevertheless, the researchers demonstrated that Itch binds to and promotes the polyubiquitinylation of p63, thereby targeting the transcription factor for degradation via the 26S proteasome pathway. Through its WW domain(s), Itch binds to a conserved proline-rich PPXP motif located upstream of the SAM domain in both p63 and p73. Thus, Itch selectively targets the TA and ΔN p63α isoforms but not the β and γ variants, which lack the PY consensus sequence. Physiologically, Itch plays a crucial role in promoting keratinocyte differentiation by down regulating ΔNp63α in the skin (23, 53). As mentioned previously, ΔNp63α is predominantly localized to the basal regenerative cells of the epidermis where it is thought to maintain the self-renewal properties of the epidermis. p63 and Itch colocalize in the skin (Figure 4 B); as keratinocytes begin to differentiate and migrate into the suprabasal levels of the skin, Itch levels begin to accumulate effectively reducing ΔNp63α levels and allowing keratinocytes to terminally differentiate, as evidenced by expression of involucrin marker (23). To date, there have been no studies examining whether Itch binding is affected by the presence of EEC and AEC mutations. However, based on reports that AEC patients exhibit p63 expression in the suprabasal levels of the skin, it is conceivable that mutations in p63 may cause aberrant Itch binding and subsequent dysregulation of its degradation in the cell.
1.4.3 Modular Structure of Itch

Itch belongs to the C2-WW-HECT (homologous to the E6 carboxy-terminus) Nedd4-like E3 ligase family. Other members of this family include: Nedd4, WWP1, SMURF1 and SMURF2 (49, 50). Figure 4A depicts the modular domain structure of Itch that includes a C2, N-terminal Ca\(^{2+}\)-dependent phospholipid binding domain, four tandem WW domains which recognize proline-rich (PY) motifs on substrates, and a C-terminal HECT domain that coordinates ubiquitin transfer from the E2-ligase to a conserved cysteine residue (C830) in the HECT domain, which catalyzes the final attachment of ubiquitin to its substrate.

**Figure 4:** Modular Structure of the E3 Ubiquitin Ligase Itch and Expression Pattern in the Epidermis. (A) Modular structure of Itch including an N-terminal C2 Ca\(^{2+}\)-dependent phospholipid binding domain, four tandem WW domains which recognize proline-rich PPXY (PY) motifs on TA and ΔN p63, and a C-terminal HECT domain. (B) Immunofluorescence analysis of skin demonstrating the reciprocal colocalization of p63 in the basal layer and decreases in the suprabasal layers were Itch is present. Figure adapted from (26).
1.4.4 Proteins Implicated in p63 Degradation

Very few p63-E3 ubiquitin ligases have been identified in the ubiquitin-dependant degradation of p63. Two members of the C2-WW-HECT Nedd4-like E3 ligase family: WWP1 and Nedd4 have been identified as negative regulators of p63α. WWP1 has been proposed to have oncogenic properties due to its frequent overexpression in approximately 40% of epithelial cancers of the prostate and breast (55, 56). WWP1 has also been reported to interact with p53 promoting its polyubiquitylation and nuclear export (56). Overexpression of WWP1 augments excessive p53 accumulation in the cytoplasm, thus inhibiting its tumour suppressive functions by preventing nuclear p53 activation of downstream target genes. With respect to p63, WWP1 promotes degradation in a similar fashion to Itch. WWP1 binds to the p63 PY consensus sequence through its WW domain; an interaction that is affected by substituting phenylalanine for tyrosine 504 and 449 in TA and ΔN, respectively (55). Polyubiquitylation destabilizes p63, targeting both the TA and ΔN isoforms for proteasomal degradation.

Another p63-E3 ligase, Nedd4, was identified by a yeast-two-hybrid screen by Bakkers et al. (2005). Nedd4 is a negative regulator of p63 in dorsoventral patterning in zebrafish (57). Nedd4, like WWP1 and Itch, binds to the C-terminal PY region of p63α and mediates ubiquitin-proteasomal degradation of the transcription factor. Bakkers et al. reported that Nedd4 was important for regulating the spatial expression of the ectoderm during embryonic development, essentially restricting neural development within the forebrain and not the dorsal region due to Nedd4 destabilization of p63. RACK1 has also been shown to regulate p63 levels. RACK1, a scaffold protein and component of the activated protein C kinase-Elongin C/B E3 ubiquitin ligase complex, promotes the ubiquitylation-mediated degradation of ΔNp63 (58).

In addition to ubiquitin-mediated degradation, other post-translational modifications such as sumoylation affect p63 stability and transcriptional activity within the cell (50, 57, 59, 60). Sumoylation is a post-translational modification that covalently links SUMO (small ubiquitin-like modifier, a 10 kDa protein) to a given substrate. Similar to ubiquitylation, sumoylation tags specific proteins, which can modify their sub-cellular localization, transcriptional activity and/or stability within the cell (50). The C-terminus of p63α contains a sumoylation consensus sequence (ψKXE; where ψ represents a hydrophobic residue and X is any amino acid).
downstream of the SAM domain (IKEE). This sequence facilitates the association of p63 with Ubc9, the catalyzing enzyme that covalently attaches SUMO-1 to K637 or at a secondary site K549 on the alpha isoforms exclusively (59, 60). The β and γ isoforms do not contain the C-terminal consensus sequence and thus do not get sumoylated. Ghioni et al. (2005) and Huang et al. (2004) determined that SUMO-1 conjugation effectively destabilizes p63α by targeting it for proteasomal degradation but does not affect its subcellular localization. Moreover, mutations that abolish p63α sumoylation, such as K549E and K637E/R or naturally occurring mutations found in split hand/feet malformations (K634X), results in a dramatic increase in transcriptional activity compared to wild type. Sumoylation-defective mutants potentiate an even stronger transactivation potential on genes that are upregulated by p63. This is attributed to p63 mutants’ stabilization within the cell, which causes genes to be continuously activated. Moreover, Huang et al. (2004) demonstrated that genes involved in bone development and osteoblast differentiation, such as RUNX2 and MINT, are improperly regulated by sumoylation-deficient TA p63α mutants, suggesting that aberrant p63 stability and/or transactivation by sumoylation can lead to a subset of ectodermal dysplasia (60).

1.4.5 MDM2 and p63

The tumour suppressor p53 is stabilized and activated to induce genes involved in cell-cycle arrest or apoptosis under various cellular stresses such as DNA damage and hypoxia (10, 66). Under normal unstressed conditions, the activity and levels of p53 are kept low, mainly through its association with the RING finger E3 ubiquitin ligase MDM2 (65). Initial studies demonstrated that MDM2 inhibited p53 function by physically interacting with the N-terminal transactivation domain of p53 thereby preventing the transcription of downstream target genes that suppress tumour formation (50). Later studies identified that MDM2 also destabilizes p53 via its E3 ligase activity that promotes the poly-ubiquitinylation of p53 which targets it for proteasomal-mediated degradation (50, 65). During cellular stress, MDM2 association with p53 is inhibited by ARF which binds to MDM2 leading to increased p53 stabilization and subsequent activation of cell-cycle arrest or apoptosis. Interestingly, p53 is able to upregulate the expression of its negative regulator by directly binding to a p53 consensus sequence in the MDM2 promoter and inducing its expression (50, 65, 66). As a consequence, p53 is able to control its own levels by upregulating MDM2 thus forming a negative autofeedback loop mechanism that keeps p53
levels low. Mutations that affect the DBD of p53 are known to stabilize the p53 protein at least in part through the disruption of MDM2-p53 feedback pathway (66).

Given the importance of MDM2 in regulating the activity and protein levels of p53, several groups investigated whether MDM2 had an effect on the other p53 family members p73 and p63. Studies involving p73 demonstrated that MDM2 retained its ability to bind to the N-terminal transactivation domain of p73 through three conserved p73 residues that directly contact MDM2 in p53. However, unlike p53, overexpression of MDM2 leads to p73 stabilization instead of promoting its degradation. This difference was concluded to be a consequence of additional residues that are responsible for MDM2 mediated degradation of p53 being absent in p73. Studies investigating MDM2 and its affect on p63 transactivation and protein stability have reported conflicting data. Little and Jochemsen (2001) reported that MDM2 was unable to physically associate with either the TA or ΔN p63 (74). Furthermore, unlike p53, MDM2 was unable to repress p63 transactivation of the p21 promoter and had no influence on the half-life of p63 which was also demonstrated independently by another research group (73,74). In contrast to these findings, Calabro et al. (2002) concluded that p63 retains its ability to bind to MDM2 (75). This interaction stabilizes p63 enhancing its transactivation of various target genes. Furthermore, these effects were augmented when the MDM2 inhibitor ARF was co-transfected with p63 and MDM2, leading to p63 destabilization and a reduced transactivation (75). More recently, MDM2 was shown to indirectly facilitate p63 degradation by cooperating with the E3 ligase Fbw7 to promote its degradation (76). The researchers demonstrate that MDM2 binds to nuclear p63 and facilitates its export out of the nucleus. Once p63 is localized in the cytoplasm Fbw7 targets p63 for degradation via ubiquitin mediated proteolysis. Mutation of the MDM2 nuclear localization effectively abolished p63 degradation, whether as the RING finger domain which catalyzes ubiquitin transfer to its substrates has no effect on p63 stability (76). Taken together, it remains unclear whether MDM2 has an effect on p63 stability and transactivation and warrants further studies to investigate whether MDM2 regulates p63 under physiologic conditions.
1.4.6 Potential Autofeedback Loop Mediates p63 Degradation

It is well known that p53 mutations within the DNA binding domain drastically increase the protein’s stability by disrupting the infamous MDM2-p53 autofeedback loop. p53 levels are kept low within the cell by MDM2-mediated destabilization. MDM2 is a downstream target gene of p53, thus the tumour suppressor regulates its own levels by upregulating its negative regulator, thus completing the feedback loop. Similar to p53, Ying et al. (2005) showed that EEC mutations in the DNA binding domain give rise to DNA binding deficient, highly stable proteins, reminiscent of p53 DBD mutants. The researchers demonstrated that overexpression of TAp63γ was able to act in trans to promote the degradation of the EEC R304W TAp63γ, but not vice versa suggesting that retention of p63’s DNA binding capacity is essential for its degradation and that an unknown negative regulator may possibly form an autofeedback loop reminiscent of p53.
2 AIMS, HYPOTHESIS AND RATIONALE

Genetic mutations in the \( p63 \) gene give rise to the rare disease known as ectodermal dysplasia; however the mechanisms by which these mutant proteins disrupt \( p63 \) function have not been elucidated. Studies have shown that both EEC and AEC mutations are typically transcriptionally inactive, however it is unknown whether \( p63 \) proteins lose their transactivation ability due to structural alterations that disrupt DNA binding or whether the mutants affect wild type \( p63 \) ability to transactivate target genes. Furthermore, it remains to be determined whether mutations in \( p63 \) alter other properties of the protein including its subcellular localization and stability. We hypothesize that EEC and AEC mutations alter the structure and function of \( p63 \) which contributes to the clinical phenotypes associated with the disease. To address this hypothesis, we have identified five specific aims to investigate. Our first aim was to determine whether the apparent loss of transcriptional activity was due to affects on the ability of \( p63 \) mutants to bind DNA in target gene promoters. Studies have shown that both EEC and AEC mutants cannot transcriptionally activate certain \( p63 \) target genes. Given that the point mutations in most EEC mutants are predicted to affect key residues that contact DNA, we hypothesize that the EEC mutants will be unable to bind to a \( p63 \) response element while AEC mutant proteins, which have an unaltered DBD, will retain their ability to bind to the response element. Our second aim was to determine whether the EEC and AEC mutants are able to transactivate the \( PERP \) promoter, and whether they act in a dominant negative manner towards wild type \( p63 \). In light of the fact that the EEC and AEC syndromes exhibit autosomal dominant modes of inheritance, we hypothesize that mutant EEC and AEC mutant \( p63 \) proteins inhibit the function of wild type \( p63 \) in a dominant negative manner through the formation of inactive hetero-oligomers. Our third aim was to determine whether mutations in the DBD domain or the SAM domain affect the stability of \( p63 \) in the cell. McGrath et al. demonstrated that in contrast to wild type \( p63 \), AEC mutant \( p63 \) is no longer exclusively localized to the basal regenerative layer of the epidermis in AEC patients. Instead AEC mutant \( p63 \) is also detected in the suprabasal layer. We hypothesize that the presence of mutant \( p63 \) in the suprabasal may be due to aberrant \( p63 \) turnover which results in prolonged stabilization in the upper layers of the epidermis. Similarly, mutations in p53 result in enhanced stability and prolonged half life. Taken together, we hypothesize that both EEC and AEC mutants may have prolonged half-lives compared to wild type \( p63 \). Our fourth
**aim** was to determine whether EEC and AEC mutations in the DBD or SAM domain respectively affect the subcellular localization of the proteins. Since p63 must be nuclear in order to bind promoters of target genes, if p63 mutations result in aberrant localization to the cytoplasm p63 will be unable to regulate target genes, including those involved in the development and maintenance of ectodermal structures and tissues. We hypothesize that p63 EEC and AEC mutants retain their ability to translocate into the nucleus based on our preliminary sequence studies that indicate that these mutations do not interfere with putative nuclear export or nuclear import sequences. Our **fifth and final aim** was to investigate the putative intramolecular interaction between the OD and SAM domain in both p63 and p73. A structural NMR investigation of the *C. elegans* p53-like homologue (CEP-1) has shown that the SAM domain helps to stabilize the transcription factor through key contacts with the oligomerization domain (9). In light of the sequence similarity and overall structural topology between the SAM domains of p63, p73 and CEP-1, we hypothesized that these structural elements serve a stabilizing role in human p63 and p73 through interactions between the OD and SAM as well. Together, the experiments performed during my Masters studies have provided insight into the mechanisms by which the altered activities of p63$^{EEC}$ and p63$^{AEC}$ may lead to the clinical phenotypes associated with ectodermal dysplasia.
3 MATERIALS AND METHODS

3.1 Cell Lines and Transfections

H1299 lung carcinoma, HEK 293A human embryonic kidney and Saos-2 osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco’s modified Eagles medium (Giboco; Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HyClone; Logan, UT) at 37°C in a humidified 5% CO₂ atmosphere. Plasmid DNA was introduced into cell monolayers (~80% confluent) by transfecting 3-10 ug of plasmid DNA for 24 hours using the PEI (polyethylenimine) method. Fresh media was replaced 24 hours later. Experiments and assays were performed approximately 48 hours post-transfection. For all experiments, equal amounts of DNA were used for each sample.

3.2 Plasmids

pcDNA3.1 (Zeo⁺) mammalian expression plasmids encoding T7-ΔNp63α and 3xFlag-ΔNp63α (herein called 3F-ΔNp63α) were recently described (61). The 3F-ΔNp63α mutants were created by site-directed mutagenesis and were sequenced to ensure the absence of secondary point mutations. These mutations were previously generated by a PhD student in the lab, J.Chung. The ΔOD construct (residues 1-285 of ΔNp63α) was also created by J.Chung. The pRK5-myc-Itch and catalytically inactive pRK5-myc-Itch C830A was previously described and generously provided by Dr. Tony Pawson (62), the identity of the plasmids were confirmed by sequencing. pcDNA3-HA-Ubiquitin was a gift from Dr. Michael Ohh. The PERP luciferase reporter plasmid, pPerpLucPS was described previously and kindly provided by Dr. Laura Attardi (20). The pET15b vector was provided to us by Dr. Cheryl Arrowsmith (21).
3.3 Antibodies

The following commercial antibodies were used:

<table>
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<th>Antibody</th>
<th>Company</th>
<th>Clone</th>
<th>Species</th>
<th>Dilution</th>
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3.4 Immunoprecipitation and Immunoblotting

Cell monolayers grown in 6-well or 10 cm tissue culture plates were scraped from the surface of the dishes and collected by centrifugation. Cells were then washed in ice-cold PBS and lysed in EBC buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% [v/v] Nonident P-40) supplemented with complete protease inhibitors (contains aprotinin, bestatin, calpain inhibitor I and II, chymostatin, E-64, leupeptin, α2-macroglobulin, pefabloc SC, pepstatin, PMSF, TLCK-HCl and trypsin inhibitor) (Roche; Indianapolis, IN) and rocked at 4°C for 30 minutes. Cell debris was cleared by centrifugation and protein concentrations were quantified by the Bradford method (Bio-Rad; Hercules, CA). Immunoprecipitation and immunoblot analysis procedures were previously described (63). In short, immunoprecipitations were performed with whole cell lysates with the indicated antibody for 2 hours at 4°C rocking with protein A-Sepharose beads (Amersham Biosciences; Sweden). The immunoprecipitates were then washed five times with NETN buffer (2 M Tris [pH 8.0], 5 M NaCl, 0.5 M EDTA [pH 8.0], 0.5% [v/v] NP-40) to remove non-specific proteins. The beads were boiled with SDS sample buffer to elute the proteins which were then resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad) for immunoblot analysis as described previously (63). In some experiments, Western blots were developed using the LiCor Odyssey infrared image system (LiCor; Lincoln, NE) as
directed by the manufacturer’s protocol. Briefly, nitrocellulose membranes were blocked with Odyssey Blocking Buffer (LiCor) for an hour at room temperature. Primary antibodies were diluted in Odyssey Blocking Buffer and rocked overnight at 4°C. Membranes were washed five times at room temperature with TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% [v/v] Tween 20 [Sigma; St.Louis,MO]) and incubated with either mouse or rabbit Infrared IRDye-labeled secondary antibodies (LiCor) diluted in a 1:1 ratio of TBST and Odyssey Blocking Buffer. After 1 hour incubation period at room temperature, membranes were washed five times with TBST and bound secondary antibodies were detected using the LiCor Odyssey infrared image system.

3.5 Ubiquitinylation Assays

Cell monolayers were grown in 10cm tissue culture plates and were pre-treated with MG132 (20 μM) for 4 hours prior to harvesting to accumulate ubiquitinated proteins of interest. Cells were harvested from dishes by scraping and were washed once with cold PBS and resuspended in 10% PBS and lysed in 90% lysis buffer (20 mM Tris [pH 7.35], 250 mM NaCl, 3 mM EDTA, 3mM EGTA, 0.5% [v/v] NP-40, 2 mM DTT, 5 mM NEM, 2mM iodoacetamide, 1% SDS [v/v]) supplemented with complete proteosome inhibitor (Roche). Samples were briefly vortexed followed by a 20 minute incubation at 100°C to denature proteins. Insoluble cell debris was removed by centrifugation at 13,200 rpm for 10 minutes. 10% of the sample by volume was collected for sample inputs; the remaining sample volume was diluted 10 times with lysis buffer prepared without SDS. Immunoprecipitation and immunoblotting was performed as described in the previous section.

3.6 Protein Degradation: Cycloheximide Chase Assay

H1299 cells were transfected with equal amounts of plasmids encoding 3F-ΔNp63α and myc-Itch or pcDNA3.1. 48 hours post-transfection cells were treated with 40 ug/mL cycloheximde (Sigma) and harvested at 0, 4, 8 and 12 hours post-treatment. Cells were lysed and subjected to p63 and Itch immunoblot analysis with anti-Flag and anti-Itch antibodies respectively using the LiCor protocol described earlier. Protein levels were quantified using the LiCor Odyssey infrared image system software and were expressed as a percentage of the untreated 0-hour time point.
3.7 In Vitro Translation

In vitro translation was performed using the TNT translation system as described by the manufacturer’s protocol. Proteins were synthesized by incubating 1 ug of the indicated plasmid per 10 uL of TNT mix and 1 uL of 1 mM methionine for 1.5 hours at 30 ºC.

3.8 Electrophoretic Mobility Shift Assay

DNA-binding experiments were performed using a radiolabeled 23-oligomer probe containing a degenerative p53-binding site also predicted to bind p63 (herein referred to as the p63 DNA binding site (63). Briefly, the synthetic oligonucleotide (sense strand 5’-AGCTTAGCATGTCTAGGCAT) was annealed to its reverse compliment and end-radiolabeled with [γ-32P] adenosine 5’-triphosphate (Perkin-Elmer; Waltham, MA) using T4 polynucleotide kinase (New England Biolabs; Ipswich, MA) as described by the manufacturer. The DNA binding reaction was comprised of 3 uL of the indicated TNT protein product (or empty vector), 2.5 uL 10X binding buffer (50 mM Tris [pH 7.5], 40% glycerol, 100 mM KCl, 10 mM DTT, 2 mg/mL bovine serum albumin and 0.2% [v/v] Triton X-100), 1 uL poly dI-dC (1 ug/uL) and 1 ng of 32P radiolabeled p63 binding site probe (50,000 – 100,000 cpm) for a final volume of 25 uL. Where indicated, control binding reactions were also carried out in the presence of a vast molar excess (300 – 350X) of unlabelled specific or scrambled nonspecific (sense strand 5’-CCAGCTAGCAGGCACATCA) competitors as controls. Binding reactions were incubated at room temperature for 15 minutes. For antibody super-shift experiments, 2 uL of the indicated antibody was added to the reaction mixture and was incubated for an additional 15 minutes at room temperature. DNA-protein complexes were separated on a 5% native PAGE gel that was pre-run with 0.5X Tris-borate-EDTA (pH 8.0) buffer. The gel was run in a 4ºC cold room for 4-5 hours at 200V (~15mA). The gel was dried at 72ºC for 1 hour and autoradiography was performed at ~80ºC in the presence of an intensifier screen. To estimate the size of the protein-DNA complexes in the native gel we followed a protocol that was described previously (64). In brief, a native gel size ladder was run in tandem with the EMSA experiment. Once the proteins were resolved by electrophoresis, the portion of the gel containing the size ladder was
carefully excised from the rest of the gel. The length of the gel was measured before and after staining/destaining in order to account for the shrinkage that occurs during the destaining process used to visualize the protein standards. The reference points were measured from the bottom of the wells to the end of the gel. The distance travelled by the protein standards were measured (and then corrected with the calculated shrinkage factor) and plotted as a function of their respective molecular weight to produce a standard curve and an equation for which to estimate the mass of the protein-DNA complexes in the EMSA. The mass of the DNA probe was subtracted from the calculated mass of the protein complex assuming that protein complex and DNA probe was in a 1:1 ratio.

### 3.9 Dual Luciferase Assay

H1299 cells were grown in 6-well tissue culture plates to ~80% confluence. Triplicate samples were transfected with 500 ng of either wild type T7-ΔNp63α or mutant 3F-ΔNp63α in presence of 500 ng of PERP luciferase reporter plasmid (pPerpLucPS) and 250 pg of renilla. The total amount of transfected DNA was equilibrated to 3 μg with pcDNA3.1 vector. Master mixtures of the reporter plasmids were used 48-hours post transfection luciferase assays were performed using the Dual-Luciferase Reporter Assay (Promega; Madison, WI). Luciferase activity was normalized to renilla activity and reported as mean activation with a standard deviation. Dominant negative experiments were performed by transfecting 500 ng T7-Np63α in the presence of increasing amounts (0, 200, 500 and 2000 ng) of EEC (R304W) or L514F (AEC) mutant 3F-ΔNp63α . Lysates were pooled from the triplicate samples for Western blot analysis.

### 3.10 Immunofluorescence Microscopy

H1299 cells were seeded into 4-well chamber slides at a concentration of 2x10^5 cells per chamber. The following day cells were transfected with 500 ng of plasmid encoding wild type or mutant 3F-ΔNp63α as described previously. 48 hours post-transfection media was aspirated and slides were air-dried for 5 minutes and then washed three times with PBS. Cells were fixed with 4% paraformaldehyde (v/v) dissolved in PBS for 15 minutes at room temperature and then washed three times with PBS. Cells were permeabilized with 0.2% Triton X-100 (v/v) for 5
minutes, washed three times with PBS, and blocked for 1 hour at room temperature with 6\% normal goat serum (v/v) and 0.5\% bovine serum albumin (w/v) in PBS. Primary anti-mouse Flag antibody was then added in PBS containing 3\% normal goat serum (v/v) and 0.25\% BSA (w/v) and left overnight at 4°C. Primary antibody was removed, cells were washed three times with PBS, and incubated for 1 hour at room temperature with Alexa 555-conjugated goat-anti-mouse (1:1,000; Molecular Probes). Cells were washed three times for 5 minutes with PBS, stained with DAPI, and visualized using a Zeiss Axioptan upright microscope.

### 3.11 NMR Spectroscopy and Protein Purification

Isolated C-terminal constructs of the human p63 oligomerization domain (residues 290-350 and 290-357) and the SAM domain (447-518) were synthesized using the PCR method with the following primers:

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<td>5’GCTACTCTCGAGCCTCCGTATCCCAACGATTC</td>
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PCR fragments were subcloned into the pET15b vector (Novagen, San Dieago, CA) which contains an N-terminal hexahistidine tag upstream of a thrombin cleavage site. Recombinant proteins were expressed in *Escherichia coli* grown in M9 media and were purified using Ni\(^{2+}\) resin chromatography. NMR protein samples were prepared in 25 mM sodium phosphate [pH 7.0] containing 150 mM NaCl, 1 mM tris-2-carboxyethyl phosphine and 1 mM benzamidine; the final NMR samples contained 90\% H\(_2\)O, 10\% D\(_2\)O with a protein concentration ranging between 0.4 and 0.5mM. Aliquots of p63-OD domains were titrated into the \(^{15}\)N labeled p63-SAM domain in molar ratios 1:1, 1:3, 1:5 and 1:10. Chemical shifts were detected in the 1H-15N HSQC spectra which were acquired and analyzed after each addition of ligand. All spectra were
recorded at 25°C on Bruker Avance 800 MHz spectrometer equipped with $^1$H/$^{13}$C/$^{15}$N triple resonance cryoprobe. Two dimensional, gradient-enhanced $^1$H/$^{15}$N HSQC spectra were processed with NMRPipe software and analyzed with SPARKY program. p63 NMR data was collected with the assistance of Dr. Lilia Kaustov in collaboration with Dr. Cheryl Arrowsmith. p73 NMR data was previously collected by Jacky Chung (a PhD student in the Irwin lab) along with Dr. Lilia Kaustov (Arrowsmith lab).
4 RESULTS

4.1 AEC but not EEC Mutants Can Bind to a p63 Response Element

p63 interacts with target gene promoters through contacts within the DNA binding domain (see Figure 1) and possibly through residues located in other domains of the protein. The ability of p63 to bind DNA is linked to its activity as a transcription factor. Given that EEC mutations generally fall within the DNA binding domain of p63, we hypothesized that these point mutations disrupt protein-DNA interactions and prevent p63 from properly binding to target gene promoters. Furthermore, it is possible that SAM domain mutations may also alter the ability of p63 to physically interact with DNA consensus sequences. To determine whether p63 mutations affect DNA binding, we performed electromobility shift assays (EMSA) using a 23mer radiolabeled probe that contains a canonical p53 DNA-binding site which p73 has also been shown to bind (63). Using EMSA, we investigated the ability of wild type ΔNp63α, four ΔNp63α mutants, two EEC (R204W and R304W) and two AEC mutants (L514F and G530V) to bind DNA (Figure 5). As expected, both the EEC mutants R204W and R304W were unable to bind to the p63 response element which was previously shown for TAp63γ R204W and R304W (Figure 5 A; lanes 6-11) (46). In contrast, wild type p63 and the two AEC mutants L514F and G530V were able to bind the DNA binding sequence. With the addition of Flag antibodies we were able to supershift these complexes indicating they contained p63 protein (Figure 5 A; lanes 5,8,11,14 and 17). Interestingly, in contrast to wild type p63, the AEC mutant binding produced three distinct bands; wild type p63 resulted in only one. The two additional bands ran slightly below where the protein-DNA complex that was observed in the wild type lane (Figure 5 A; lanes 2). We speculated that the additional complexes may be different oligomeric states which retain their ability to bind to the p63 response element. To test this, we performed EMSAs employing a technique in which native gel protein size markers were used to estimate the molecular masses of the various AEC DNA-protein complexes (Figure 6). As expected, the estimated mass of the wild type complex was 262.9 kDa which correlates favourably to the theoretical mass of 275.2 kDa for tetrameric 3F-ΔNp63α (Figure 6 A; lane 2 and B). Similarly,
the top bands of both the L514F and G530V variants correlate to the mass of a tetrameric p63 complex as indicative of the estimated ~278 kDa and 295 kDa masses respectively. The estimated sizes of the middle and lower complexes of the AEC mutants correlate to dimeric and monomeric p63 states (Figure 6A; lane 6 and 9 respectively and B). The mutant I378P, which is unable to form oligomers, was unable to bind DNA (Figure 7B; lane 6-8). Given that the AEC mutations reside within the SAM domain, we asked whether a truncated p63 construct which lacks residues downstream of the oligomerization domain (ΔOD), including the SAM domain, would have a similar effect to point mutations in the SAM domain. In Figure 7A, lane 6 the ΔOD construct exhibits a similar banding pattern reminiscent of the AEC mutants; however, two complexes are observed for ΔOD compared to three observed with AEC mutants. Taken together, the SAM domain may have a stabilizing effect on the oligomeric state of p63 whereas introducing point mutations or eliminating the domain altogether may affect the quaternary state of the protein.
Figure 5: AEC (SAM Domain) mutants but not EEC (DNA-Binding Domain) mutants bind to a p63 binding-site. (A) A $^{32}$P-radiolabelled p63 DNA-binding site was incubated with in vitro translated 3F-ΔNp63α (A, lane 2-5), EEC mutants: R204W (A, lane 6-8), R304W (A, lane 9-11), AEC mutants: L514F (A, lane 12-14), G530V (A, lane 15-17) or vector reticulocyte lysate (A, lane 1) and subjected to electrophoretic mobility shift analysis in a 5% native PAGE gel. Binding reactions were carried out in the presence of a vast molar excess of unlabelled specific or scrambled (Scr) nonspecific competitor DNA where indicated. Anti-T7 (wild type p63) or anti-Flag (mutant p63) was incubated with the complex prior to running the gel as indicated. (B) Western blot inputs of in vitro translated ΔNp63α (10% by volume). N=2.
Figure 6: AEC mutants produce multiple protein-DNA complexes that correlate to molecular weights of tetrameric, dimeric and monomeric ΔNp63α. (A) A $^{32}$P-radiolabelled p63 DNA-binding site was incubated with in vitro translated 3F-ΔNp63α (A, lane 2-5), AEC mutants: L514F (A, lane 6-8), G530V (A, lane 9-11) or vector control reticulocyte lysate (A, lane 1) and subjected to electrophoretic mobility shift analysis in a 5% native PAGE gel. The AEC mutant-DNA complexes are indicated by asterisks (*). (B) Top Panel: Standard curve to estimate the molecular mass of the protein-DNA complexes. The migration distance of native gel size ladder standards plotted as a function of their respective molecular weight. A line of best fit was added; the regression equation and $R^2$ value are also displayed. Lower Panel: Estimated molecular mass (corrected for the mass of the probe$^2$) and oligomeric stoichiometry of the wild type and AEC protein-DNA complexes. (C) Western blot inputs of in vitro translated ΔNp63α (10% by volume). N=1.

$^2$ With the assumption that the protein complex to DNA probe is in a 1:1 binding ratio.
Figure 7: Deletion of the SAM domain causes differential banding reminiscent of AEC mutants (A) and oligomeric defective mutant p63 (I378P) is unable to bind DNA (B). A $^{32}$P-radiolabelled p63 DNA-binding site was incubated with in vitro translated 3F-ΔNp63-ΔOD (A, lane 6-8), monomeric mutant I378P (B, lane 6-8) and subjected to electrophoretic mobility shift analysis in a 5% native PAGE gel (C) Western blot inputs of in vitro translated 3F-ΔNp63α (10% by volume). In both (A) and (B) the two panels were conducted within the same experiment. N=1.
4.2 EEC and AEC Mutants are Transcriptionally Inactive and Form Dominant Negative Complexes with Wild Type p63

We hypothesized that mutations in the DBD and SAM domains of p63 may affect the transcriptional activation of known p63 target genes and may hetero-oligomerize with wild type p63 to affect its activity. To address these questions we performed dual luciferase assays with a plasmid in which the promoter region of PERP, a p63 target gene involved in desmosomal adhesive complexes in the epithelia, was cloned upstream of luciferase (20). We first examined whether the EEC mutants R204W and R304W and AEC mutants L514F and G530V were able to transactivate the PERP promoter in p53−/− H1299 cells

**Figure 8 A**. As expected, wild type ΔNp63α was able to transactivate the PERP promoter; however none of the EEC or AEC mutants tested were able to significantly active transcription. We then performed titration experiments in which we transfected increasing amounts of either, EEC R304W **Figure 8 B** or AEC L514F **Figure 8 C** to investigate whether these mutant p63 proteins can act as dominant negatives over wild type p63. As previously reported (19), both the EEC and AEC mutants were unable to transactivate the PERP promoter compared to the wild type. Luciferase activity decreased in a dose-dependant manner when either R304W or L514F was added with wild type ΔNp63α. To validate that neither the T7 nor 3F tag differentially affect transactivation of the PERP reporter construct, we compared their activity in a single experiment and found no appreciable difference (**Figure 8 D**). However, compared to the PERP promoter activity observed in Figures A-C, we noted lower levels of arbitrary PERP promoter units, albeit the relative levels of transactivation were the same.

To determine whether mutant p63 and wild type p63 can form hetero-oligomers to perhaps explain the dominant negative activity, we performed co-immunoprecipitation experiments. Co-immunoprecipitation confirmed binding between wild type and mutant p63 (R204W, R304W, L514F and G530V) suggesting that the formation of inhibitory complexes are likely responsible for the dominant negative phenotype (**Figure 9**). In summary, both the EEC and AEC mutants that were tested demonstrate a dominant negative phenotype over wild type
p63. In the case of the EEC mutant, it is believed that R304W inactivates wild type solely by interacting and sequestering its transactivation activity where as the AEC mutant likely binds to and inactivates p63, but may also compete with the wild type for promoter binding sites.
Figure 8: EEC and AEC mutants are transcriptionally inactive and exert a dominant negative affect over wild type p63. PERP reporter constructs were transfected into H1299 (null of p63 expression) along with wild type or mutant ΔNp63α; graph depicts the mean induction activity from triplicate experiments along with ± SD, and P values calculated by student unpaired t-tests relative to vector (***p<0.001, **p<0.005, *p<0.03). (A) Single transfections with 3F-ΔNp63α wild type, EEC (R204W and R304W) or AEC (L514F and G530V) mutants resulting in a loss of transactivation by the various p63 mutants. N=2 (B) and (C) PERP promoter was transfected with or without T7-wild type ΔNp63α in presence of increasing amounts of 3F-ΔNp63α mutant EEC R304W (B) or AEC L514F (C) (0:0.2, 1:1 and 4:1) demonstrating a dominant negative effect over wild type p63. N=2 for both EEC R304W and AEC L514F. (D) T7-and 3F-ΔNp63α wild type control experiment demonstrating that neither tag significantly interferes with transactivation of the PERP promoter construct.
Figure 9: EEC and AEC Mutant p63 is able to form hetero-oligomers with wild type p63. H1299 cells were transfected with equal amounts of Flag-tagged mutant and T7-tagged wild type plasmids. Cells were lysed and equal amounts of proteins were immunoprecipitated with anti-Flag and analyzed by Western blot analysis as indicated. Control immunoprecipitations shown on the left were conducted in the same experiment and are from the same blot. Mock and single transfections with either T7-wild type p63 or 3F-wild type p63 alone were immunoprecipitated demonstrating that neither tag results in a non-specific band being pulled down. 10% input controls are shown below their respective lanes of the co-immunoprecipitation. EEC and AEC mutant p63 is able to form dominant negative heterooligomers with wild type p63. N=2
4.3 Nuclear Localization is Unaffected by EEC and AEC Mutations

Cellular trafficking of proteins is essential for proper gene regulation and overall physiological function within the cell. Transcription factors must be imported into the nucleus after translation in the cytoplasm in order to modulate target gene expression. Conversely, export of these proteins out of the nucleus is equally as important (65). We sought to determine whether point mutations in the DBD or SAM domain may affect transcriptional activation due to changes in the subcellular localization of p63. This may explain why the EEC and AEC mutants were unable to activate the PERP promoter tested in Figure 8, as well as other reported target genes (15, 19, 36). If in contrast to wild type p63, mutant p63 is not able to properly enter the nucleus and accumulates in the cytoplasm, it will be unable to modulate downstream target genes. We investigated four ΔNp63α point mutants, two EEC mutants (R204W and R304) and two AEC SAM domain mutants (L514F and G530V). All of these proteins, including wild type p63 were Flag tagged at the N-termini, allowing us to visualize the location of transfected proteins in the cell by immunofluorescence microscopy with an anti-Flag antibody. We chose to overexpress these proteins in H1299 cells in which p63 is not detectable by Western blot analysis (data not shown). Our results indicate that, similar to wild type ΔNp63α, both the EEC mutants R204W and R304W (Figure 10 A) and the AEC mutants L514F and G530V (Figure 10 B) localize to the nucleus. We also observed that a few cells expressing the R304W mutant resulted in cytoplasmic localization of p63 (right panel). However, the majority (>95%) of the R304W mutant was nuclear. We suspect that the observed cytoplasmic localization is an artifact of overexpression and is not a result of the mutation itself.
Figure 10: Nuclear Localization Is Unaffected by EEC and AEC Mutations.
Immunofluorescence analysis in H1299 cells using an anti-Flag antibody to stain Flag-tagged mutant or wild type ΔNp63α (red) and DAPI staining of the nuclei (blue). (A) EEC mutants and (B) AEC mutants do not alter the nuclear localization of ΔNp63α. N=1.
4.4 EEC and AEC mutations increase p63 stability

Certain mutations in p53 (e.g. in the DNA binding domain) result in increased p53 half-life within the cell (66). We hypothesized that similar to p53, some p63 mutations may have effects on protein stability. Another reason that we suspected p63 half-life may be enhanced by p63 mutations was based on skin biopsies of AEC patients that revealed that, in contrast wild type p63, AEC mutant p63 proteins are detected in the suprabasal levels of the skin which we hypothesize may be due to aberrant p63 degradation (42). Moreover, Rossi et al. (2006) has shown that Itch, an ubiquitin E3 ligase is an important negative regulator of p63 levels in the epidermis. Itch binds p63 through the PY motif located upstream of the SAM domain. Given that AEC mutations in the SAM domain are located a few residues downstream of the PY motif, this may alter Itch from binding to AEC mutant proteins, thus preventing their degradation. Thus, we hypothesized that mutations of p63\textsuperscript{EEC} or p63\textsuperscript{AEC} may alter the ability of Itch to promote its degradation, effectively leading to increased levels of the mutants within the cell. To test this we asked whether: (1) Itch affected wild type or mutant p63 half-life and (2) whether Itch bound mutant forms of p63. We have performed cycloheximide assays to determine and compare the half-lives of wild-type, EEC R204W and R304W and AEC L514F and G530V ΔNp63α both in the presence and absence of Itch.

**Figure 11.** In the absence of Itch, the $t_{1/2}$ of wild type p63 was 6 hours, which is similar to the G530V mutant (5 hours). In contrast, both the EEC mutants R204W and R304W and L514F mutations stabilize p63, with L514F showing a 33% increase in half life (9 hours) and a double increase in R204W and R304W $t_{1/2}$ (12 and >12 hours respectively). In the presence of Itch, we found that all of the mutants are destabilized, although to varying degrees. As expected, wild type p63 is degraded by Itch reducing its half life from 6 to 3 hours. Similar to wild type, the levels of the two AEC variants are drastically reduced by the presence of Itch ($t_{1/2} = 3$ hours for both L514F and G530V). In comparison to wild type and the AEC mutants, the EEC associated R204W and R304W had delayed degradation in the presence of Itch (8 hours and 11.5 hours respectively). We asked whether the stability of the EEC and AEC mutants could be attributed to altered Itch binding and/or ubiquitinylation of p63. **Figure 12** shows that neither the DBD nor SAM domain mutations affect the ability of p63 to bind to Itch. Furthermore, all of the mutants...
examined are polyubiquitinated by Itch as evident by the presence of higher migrating bands in the presence of ubiquitin seen in Figure 13. Higher migrating p63 bands can also be seen at 95 kDa and slightly below 130 kDa for wild type and all of the mutants. However, there were no discernable differences between the pattern of modification/ubiquitinylation between wild type and mutant p63 suggesting the mutants’ stability is not due to aberrant Itch ubiquitin-mediated degradation.
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<tr>
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| ii) | R204W | + myc-Itch |
| Hour | 0 | 4 | 8 | 12 | 0 | 4 | 8 | 12 |
| Itch | IB: Itch |
| p63 | IB: Flag |
| β-Actin | IB: β-Actin |

| iii) | R304W | + myc-Itch |
| Hour | 0 | 4 | 8 | 12 | 0 | 4 | 8 | 12 |
| Itch | IB: Itch |
| p63 | IB: Flag |
| β-Actin | IB: β-Actin |

| iv) | L514F | + myc-Itch |
| Hour | 0 | 4 | 8 | 12 | 0 | 4 | 8 | 12 |
| Itch | IB: Itch |
| p63 | IB: Flag |
| β-Actin | IB: β-Actin |

| v) | G530V | + myc-Itch |
| Hour | 0 | 4 | 8 | 12 | 0 | 4 | 8 | 12 |
| Itch | IB: Itch |
| p63 | IB: Flag |
| β-Actin | IB: β-Actin |
Figure 11: Protein stability of the EEC and AEC mutants in the presence of Itch. H1299 (null of p63 expression) were transfected with 3F- ΔNp63α wild type (i), R204W (ii), R304W (iii), L514F (iv) or G530V (v) in the presence and absence of myc-Itch. 48h post-transfection, cells were treated with 40 μg/mL of cycloheximide and lysed at the indicated times. Equal amounts of whole cell extract were resolved on a SDS-PAGE gel and analyzed by Western blot analysis (left panels). Protein levels were quantified by densitometry using Licor Odyssey software and expressed as a percentage of the no treatment baseline (right panels). N=2.
**Figure 12: Itch binds to both EEC and AEC mutants.** H1299 cells were transfected with equal amounts of plasmids encoding 3F-\(\Delta\)Np63\(\alpha\) and myc- Itch plasmids. Cells were lysed and equal amounts of whole cell extracts were immunoprecipitated with anti-Itch antibody and analyzed by Western blot analysis with the indicated antibodies. 10% lysate input controls are shown below their respective co-immunoprecipitations. Control immunoprecipitations with mock and single transfections with either myc-Itch or 3F-wild type p63. In the 3F-wild type control lane we see p63 being pulled down in the absence of myc-Itch. This is due to endogenous Itch being immunoprecipitated by the Itch antibody. However, in the mock control where neither myc-Itch or 3F-wild type p63 were transfected immunoprecipitation with the Itch antibody did not pull down N=1.
Figure 13: Itch ubiquitinylates EEC (A) and AEC mutants (B) Ubiquitin assays of EEC and AEC mutants with Itch. H1299 cells were transfected with 3F-Np63α in the absence (“-”) or presence (“+”) of myc-Itch and HA-Ubiquitin (Ub) plasmids. As controls, mock lanes with vector 48 hours post-transfection cells were pre-treated for 4 hours with 20 μM of the proteasome inhibitor MG132 to accumulate ubiquitinylated proteins prior to harvesting cells. p63 was immunoprecipitated with anti-Flag antibody and HA-ubiquitin (red) and 3F-ΔNp63α (green) was detected with the indicated antibodies. 10% input controls are shown below their respective co-immunoprecipitation lanes. No discernable difference between wild type and mutant ubiquitinylation was observed. N=2.
4.5 Oligomerization domain and SAM domain of p73 but not p63 interact and residues involved are not conserved in p63

Structural NMR investigations of the p53-like homologue in *C.elegans* (CEP-1) has shown that the SAM domain helps to stabilize the transcription factor through key contacts with the oligomerization domain; the protein module essential for oligomerization (9). In light of the sequence similarity and overall structural topology between the SAM domains of p63, p73 and CEP-1, we hypothesized that these structural elements serve a stabilizing role in human p63 and p73 as well. We utilized $^{15}$N HSQC NMR spectroscopy to validate the putative OD-SAM interaction of both p63 (*Figure 14 A*) and p73 (*Figure 14 B*) by titrating $^{15}$N/$^1$H labeled SAM domain with their respective OD domain. We observed chemical shifts in the p73 spectra of the labeled SAM domain when we titrated in increasing amounts of the OD indicating that these two domains physically interact with one another; the residues involved at this interface are highlighted in bold (*Figure 14 C*). Surprisingly, we did not observe any perturbations in the HSQC spectra when we titrated the OD of p63 with the SAM domain even at a molar ratio of (10:1 OD:SAM) suggesting that unlike p73, the OD and SAM domain of p63 do not interact with one another. We performed a sequence alignment of the p73 and p63 SAM domains and found that the majority of residues involved in the p73 interactions are not conserved in the SAM domain of p63, thus explaining the absence of the interaction (*Figure 14 C*). With also noted that none of the residues of the p73 SAM domain that make contact with its OD domain are found in CEP-1 suggesting that this interaction is conserved in *C.elegans* using a different set of residues.
Figure 14: p73 residues involved with OD-SAM interaction are not conserved in p63.

$^1$H-$^1$N HSQC spectra of (A) p63-SAM domain in the absence (blue) and presence (red) of p63-OD domains (10:1) (B) p73-SAM domain in the absence (blue) and presence (red) of p73-OD domains (4:1) No interactions have been detected for the p63-OD with p63-SAM domain. Interaction is observed in between the p73-OD with the p73-SAM at a ratio of 4:1 respectively (C) Sequence alignment between p73, p63 and Cep1 SAM domains. Amino acids highlighted in bold indicate the residues involved in the p73 OD-SAM interaction and the analogous positions in p63 and CEP-1; asterisks (*) indicate residues that are not conserved between the three SAM domains. Alignments were performed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw/). N=1
5 DISCUSSION

5.1 p63 Mutants Exhibit Differential Binding of a p63 Response Element

p63 interacts with target gene promoters via contacts through its DBD and perhaps through residues located in other domains of the protein. The majority of studies have shown that EEC mutants are transcriptionally defective due to mutations that abolish their ability to bind to DNA (43, 44, 46, 48). In contrast, mutations associated with AEC do not affect the core DBD, yet they often fail to properly drive the expression, or repression of various downstream target genes (14, 15, 19, 31, 33, 42). We asked whether AEC-associated mutations in the SAM domain affect the ability of p63 to physically interact with DNA containing a p63 response element using EMSA assays. As expected, wild type p63 but neither of the EEC mutants, R204W or R304W were able to retain the capacity to bind DNA (Figure 5), consistent with previously published data (46). Here, we have shown for the first time that the AEC mutants L514F and G530V retain their ability to bind to DNA. We also documented two additional protein-DNA complexes in both the L514F and G530V lanes. These complexes migrated slightly faster in the gel compared to the single complex observed in the wild type lane. We estimated the molecular mass of the individual protein complexes and found that the single wild type band and the highest migrating bands of both the AEC mutants corresponded to a tetramer. Interestingly, the additional two complexes (middle and bottom) observed in the L514F and G530V lanes correspond to the theoretical masses of dimeric and monomeric p63 complexes, respectively. This data suggest that AEC mutants may exist in an equilibrium of tetrameric, dimeric and monomeric states, in which the SAM domain has a direct stabilizing effect on the quaternary structure of p63 that favours the tetrameric arrangement.

Figure 15). In support of this, deletion of the SAM domain produced a banding pattern reminiscent of AEC-derived variants. In the model organism Caenorhabditis elegans, the SAM domain of the p53-like protein CEP-1 stabilizes the transcription factor through interactions between the OD and the SAM domain (9). Deletion of the CEP-1 SAM domain destabilizes the
structure leading to conformational instability. It is conceivable to speculate that in humans, the SAM domain of p63 has a similar role in stabilizing the protein, specifically its oligomeric state. However, our NMR studies revealed that the SAM domain of human p63 does not contact its OD domain, thus this SAM-OD interaction is less likely to explain the destabilization of the oligomeric structure we observed here. Perhaps the SAM domain imposes a specific fold or structural conformation that allows itself, or specific binding partners to influence its oligomeric state. The S100 family of proteins has recently been shown to play a role in influencing the tetramerization of all p53 family members (67). Given that the L514F mutation is proposed to cause major structural changes to the SAM domain, and that the G530V variant destabilizes a putative interaction site for SAM domain binding partners, it is conceivable that these mutations may prevent proper intramolecular interactions, or intermolecular interactions with binding partners that promote the formation of tetramers. We propose that the SAM domain may confer a stabilizing effect on the oligomeric state of p63. Point mutations that destabilize the structure of the SAM domain or elimination of this domain altogether, affects the quaternary state of the protein, thus shifting the tetrameric state towards alternative oligomeric arrangements such as dimers or even monomers.

Figure 15). Indeed, p53 mutations that inhibit tetramerization exhibit a diminished transactivation potential (68). Similarly, we propose that AEC mutations that shift the tetramer to other oligomeric states may explain why the AEC mutants have a diminished capacity to transactivate target genes, thus contributing in part to the etiology of the disease.

Our EMSA data sheds light towards understanding the minimal oligomerization state required for p63 to physically associate with DNA. We observed that the SAM domain mutants retain their ability to bind to DNA as tetramers, dimers and monomers suggesting that p63 does not require oligomerization to interact with DNA. On the other hand, the monomeric I378P mutant which is unable to form oligomers fails to retain its DNA binding capacity which is contradictory to the data collected with the SAM domain mutants. It is possible that the I378P mutation may impose a structural change that prevents oligomerization as well as affecting the fold of the DNA binding domain. To address this question in the future, one might create a truncated p63 protein which lacks the OD domain to see whether oligomerization is required for p63 to bind to DNA. Based on these discrepancies, we are unable to conclude that monomeric p63 retains its ability to bind DNA, but within the limitations of this study one may infer that the minimal p63 oligomer
required to bind DNA is a dimer. In the future, these experiments warrant further investigation either by gel filtration analysis or analytical ultracentrifugation sedimentation to determine if indeed the SAM domain mutants characterized herein affect p63 oligomerization. Our technique for estimating the molecular size of protein complexes provided an approximation. Unlike SDS-PAGE gels, where protein migration is directly proportional to its mass, the migratory distance of a species in native gels depends on additional factors such as charge, conformation, as well as size. Thus, we cannot rule out that these bands are due to alternative conformations.

Figure 15: Mutations in the SAM domain may alter the oligomeric equilibrium of p63, shifting the tetrameric archetype in favour of lesser oligomeric states.
5.2 EEC and AEC Mutants Are Transcriptionally Inactive and Can Form Dominant Negative Hetero-oligomeric Complexes with Wild Type p63

Given that both EEC and AEC syndromes exhibit autosomal dominant modes of inheritance, we were interested in determining whether the observed dominant negative effect could be due to the expression of the mutant allele of p63 resulting in a mutant p63 protein that sequesters the transcriptionally active p63 protein. To mimic this in vitro we performed dual-luciferase assays with a PERP reporter construct and titrated increasing amounts of the mutant to wild type p63 (20). We first performed single transfections with the p63 variants and found that none of the EEC mutants (R204W and R304W) or AEC mutants (L514F and G530V) were able to transactivate the PERP promoter. Together with our EMSA data we conclude that the EEC mutants are transcriptionally inactive due to their inability to bind to the PERP promoter. However, the AEC mutants were also unable to transactivate the PERP promoter despite the fact that our previous data indicated that they retained their ability to bind DNA. Similar results were obtained by Beaudry et al. who showed that the TAp63α mutants L514F and to a lesser degree, G530V are transcriptionally inert, in contrast to the Q536L mutant, which was able to activate the PERP promoter to wild type levels. We speculate that the mutants tested were unable to activate the PERP promoter for two reasons. First, activation of PERP may require an essential cofactor for its activation, which likely binds to the SAM domain of p63. Since the L514F variant is known to destabilize the structure of the SAM domain, and the G530V mutation is predicted to interfere with a putative protein docking site, we speculate that these structural alterations may prevent the association of p63 with an essential cofactor. Second, we hypothesize that SAM domain mutations alter p63 tetramerization, which prevents its full transactivation potential. In addition, our titration experiments demonstrate that both the EEC R304W and AEC L514F variants exert a dominant negative effect towards wild type p63. We go on to show that wild type p63 is able to form hetero-oligomers with mutant p63 (R204W, R304W, L514F and G530V) suggesting that inhibitory complexes are responsible in part for the dominant negative phenotype observed in our reporter assays (Figure 9). In the case of the EEC mutant, R304W
inactivates wild type solely by interacting and sequestering wild type p63 transactivation, whereas the AEC mutant may bind to and inactivate p63, or could also compete with wild type for promoter binding sites. In this study we show that both EEC and AEC mutants compromise the expression of PERP by acting in a dominant negative fashion to wild type p63 and this is likely to occur through the formation of an inhibitory complex composed of hetero-oligomers with mutant p63. Since PERP is essential for maintaining the structural integrity of various epithelialized tissues in our body, dysregulation of this gene by AEC and EEC p63 mutations likely contributes to some of the abnormal phenotypes observed in patients with these two syndromes. For instance, PERP null mice exhibit similar phenotypes to EEC and AEC patients such as dystrophic nails, as well as abnormalities of the hair and skin (42, 43, 69). Furthermore, AEC patients exhibit aberrant PERP expression in the skin and together with our data we may conclude that the dominant negative effect of AEC over the wild type protein may contribute to the skin fragility phenotype by preventing wild type p63 from expressing PERP. Based on our findings for PERP, we hypothesize that EEC and AEC mutants may act in a dominant-negative fashion or perhaps a gain-of-function activity for other genes regulated by p63. Indeed, during our studies Koster et al. (2009) reported that AEC mutants L514F and Q536L function as dominant negatives towards wild type p63 on the IKKα promoter (35). It is also important to consider that different members of the p53 family are able to hetero-oligomerize with one another. In a recent study, Coutandin et al. (2009) demonstrated that p63 and p73 preferentially form hetero-oligomers consisting of two homodimers that we found to be more stable than p63 or p73 homotetramers. Moreover, the researchers demonstrated that p63 and p73 are co-expressed together in the skin suggesting that p73 may also have a role in epithelial tissue that may require hetero-oligomerization with p63. In our studies we used the H1299 cell line which is null of p63, an express undetectable amounts of p63 and p73 at the protein level thus expression of the mutants is likely not confounded by contaminant expression of endogenous p53 family members. It is intriguing to speculate that if p73 was expressed in 1299 cells whether or not we may obtain the same results. For instance, perhaps mutant p63 may have a gain-of-function on the PERP promoter when p73 is present in the cell. p53 normally is unable to hetero-oligomerize with p63 or p73. However, certain p53 mutations gain the ability to physically interact with the other p53 family members and negatively regulate their proapoptotic function (79). Researchers speculate that p53 mutations may exert a gain-of-function phenotype possibly through the formation of hetero-oligomeric complexes with p63 and p73. Similarly, p63 mutations associated
with EEC and AEC may disrupt cross-talk between p53 and p73 proteins. Thus, understanding how the p63 mutants interact with and modulate downstream target genes as well as their interactions with the other p53 family members will help elucidate their contribution to the clinical manifestations of the disease.

5.3 EEC and AEC mutations have a stabilizing effect on p63

Mutations in p53, particularly in the DBD, are known to cause aberrant p53 degradation that consequently prolongs its half life within the cell (66). We hypothesized that similar to p53; EEC and AEC-derived mutations may have a comparable stabilizing effect on p63 due to aberrant degradation. We performed cycloheximide chase-assays and found that both of the EEC mutants, R204W and R304W, as well as the AEC-derived L514F mutation confer a stabilizing effect on p63, whereas the G530V variant does not (Figure 11). Notably, the EEC mutants exhibited the most robust stabilization of p63 with calculated half-lives that effectively doubled that of wild type. Stabilization of mutant p63 leads to its accumulation in the cell, where it may further contribute to the disease phenotypes by causing abnormal activation or repression of genes. Furthermore, increasing the levels of mutant p63 will likely create a dominant negative effect over wild type through competitive binding of promoter DNA sequences. In addition to the formation of hetero-oligomeric complexes, which sequester wild type p63 function from various downstream target genes which thereby disrupt key signaling pathways resulting in clinical manifestations of the diseases. For instance, analysis of skin biopsies from AEC patients reveals keratinocytes abnormally proliferating in the suprabasal levels of the skin: a region where keratinocytes should normally cease mitotic divisions and begin to terminally differentiate. Interestingly, earlier studies have documented strong p63 staining in AEC patient skin biopsies within the upper layers of the epidermis where p63 is normally absent (42). It is interesting to speculate that the presence of proliferating cells in the upper layer may be due to the aberrant turnover of mutant p63. As a consequence, accumulated levels of p63 in the suprabasal levels may inadvertently prevent cell cycle withdrawal through its continual activation of cell-cycle progression genes such as p57kip2, thus explaining the presence of proliferating keratinocytes in layers of the skin where they are normally absent. It is intriguing to speculate that ectodermal
dysplasia may arise from a hyper-stabilized p63 molecule that is not mutated. The mechanisms underlying ectodermal dysplasia are far from being understood, thus it is plausible that the function or expression of proteins that affect the stability of p63 may be altered due to mutation or epigenetic factors such as hyper-methylation. For instance, it is possible that mutations in Itch may prevent the enzyme from properly degrading p63 possibly leading to abnormally high gene activation or repression by p63 which may clinically manifest as ectodermal dysplasia. Studies have shown that mutating Y504 TAp63 (or Y449 in ΔNp63) artificially stabilizes the p63 by preventing E3 ligases such as Itch, WWP1 and Nedd4 from binding and degrading p63 (54,55,57). To test whether hyper-stabilized p63 can lead to ectodermal dysplasia, one might use knock in mice that expressing a hyper-stabilized form of p63 to determine whether the resulting phenotype resembles those associated with ectodermal dysplasia.

Given that the EEC and AEC mutants were more stable, we were interested to see whether the enhanced stability of the mutants was due to aberrant degradation by Itch, an E3 ligase that marks p63 for ubiquitylation-mediated degradation in the skin. To our surprise, all of the mutants were destabilized by the E3 ligase, albeit to varying degrees. The levels of AEC mutant proteins, L514F and G530V, were decreased by the Itch E3 ligase, and they exhibited half-lives roughly equivalent to wild type. In contrast, the EEC mutants had delayed degradation. We performed co-immunoprecipitation and ubiquitylation assays to see if the observed stability of the p63 variants could be attributed to decreased Itch binding and/or Itch-mediated ubiquitylation (Figure 12 and Figure 13). Our results support that neither the DBD mutants, nor the SAM domain variants lose their ability to interact with Itch. Furthermore, we did not observe any appreciable difference between the levels of polyubiquitylation of wild type p63 and the EEC and AEC mutants tested herein. These data suggest that the enhanced stability of p63 mutants is not attributable to abnormal Itch-mediated degradation. However, we noted that the EEC mutants were the most stable and that Itch-mediated degradation is delayed. It is intriguing to speculate that the stability of the mutants may be related to their inability to transactivate their own negative regulator in an autofeedback loop mechanism reminiscent of the renowned p53-MDM2 pathway. Indeed, Ying et al. (2005) showed that EEC mutations in the DBD give rise to DNA binding deficiency and highly stable proteins. They demonstrated that overexpression of TAp63γ was able to act in trans to promote the degradation of the EEC R304W TAp63γ but not vice versa suggesting that retention of p63’s DNA binding capacity is
essential for its degradation. We believe that an unidentified negative regulator may possibly form a p63-direct, or indirect autofeedback loop analogous to p53 (Figure 16). We have begun to investigate WWP1 as a potential candidate for our proposed faulty p63 autoinhibitory loop for several reasons. First, WWP1 is expressed in epithelial tissues and is known to bind and ubiquitinylate p63 which promotes its degradation (55). Furthermore, WWP1 contains a p53/p63/p73 response element in its promoter region and has been shown to be upregulated by both the TA and ΔN p63 isoforms (70). If indeed WWP1 is a downstream target gene of p63, based on our DNA binding and transactivation studies, we speculate that the EEC and AEC mutants will prevent WWP1 expression thereby leading to increased levels of mutant p63 in the cell. As a consequence, increasing relative levels of cellular mutant p63 will affect downstream target gene regulation, increase the likelihood of wild type p63 inhibition and will also lead to outcompeting of the functional p63 protein for promoter binding sites, which together, may enhance the disease phenotypes of both EEC and AEC syndromes.
Figure 16: Proposed p63-negative regulator autoinhibitory loop. Upper Half: Wild type p63 directly or indirectly induces the expression of its negative regulator” X”. X in turn targets p63 for degradation reducing its levels in the cell and turns off expression of X. Lower Half: Mutant p63 is unable to drive the expression of its negative regulator. Mutant p63 is stabilized and accumulates in the cell which prevents downstream target gene activation, inhibits wild type p63 and also outcompetes it at promoter binding sites which leads to aberrant gene modulation and phenotypes of EEC and AEC syndrome.
5.4 Oligomerization and SAM domains of p73 but not p63 interact and residues involved are not conserved in p63

Ou et al. (2007) demonstrated that the OD of the p53-like protein CEP-1 structurally interacts with its SAM domain. This interaction is important for providing CEP-1 with conformational stability. Considering the high sequence similarity and overall structural topology between the SAM domains of p63, p73, and CEP-1, we speculated that there may be a similar OD-SAM interaction in human p63 and p73. We utilized $^{15}$N HSQC NMR spectroscopy to validate the putative OD-SAM interaction of both p63 (Figure 14 A) and p73 (Figure 14 B) by titrating $^{15}$N/$^1$H-labeled SAM domains with their respective OD domains. We found that the OD and SAM domain of p73 physically interact and that this is mediated by 11 residues in the SAM domain. To our surprise, the OD of p63 does not interact with its SAM domain, despite the high sequence identity between the OD and SAM domains of p63 and p73 (60% and 50%, respectively). Upon further investigation, sequence alignments of the p73 and p63 SAM domains reveal that only 3 of the 11 residues involved in the p73 interaction are conserved in p63, thus potentially explaining the absence of this interaction in the latter even at high molar ratios of OD:SAM. The biological significance of this finding has yet to be determined, however, given the differential roles that p63 and p73 have in development and cancer, it is intriguing to speculate that this interaction may serve as a means for potential specificity of binding partners to discriminate between p63 and p73 thus allowing these proteins to preferentially modulate different target genes. To investigate this concept, one could splice the SAM domain of p73 into the p63 protein and test whether this alteration would have an affect on transactivation of p63-specific target genes such as JAG-1 or alter the association of p63 with p63 specific SAM domain binding partners such as the RNA splicing protein apobec-1 binding protein-1 (ABBP1) (77). To determine whether there is a different role of p63 and p73 SAM domains, in vivo generation and examination of knock in mice in which wild type p63 has been replaced with the p63-p73 SAM hybrid may be performed. If this substitution severely alters the function of p63 this would highlight the potential role that the individual SAM domains of p63 and p73 have in different developmental processes. Moreover, an additional helix, C-terminal to the OD domain, was recently identified in p63 and p73, which stabilizes the tetramer (71). In p73, this helix serves as a second TA domain that preferentially upregulates genes involved in cell-cell cycle
arrest as opposed to those involved in apoptosis (72). Although we did not perform experiments that allowed us to identify which residues in the OD of p73 are involved in the SAM domain interaction; it is interesting to speculate that the SAM domain may serve as a molecular switch to preferentially target cell-cycle as opposed to proapoptotic genes via different conformational states (Figure 17). In the “closed” conformation, the SAM domain masks residues that are important for the second transactivation domain. This allows p73 to upregulate proapoptotic genes as opposed to those involved in cell cycle arrest. In the event of stress or post-translational stimuli, p73 undergoes a conformational change into the “open” state, which exposes the second transactivation domain allowing it to drive the transcription of genes that promote cell-cycle withdrawal.

Figure 17: Putative masking of residues by the SAM-OD interaction in p73 may be necessary for gene modulation and function on various p73 target promoters.
Coutandin et al. (2009) demonstrated that p63 and p73 preferentially form hetero-oligomers consisting of two homodimers. In our study, the p63 OD-SAM did not interact, however, it is conceivable that the SAM domain of p63 makes contacts with the OD of p73 and vice versa, which may play a role in the apparent stability of the heterotetramer. We did not map the residues in the p73 oligomerization domain that make contact with the SAM domain, but perhaps these residues are conserved in both the OD of p73 and p63 for this interaction. Nevertheless, we demonstrate a novel intra-domain interaction between the OD and SAM of p73, which is not conserved in p63. This interaction may have an important role in modulating unique p63 or p73 target genes through preferential binding with specific cofactors.
6 CONCLUSIONS AND FUTURE DIRECTIONS

With the discovery of the ancestral member of the p53 family over a decade ago, p63 has created an exciting and complex field of study with regards to understanding the development and maintenance of several ectodermal-derived structures and tissues. Specifically, p63 is involved in complex epithelial-mesenchymal interactions that give rise to ectodermal-derived appendages such as limbs, teeth, hair, skin and several glands during development. In the mature skin, p63 is crucial for maintaining proliferative stem cells for tissue homeostasis, as well as committing keratinocytes for terminal differentiation in the upper stratum of the skin. In recent years, studies identifying specific p63 targets have begun to elucidate the fine network of signaling that directs the formation and maintenance of the epidermis and ectodermal appendages. Molecular defects in the \( p63 \) gene give rise to severe physiological abnormalities in patients with ectodermal dysplasia, however the precise etiology of how these mutant proteins disrupt the various p63 pathways is far from being understood.

Clinical variability is a hallmark of ectodermal dysplasia. As p63 signaling cascades and downstream target genes continue to be identified it will be crucial to elucidate how dysregulation of these genes by the individual p63 mutations contribute to the disease pathology of EEC and AEC syndromes. In our study we demonstrate that EEC and AEC mutants may contribute to the skin fragility and other phenotypes of ectodermal dysplasia through the ability of mutant p63 to form dominant negatives with wild type p63. However, these mutants may have a gain-of-function or exert no aberrant function on other genes, therefore understanding how different p63 mutations differentially regulate downstream target genes will allow us to better understand the etiology of the disease and also help design novel therapeutics to treat patients affected by it. In our studies we investigated the p63 target gene \( PERP \), an important desmosomal membrane protein that is important for cell-cell adhesion that helps support the integrity of the skin. We showed that EEC and AEC disease causing mutations prevent p63 transactivation of \( PERP \), which we concluded may contribute to the defective skin phenotypes observed in patients with these EEC and AEC syndrome. Clinical manifestation of p63 derived ectodermal dysplasia is undoubtedly a cumulative effect of several p63 target genes being abnormally regulated. In future studies, one may investigate p63 target gene promoters of genes
that are essential to maintaining the structural and tensile strength of epithelial tissues such as *ALOX12*, *claudin-1* and *FRAS-1* which may be dysregulated by mutant p63 proteins and contribute to the disease phenotypes associated with the skin, hair and other epithelial structures. Given that p63 is involved in maintaining the proliferative capacity of stem cells in the epidermis and also plays a role in stimulating keratinocyte commitment to terminal differentiation, additional target genes such as *notch1*, *IKKα*, *Ets-1* and *p57kip2* will also be important to study in order to determine whether dysregulation of these genes is responsible for aberrant development of the epidermis as well as other structures derived from the ectoderm. To investigate these target gene promoters, it would be advantageous to obtain EEC or AEC derived patient tissue samples or create stable cell lines expressing mutant p63 proteins in primary keratinocytes or SKPs to study possible differential p63 transactivation of these target genes. Techniques such as chromatin immunoprecipitation (ChIP) and Real-Time PCR will be useful to determine whether EEC and AEC p63 mutants retain their ability to bind to specific promoters and will also allow one to quantify the relative amounts of gene expression by the p63 mutant proteins. In our studies, we employed dual-luciferase assays to assess the transcriptional activity of the various EEC and AEC mutants on the *PERP* promoter. Although dual luciferase assays are extremely useful molecular tools, they also have their limitations. Our *PERP* reporter construct contained only a portion of the native gene promoter. It is possible that other enhancer or repressor regions located in the full genomic promoter may also influence gene transactivation which were occluded in our construct. Thus, additional experiments such as Real-Time PCR and ChIP with p63 and the mutants should also be performed in the future to compliment our dual luciferase assay data.

The majority of our studies focused on how aberrant p63 transactivation and DNA binding may contribute to the disease phenotypes associated with EEC and AEC syndrome. Although it is important to understand how the various mutations may disrupt proper gene modulation through direct transactivation, other important cellular events such as protein-protein interactions and epigenetic factors are additional mechanisms that likely contribute to the etiology of ectodermal dysplasia must not be overlooked. For instance, Fomenkov *et al.* (2003) demonstrated that the RNA splicing enzyme apobec-1-binding protein-1 (ABBP1) specifically binds to the SAM domain of p63α and promotes the splicing of *FGFR2* from the mesenchymal differentiation isoform BEK towards the K-SAM variant required for keratinocyte differentiation. The
researchers showed that SAM domain mutations associated with AEC prevent ABBP1 from binding to p63α causing aberrant splicing of FGFR2 preventing the K-SAM isoform from being expressed. This event is hypothesized to inhibit epithelial differentiation and is thought to help explain the AEC skin phenotype. In addition, this may also explain the observation of proliferating keratinocytes in the suprabasal layers of AEC patients (77). Moreover, epigenetic factors may also contribute to EEC and AEC syndrome. p63 is known to recruit and bind proteins involved in chromatin remodeling including the histone acetyl-transferase protein p300 (78). It is possible that aberrant interactions between mutant p63 and p300 or other unidentified epigenetic modifiers of chromatin structure (DNA methylases and acetylases) may cause atypical gene silencing or gene expression that disrupt p63 signaling pathways which contribute to the etiology of ectodermal dysplasia. Thus, identifying novel p63 binding partners and understanding how their association with mutant may lead to dysregulation of p63 signaling pathways is of great importance. In particular, cofactors that interact with the α-isoforms, given that these proteins are the predominant transcripts expressed during development and into adulthood and the only isoforms that include the SAM domain. In terms of the AEC mutants, their deviant transactivation of downstream target genes is thought to be due in part, as a result of their improper binding to essential co-modulators that comprise the proper transcriptional machinery at the promoter. Thus, identifying these partners and understanding how their association may be affected by the different p63 mutants, will help to elucidate the molecular causes of the various disease phenotypes associated with EEC and AEC syndrome. In order to search for these binding partners, one may employ large scale co-immunoprecipitation or GST-pull down assays to identify novel proteins that associate with p63. For example, the p63 interacting protein SATB2 was discovered in our lab using a large scale immunoprecipitation method (61). Pull down assays may also be performed with EEC and AEC mutant p63 proteins in order to investigate whether proteins identified to interact with wild type retain their ability to interact with mutant p63. Careful selection of tissue or cell lines will be essential to discover these unidentified binding partners. Here, we suggest using cells that express p63α, which will increase the likelihood that essential cofactors specific to this isoform are present. These cells include primary keratinocytes, SKPs, or embryonic mouse tissue derived from ectodermal layers. In addition to pull down assays, recent improvements to phage display technology offers a high-throughput approach to identifying novel p63 protein-binding partners as well.
We also demonstrated that EEC and AEC mutants (with the exception of G530V) are more stable than wild type p63, leading to relatively increased levels of the mutants and the formation of inhibitory complexes with wild type that may outcompete wild type p63 at the promoter. Identifying the negative regulator(s) responsible for the inherent stability of the mutants will help elucidate mechanisms to preferentially degrade the aberrant proteins in patients. To determine whether protein stability of EEC and AEC mutant p63 is due to aberrant ubiquitin-mediated degradation, experiments using proteasome inhibitors such as lactascystin or MG132 may be employed to examine whether p63 mutant proteins are stabilized in the presence of these inhibitors. Similarly, non-ubiquitin-dependant degradation of p63 may also be useful to examine including sumoylation or phosphorylation pathways. We are currently investigating WWP1, which we believe may be a potential candidate responsible for the differential stability of the mutants. If WWP1 is not responsible for the stability of the p63 mutants, one may look at other members of the Nedd4-like C2-WW-HECT family of E3 ligases such as Nedd4, SMURF1 and SMURF2 which contain WW domains that interact with the PY motif of p63 similar to Itch and WWP1.

In the future, one may investigate the potential predisposition of EEC and AEC patients to cancer malignancies. There is considerable evidence that disruption of p63 contributes tumourigenesis. For instance, p63 heterozygous mice are more prone to malignant lesions then wild type mice (83). Tumourigenesis was associated with concomitant loss of heterozygosity. Given that EEC and AEC patients have a mutant allele of p63 which typically is associated with improper gene modulation, it is possible that disruption of the remaining wild type p63 allele may result in the development of a neoplasm. Moreover, overexpression of p63 (particularly the anti-apoptotic isoform ΔNp63) can promote the development of a variety of squamous cell carcinomas including malignancies of the esophagus, head and neck, bladder, breast and prostate which is thought to augment TAp73-dependant apoptosis (61, 84). From our studies we have shown that mutations associated with EEC and AEC are more stable, thus leading to their accumulation in the cell. It is intriguing to speculate that the enhanced stability of mutant p63 may be analogous to overexpression of the wild type ΔNp63, thus predisposing patients with EEC and AEC syndrome to cancer. To date this has not been addressed in the literature and is likely due to the low incidence of this ectodermal dysplasia, however a correlation may exist between the two diseases.
Finally, we proposed that AEC-derived p63 mutations destabilize the quaternary structure of the transcription factor and shift the oligomeric equilibrium in favor of dimeric and monomeric states. In future studies, we will need to confirm this hypothesis by employing techniques such as gel filtration chromatography and/or analytical ultracentrifugation, which will help determine whether mutations in the SAM domain indeed affect p63 oligomerization.

In conclusion, these studies provide new insight into the mechanisms by which mutations associated with EEC and AEC affect the activities of p63 and potentially lead to some of the associated clinical phenotypes. Current data suggests that p63 is both critical for the maintenance of epidermal stem cells and initiation and progression of terminal keratinocyte differentiation. Recent studies analyzing patient samples from AEC patients and murine knock-in models that are heterozygous for wild-type and EEC or AEC derived mutant p63 have demonstrated that suprabasal proliferating cells in the upper skin layers continuously express p63. In addition, in these models mutations in p63 also affect keratinocyte differentiation causing a delay in terminal differentiation and aberrant expression of differentiation markers (42, 85). Our data shows that EEC and AEC mutant p63 proteins have lost at least one measure of their wild-type function (transactivation of PERP) and are considerably more stable. This loss of function may contribute to the phenotype of proliferating cells in the suprabasal layer of the skin by preventing the activation of other target genes such as p57 that to promote cell cycle arrest or those involved in differentiation such as the keratin filament K1. p63 mutants that are very stable likely disrupt several aspects of p63 signaling by altering the wild-type function of p63, many of which may contribute to the disease phenotype. This observation leads to a possible treatment avenue to be developed in the future that specifically focuses on targeting mutant p63 for degradation or designing specific inhibitors preventing mutant p63 hetero-oligomerization with wild type p63. Moreover, it is possible that having excessive amounts of p63 (wild type or mutant) can give rise to ectodermal phenotypes. Thus, mechanisms that silence p63 at the transcriptional level, such expression of miR203 microRNA, or promote p63 degradation at the protein level by enhancing the expression of p63 negative regulators such as the E3 ubiquitin ligases Itch or WWP1, may be effective targets leading to novel treatments of these rare syndromes (23, 55, 86). Understanding how each individual mutation affects p63 binding to DNA, transactivation activity, oligomerization with wild-type p63 as well as changes in protein stability and nuclear
localization will help to define the underlying mechanisms of the disease and contribute towards the develop of novel therapies to treat patients affected by this spectrum of disorders.
7 REFERENCES


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