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The Importance of the Transcription Factor p63 in Epidermal Development and Disease

Isoform-based analysis of p63 in keratinocytes

Sean Hockney | MSc by Research- Biological Sciences | Sept 2017- Jan 2019

The above author confirms that, unless stated otherwise, that they completed all work. Any other work, which was completed by others, is fully acknowledged and referenced.

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Abstract

The transcription factor p63 is crucial for the maintenance and development of the epidermis. The p63 protein is encoded by the *TP63* gene, which contains two distinct promoters giving rise to two major protein isoforms, termed TAp63, and Δ Np63. Mutations in the *TP63* gene can lead to severe epidermal disease such as ectrodactyly/ecto-dermal dysplasia and cleft lip/palate (EEC) and ankyloblepharon/ectodermal defects and cleft lip/palate (AEC) syndromes. Within the literature there is conflict as to spatial and temporal expression of the p63 isoforms in developing and differentiating epidermis, with TAp63 appearing to play roles in the early stages of ectodermal development with Δ Np63 thought to be the dominant isoform during the maturation and maintenance of the epidermis. Despite this, recent work has suggested a role for TAp63 in regulating the steps within terminal differentiation of keratinocytes.

To examine this possible role for TAp63 isoforms in terminal differentiation we induced differentiation in 2D cell culture of primary and immortalized keratinocyte lines. Isoform expression, alongside other differentiation markers, were quantified via qRT-PCR. Furthermore, siRNA knockdown systems were developed to knockdown total p63, all TAp63 isoforms and all Beta forms of p63 and qRT-PCR was used to assess knockdown efficiency and effects on isoforms expression and expression of other linked genes.

Results from calcium-induced differentiation of keratinocyte lines from basal cells demonstrated a mirroring expression pattern for both the p63 isoforms with Δ Np63 expression being higher during the early stages of differentiation with its expression decreasing beyond day 8. In comparison, TAp63 expression began to increase at day 8 of differentiation, with its peak expression after day 12 and 13 suggesting a role for this isoform during the terminal stages of differentiation. This was seen in one HaCaT cell line and two primary keratinocyte lines. Expression of other differentiation markers such as FLG, KLF4 and ETS-1 followed expected trends.

Knockdown of total p63, TAp63 isoforms and Beta isoforms provided varying results in two keratinocyte lines. When total p63 was targeted in basal cells, we saw 50-75% knockdown of p63 isoforms suggesting that Δ Np63 was the most prominent isoform at this stage. When TAp63 was targeted, we saw a 60% knockdown efficiency. When Beta isoforms were targeted, we saw between 60 and 80% knockdown efficiency for Δ Np63 and a 30% knockdown efficiency for TAp63. We also demonstrated that expression of ETS-1 was consistent through two keratinocyte lines when each of the siRNA knockdowns were introduced.

We have demonstrated *in vitro* that the expression of the p63 isoforms shows a clear temporal difference in differentiating keratinocytes. We have further shown that when p63 isoform expression is interfered with, downstream marker expression changes depending on the target. These findings are important for understanding the distinguished roles of each isoform in differentiating keratinocytes.

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Terminology

<i>Term</i>	<i>Definition</i>
<i>P63</i>	<i>Transcription factor encoded by the TP63 gene</i>
<i>siRNA</i>	<i>Short interfering RNA</i>
<i>KLF4</i>	<i>Kruppel-like factor 4</i>
<i>qRT-PCR</i>	<i>Real time Polymerase chain reaction</i>
<i>FLG</i>	<i>Filaggrin</i>
<i>ETS-1</i>	<i>ETS Proto-oncogene 1</i>
<i>IKK-a</i>	<i>Also known as CHUK. Conserved Helix-Loop-Helix Ubiquitous Kinase</i>

1.0 Introduction

1.1 General Introduction

The skin is the largest organ in the human body, covering the entire outer surface of the body. It has a key feature in that it is a self-renewing organ. This barrier acts to prevent the entry of pathogens and debris whilst keeping the skin from dehydrating. In order to achieve this, different cell types work in unison, each with specific functions. In order to allow the cells to have a specific function, the cells must take on certain morphological features. These morphological features are determined by the gene expression within the cells. The expression of these genes are dependent on extracellular signals which trigger an internal cascade of expression of various transcription factors which regulate certain genes within the cell.

Within the skin, there are multiple layers, each containing various cell types and cells in different stages of differentiation. Each layer of the skin is crucial for its own reasons allowing the maintenance, development and function of the skin barrier.

1.2 The Skin and Epidermis

The skin is compartmentalized into three main layers - the epidermis, the dermis and the hypodermis. The dermis sits on the internal layer, the hypodermis, comprising of the connective tissue, blood vessels, nerve endings, sweat glands and hair follicles. The hypodermis itself is a subcutaneous layer of fat, which functions as insulation for the body, whilst also providing protective qualities. The epidermis is the outermost component of the skin, being the “true” skin. The cooperation between both the dermis and the epidermis achieves the functionality of the skin as a barrier, protecting the body from external insults whilst maintaining a balance of water movement. Both layers interact forming a highly specialized matrix structure

called the basement membrane. The basement membrane acts to separate the two layers whilst providing stability as well as a dynamic interface (Breitkreutz et al., 2009). Despite the organ as a whole being responsible for the active skin structure, the epidermis is the most important compartment for preventing water and other compound loss as well as a protective element from the environment.

The epidermis is a self-renewing epithelial structure that is subdivided further into

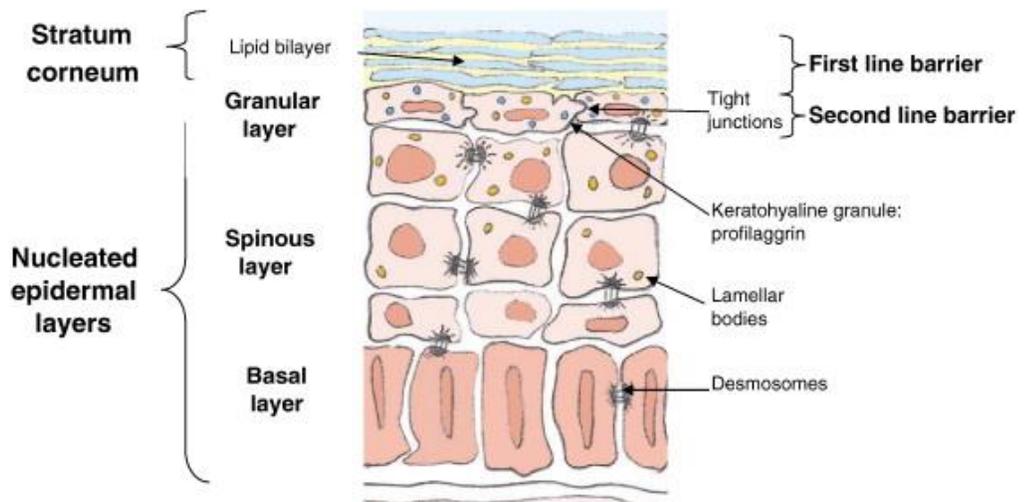


Figure 1: Human epidermal structure: The innermost layer comprising of cells with self-renewing qualities associates with the basement membrane. The spinous layer and granular layer provide the other nucleated layers with the enucleated stratum corneum on the outermost edge of the epidermis (Baroni et al., 2012)

multiple strata. The innermost layer of the epidermis, forming connections with the basement membrane, is the basal layer. Moving upwards through the epidermis, the spinous layer sits on top of the basal membrane. The outermost layer of live cells is called the granular layer containing tight junctions that seal neighboring cells in this layer. Within the layers of the nucleated cells, desmosomes interconnect adjacent cells. The outermost structures are predominantly made up of lipids and dead keratinized cells called the stratum corneum. The stratum corneum's role is to protect the rest of the skin components providing adaptive immunological, chemical and biochemical barriers (Wickett & Visscher, 2006) (Figure 1).

The cells of the basal layer are responsible for the self-renewing quality of epidermis with approximately 15% of the cells at the basal layer being part of this process, with the other cells being in a quiescent state (Baroni et al., 2012). Within the epidermis the most commonly found cell type is keratinocytes and these cells are nucleated and present from the basal layer right through to the granular layer (Proksch et al., 2008). Keratinocytes synthesize structural proteins and lipids which are key for the epidermal structures and function. Keratinocytes are also responsible for the stratum corneum, with this structure being made up of enucleated keratinocyte cells which have been differentiated throughout a migration through the epidermal layers. These cells act through keratin filaments and are surrounded by a cell envelope which is composed of cornified envelope proteins.

1.3 Skin Development in Embryogenesis and Epidermal Commitment

The current literature exploring the process of human skin development at a foetal age is limited. Despite this, the development of human skin has been studied at a morphological level using electron microscopy approaches (Peltonen et al., 2010). The development of skin in the fetal stages of development are crucial for the survival of the individual due to fetal skin having advantageous properties such as scar less wound repair (Walmsley et al., 2015).

During embryonic and fetal development, many orchestrated gene regulation pathways must take place allowing for proper organogenesis. These processes must occur in the correct spatial and temporal situations for the desired organ and systems to develop. In order for the steps to take place in the right order, these processes must be tightly controlled by specific transcription factors including p63. Historic studies of p63 have showed the importance of p63 in embryonic epidermal development as well as in keratinocyte proliferation and differentiation (Carroll et al., 2006; Pellegrini et al., 2001; Truong et al., 2006).

As shown in mouse models, p63 has an essential role in proper epidermal commitment (Mills et al., 1999). During the early stages of vertebrate development, the surface epithelium is a single layer of Keratin 8 and Keratin 18 positive cells (Aberdam, 2008). This layer has a function of allowing cellular diffusion, secretion and absorption. As the development process continues, this group of ectodermal cells develop into the keratinocyte cells, which express high levels of Keratin 5 and 14. These keratins are expression markers associated with mature epithelial cells (Soares & Zhou, 2018). This change in physiological states is known as epidermal commitment and this process is tightly controlled by p63. As a result, p63 is known as the "gatekeeper" of epidermal commitment (Shalom-Feuerstein et al., 2011)

Spatio-temporal analyses using mouse embryos have demonstrated that p63 is present at the limb buds and branchial arches initially, and then expands into the whole surface of the embryo (Zhao et al., 2015). This expression pattern correlates with the affected structures such as epidermis and limbs. Work using knock out mouse models have further confirmed the importance of p63.

Despite the importance of p63 in epidermal commitment being widely respected, the molecular pathways and cascades remain to be fully uncovered. This means work into this area is needed further to note the action of p63 and which isoforms are responsible.

1.4 Keratinocyte Lifecycle

The architecture of the stratum corneum gives rise to the permeable barrier function of the mammalian epidermis. The stratum corneum is made up of highly organized cellular compartments with lipid compartments interspaced (Houben et al., 2007). In order to maintain this barrier, there is need for constant replenishment of cells. This renewal of cells is created by a balance of loss of the outermost cells and generation of new cells at the basal level. The generation of new keratinocytes is exclusive to the most internal layer of the epidermis, the stratum basale. At this level, there are high levels of proliferation, forcing a large amount of cells to migrate towards the most external point. In doing so, these cells undergo differentiation, eventually arriving at the point when they are terminally differentiated into corneocytes.

This process takes approximately one month in human skin, but can mimicked *in vitro* over a shorter period of time. In order to reach terminal differentiation, the cells must undergo a number of morphological changes that are determined by a series of events. During the migration, the keratinocytes undergo cornification and generate extracellular lipid domains between cells. These events begin during the movement through the stratum spinosum and continues more proficiently in the stratum granulosum.

Finally, to reach terminal differentiation, the cells undergo epidermal programmed cell death in which the cells detach through loss of cell-cell adhesion (Ishikawa et al., 2015). This loss of the most terminal cells, allows the process of renewal to carry on. This process is different from the classical apoptotic pathways, in which there is programmed cell death either intrinsically or extrinsically.

1.4.1 Keratinocytes at the Basal Layer

In the basal layer of the epidermis a subpopulation exists which are responsible for maintaining the process of replacing terminally differentiated cells with new keratinocytes. This population have other roles including the repair or replenishment of damaged cells. The cells in this population maintain a slow cell cycle and hence a long life span with a high proliferative potential. Found predominantly in the bulge of hair follicles, these cells can migrate, taking up space in the basal layer between hair follicles (Jahoda & Reynolds, 2000). The epidermal stem cells found in this population replicate asymmetrically (Figure 2), meaning one single stem cell divides and gives rise to two daughter cells which may be two new cells, one stem cell and one transit amplifying cell or two transit amplifying cells (Watt, 2001). The product of the division is determined by the need of the epidermis at that time.

If a transit-amplifying cell is produced as a daughter cell, this allows the proliferation of the cells to continue. Each transit-amplifying cell is able to divide a set number of times before the progeny of this population moves towards terminal differentiation. In order to do this, they must remove themselves from the cell cycle. This process results in multiple

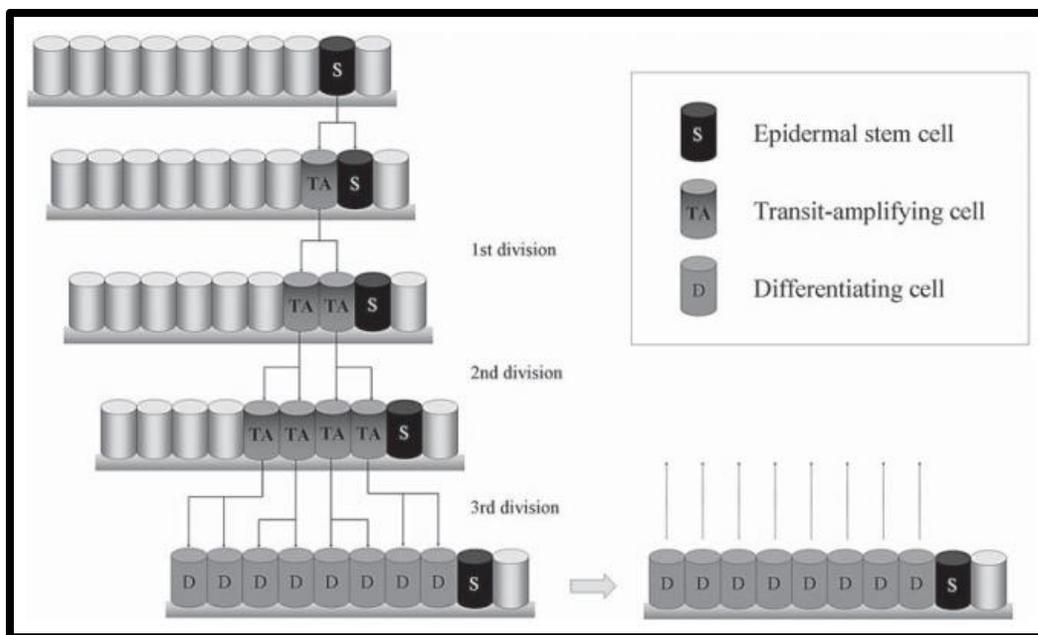


Figure 2: Schematic representation of the asymmetrical nature of stem cell division in the stratum basale. One stem cell division allows multiple daughter cells that are then allowed to proceed to terminal differentiation. Modified from (Watt, 2001).

differentiating cells from one individual stem cell. This allows maintenance of the stem cell populations at the basal layer, without affecting the number of differentiating cells produced (Watt et al., 2000).

The fate of stem cells is determined by the need of the epidermis and is controlled by expression of various markers. The signals can determine the fate of epidermal stem cells. As a result, we can use the markers to understand the cascade of events throughout the differentiation process. Also, these signals can be hijacked or artificially-induced in various therapies and used in the laboratory (Chunmeng & Tianmin, 2004). An example of a marker expressed by epidermal stem cells is β_1 -integrin. The expression of β_1 -integrin is believed to be involved with adhesion to the basement membrane. Adhesion to the basement membrane inhibits differentiation of keratinocytes as it prevents the migration through the layers of the epidermis. Additionally, other markers are expressed at this basal level. These markers are thought to be more specific to keratinocytes, many of which are isolated to the cells in this basal state.

A key marker for keratinocyte differentiation is p63. This is a transcription factor that is expressed in basal keratinocytes and throughout differentiation. This marker is the main marker that is to be investigated in this thesis and will be discussed at greater length in **Sections 1.5 and 1.6**.

1.4.2 Process of Cornification

Identification of cells, which have been released from the basal layer, is apparent due to the morphological changes to the structure. The cells, which have been released into the upper layers of the epidermis, express a number of different genes which induce these phenotypic changes. These cells also lose their replicative ability at this stage. Genes which were previously expressed at the basal layer, such as Keratins 5, 14 and 15 are down regulated, whilst upregulation of differentiation-specific keratins

such as Keratin 1 and 10 is induced (Yano et al., 2005). These keratins that are specifically expressed in the suprabasal layers can form the heteropolymers which eventually make up part of the cytoskeletal elements, along with actin filaments and microtubules. In unison, these subunits allow migration of organelles and structural roles for the cells.

Once suprabasal keratinocytes differentiate further into the granular layer, new morphological structures appear. These structures are known as keratohyalin granules (Figure 3). These granules are primarily made up of a protein called profilaggrin (Houben et al., 2007). Through proteolysis and dephosphorylation, profilaggrin is processed into the mature form of the protein- filaggrin (Brown & McLean, 2012). Filaggrin's function is to aid the aggregation of the keratin filaments at the boundary between the stratum granulosum and the stratum corneum, through a process known as the "ionic zipper hypothesis" (Mack et al., 1993). Filaggrin also has been shown to have other roles, including roles within the distribution of cytoskeletal elements including actin microfilaments (Presland et al., 2001). Furthermore, the NH₂ terminal filaggrin has been demonstrated to have nuclear activity in gene transcription of genes associated with the subsequent differentiation steps (Pearson et al., 2002) whereby it acts within the nucleus itself. The protein passes through the nuclear membrane and acts directly in gene transcription.

Another structural protein, which is isolated to the keratohyalin granules, is loricrin. This protein becomes cross-linked to the cornified envelope. The events of the formation of the cornified envelope is triggered initially by rising Ca²⁺ concentration in the differentiating keratinocytes (Kalinin et al., 2002). This begins in the upper levels of the stratum spinosum. The increasing calcium ion concentration encourages the proteins called periplakin and envoplakin to form heterotetramers to which involucrin

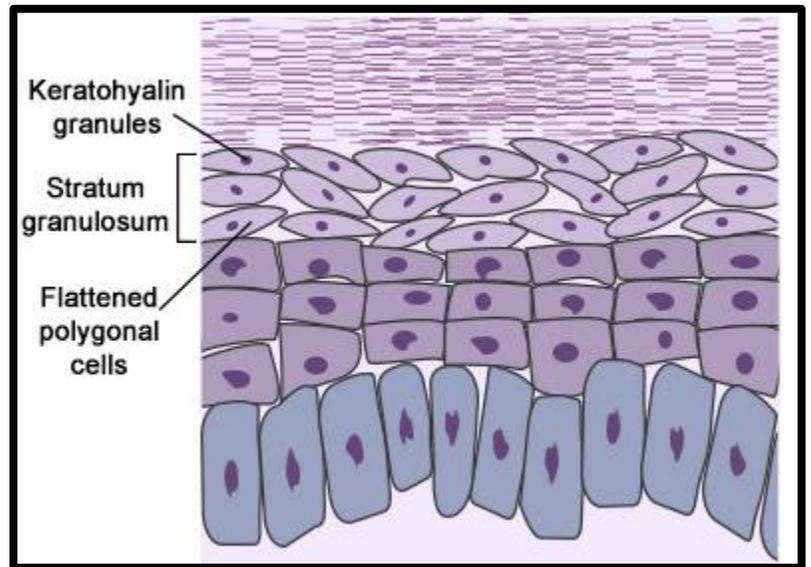


Figure 3: Cartoon representation of the epidermis with the stratum granulosum labelled. The flattened polygonal cells at this layer contain keratohyalin granules made of filaggrin filaments.

becomes bound via the action of the enzyme transglutaminase 1 (Kalinin et al., 2004). A protein scaffold, which is reinforced by a lipid envelope, makes up the extracellular matrix. A summary of the events leading to the formation of the cornified layer is depicted in Figure 4.

1.4.3 Cell death and Desquamation

The terminal differentiation of keratinocytes results in the cells dying. Given that the internal state of the cells is now mainly intermediate filaments and structural elements, the membrane-bound organelles can be destroyed and the DNA within the cell is degraded (Candi et al., 2005). During normal keratinocyte cornification, a process which is distinct from normal apoptosis is undertaken. This sequence is programmed, similar to the programmed nature of both apoptotic pathways, but this process begins as soon as the keratinocyte leaves the basal layer of the epidermis. This phenomenon is termed “epidermal programmed cell death”. The ways in which this differs from classical apoptosis is that the corneocytes are not destroyed via phagocytosis, the cytoskeleton of the cell is not degraded, but instead reorganized and the nucleus is destroyed via a different pathway during epidermal programmed cell death. Moreover, many of the caspases which are usually activated during apoptosis are not activated (Lippens et al., 2005). Instead, caspase 14, which is exclusively expressed in epidermal cells, becomes active during terminal differentiation, but not during classical apoptosis (Tschachler, 2005). Despite these cells in the cornified layer being dead, they still play a crucial role in the epidermal barrier function until they finally detach from the outermost layer of the skin through a process called desquamation.

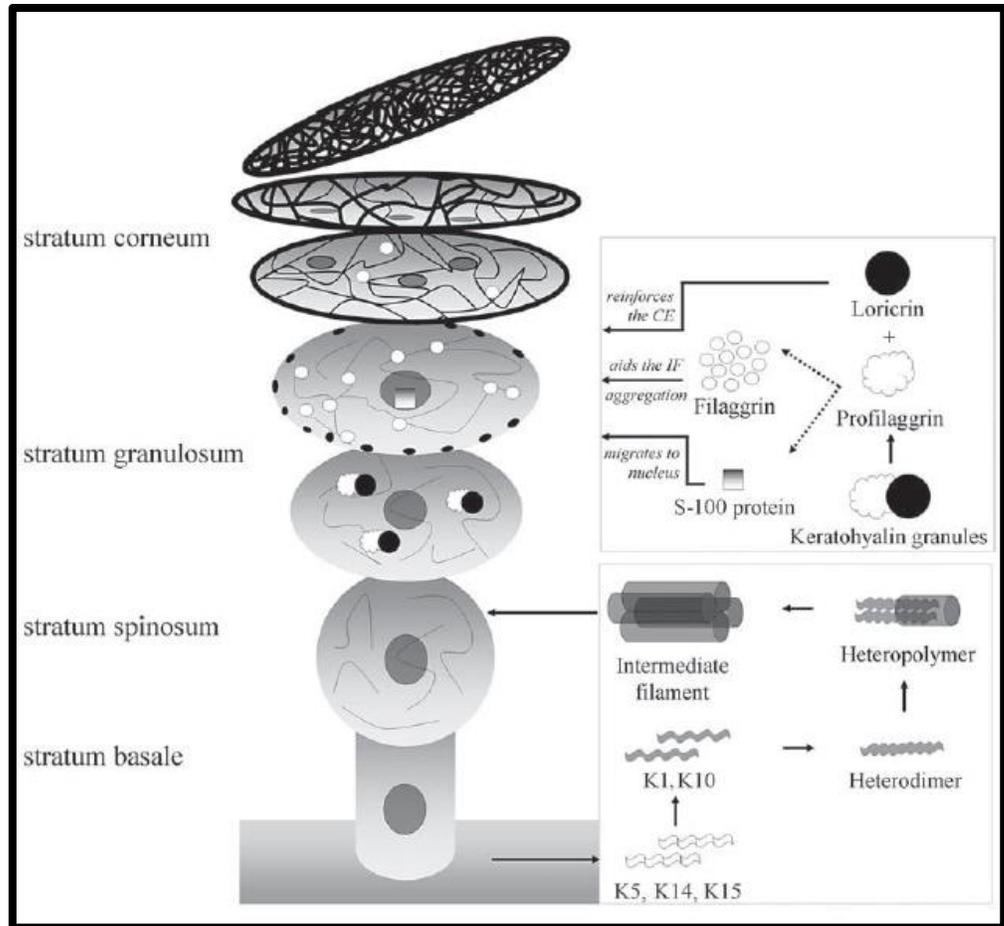


Figure 4: Schematic representation of the stages leading to the formation of the cornified layer of the epidermis. Key proteins that are expressed at these steps are shown on the right. Keratins 5, 14 and 15 are replaced by K1 and K10 allowing the intermediate filaments to form. The keratohyalin granules release Filaggrin, allowing it to aid the aggregation of other microfilaments. Loricrin is expressed to reinforce the cornified envelope resulting in complete cornification. (Houben et al., 2007)

The process of desquamation involves the degradation of desmosome, the complexes that adhere keratin filaments to one another. Desmosomes, much like the cells, undergo changes as they move through the epidermis too. In the cornified layers, the corneodesmosomes interact with multiple proteins. It is the enzymatic proteolysis of the interactions between these proteins which allow desquamation (Ishida-Yamamoto et al., 2018). The degradation of the linkages between the desmosomes and structural proteins has been extensively studied using ultrastructural microscopy (Chapman & Walsh, 1990).

In order for these sequences of events to correctly take place during differentiation, the genes involved need to be tightly controlled by transcription factors. One of the most important in epidermal differentiation is the transcription factor p63.

1.5 p63 Structure and Function

The transcription factor, p63 is known as a master regulator and is responsible for the control of expression of multiple genes. Many of the genes that are regulated by p63 are associated with development. As a transcription factor, its role is to bind to the upstream region of target genes and recruit proteins associated with the transcription initiation complex (Little & Jochemsen, 2002).

The gene encoding for the p63 protein (TP63) is mapped to the chromosomal location of 3q27-29 (Kaelin Jr, 1999). As a member of the p53 family of transcription factors, p63 shares certain homology to the other p53 family members- p53 and p73 (Melino et al., 2003). One highly conserved domain within each of the three family members is the DNA-binding domain (Figures 5 and 6). This domain is crucial for the interaction between the transcription factor and the DNA itself so any mutation in this region would be detrimental to the function of the protein (Natan & Joerger, 2012).

The gene contains two distinct promoters (Figure 6), each giving rise to different isoforms. Any mRNA transcribed using the most 5' promoter encode p63 isoforms that contain the transactivation domain. These are termed TA isoforms. Transcription of mRNA at the most 3' promoter results in products lacking the transactivation domain. These isoforms are termed ΔN forms (Figure 6). In addition to the distinct promoters, alternative splicing leads to further complexity within each class of isoform. Within the TA and ΔN classes, the alternative splicing creates three different C-termini, termed α, β and

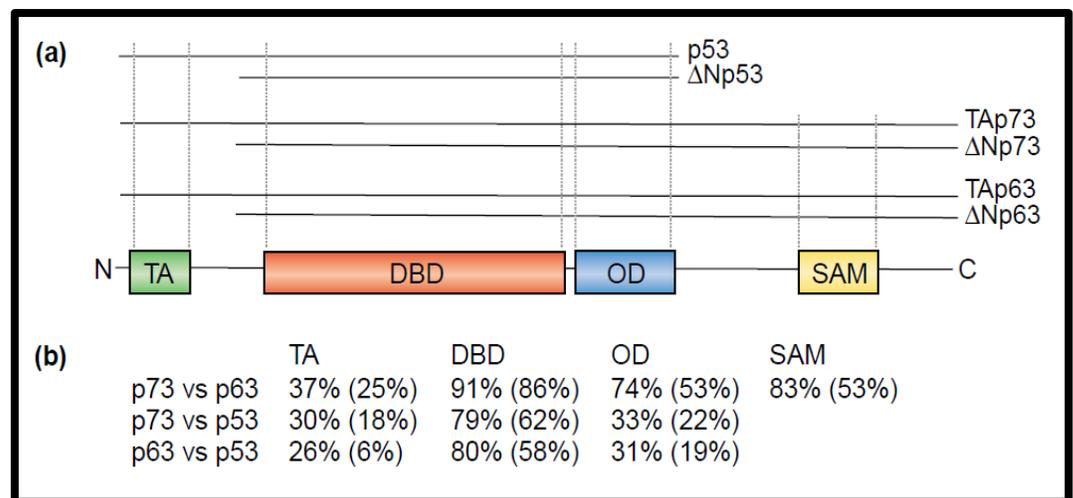


Figure 5: Schematic representation of the homology between the p53 family members. A) Features of each of the family members (TA: Transactivation domain, DBD: DNA-binding domain, OD: oligimerisation domain, SAM: sterile alpha motif). B) Percentage homology of each of the key regions of the p53 family members (Melino et al., 2003).

γ (Orzol et al., 2012). Further additional splicing of both the TA and ΔN isoforms at their C- terminal ends generate at least 10 different p63 isoforms. These species are termed α , β , γ , δ and ϵ (Mangiulli et al., 2009). The ΔN forms lack the TA domain and have a negative inhibitory effect on the transcriptional activation by the TA isoforms (Moll & Slade, 2004). $\Delta Np63$ isoforms contain an ability to activate a smaller subset of genes due to the presence of a TA₂ domain found at the C- terminal end. In addition, the sterile alpha domain (SAM) is present in the α form of $\Delta Np63$ and this is thought to be involved with protein-protein interactions (Ghioni et al., 2002). Furthermore, a transcriptional inhibitory domain (TI) can inhibit the transcriptional activity of the TA and $\Delta Np63\alpha$ isoforms (Kim & Bowie, 2003) (Figure 6).

p63 is one of the most commonly found markers in keratinocytes and is widely reported as being responsible for maintaining the proliferative potential of basal stem cells whilst also moderating and controlling the differentiation process in the epidermis.

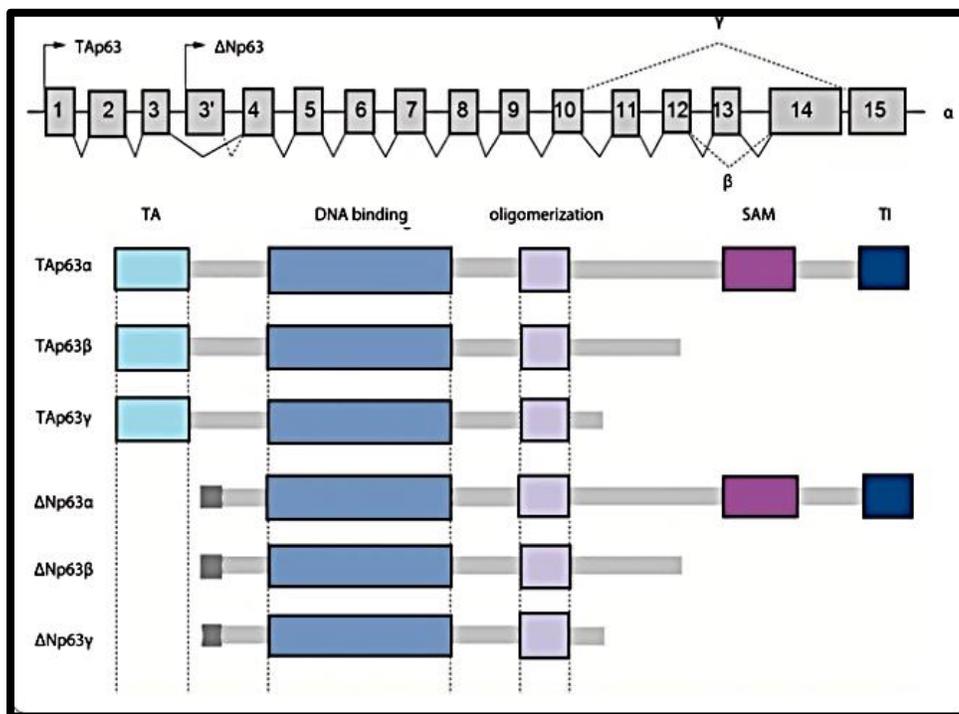


Figure 6: Varying transcripts of TP63 gene derived from two distinct promoters and alternative splicing. TAp63 subclass occur due to promoter I exon 1 allowing transcription of transactivation domain. $\Delta Np63$ subclass has absence of transactivation domain due to promoter being in 3' exon. Each transcript (α , β and γ) caused by alternative splicing at 3' end (Orzol et al., 2012).

1.6 Key Roles of p63 as a Transcription Factor

Historically, mouse models have been used for gene expression studies, however knockout models for p63 resulted in early death of the offspring, with lack of development of epidermal appendages (See 1.7). This observation suggests that p63 had clear implications in epidermal development (Mills et al., 1999; Yang et al., 1999). Moving away from mouse models and using high throughput gene expression analyses and cell culture approaches have allowed a greater depth of understanding of the expression and regulatory roles of p63.

As a transcription factor, p63 acts on a high number of target genes either resulting in upregulation or downregulation of the target. As a result, p63 plays an important role in a variety of cellular regulation processes: many involved with development. These pathways include: development of cytoskeletal elements, cytoskeletal remodeling, internal cell signaling, DNA repair and activating certain microRNAs (Botchkarev & Flores, 2014). In addition, p63 can act as a transcriptional repressor to genes that inhibit the cell cycle and transcriptional signaling (Su et al., 2013).

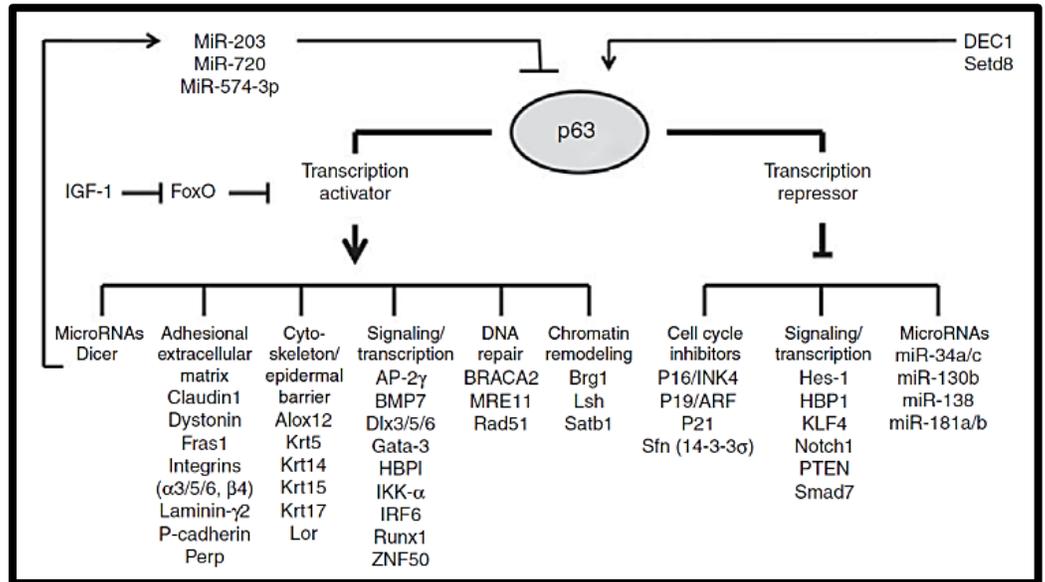


Figure 7: The transcriptional targets of p63 found in epidermal keratinocytes where p63 acts a transcriptional activator and repressor. In keratinocytes, p63 regulates specific groups of genes many of which are associated with development and maintenance. miRNAs, the transcription factor DEC1 and the methyltransferase Setd8 regulate p63 expression (Botchkarev & Flores, 2014).

As seen in Figure 7, p63 can interact with multiple targets within each of the pathways (Botchkarev & Flores, 2014). p63 itself is part of its own regulatory control as activation of certain microRNAs such as MiR-203 (Lena et al., 2008; Yi et al., 2008), MiR-720 and MiR-574-3p (Chikh et al., 2011) can in turn affect the expression of p63 in a self-regulating loop. Additionally, the *TP63* gene is regulated by other transcription factors: DEC1 and Setd8. Setd8 is a protein-lysine N-methyltransferase which acts through monomethylation of either histones or non-histone proteins while DEC1 is less known, but has been implicated with p53 family interactions (Qian et al., 2008).

Some of the key pathways for development and maintenance of the epidermal barrier are p63-regulated. Many of the keratins associated with being the key fibrous structural proteins responsible for providing the mechanical stability and protection of the epidermal barrier are regulated by p63. Terminal differentiation markers such as loricrin and filaggrin have been described as p63-regulated which suggests a role for p63 not only in the maintenance and early differentiation steps but also at the terminal stages. In order for all steps of development and maintenance to be carried out without problems, tight

signaling and transcriptional control must be in place, and again p63 plays a role here. Key signaling molecules in the epidermis such as Gata-3 (a zinc-finger nuclear transcription factor (Mertens et al., 2015)), IKK- α (an enzyme complex that forms part of the NF-kappaB signaling cascade) and IRF6 (a transcription factor associated with developmental pathways) are regulated by p63 in the epidermis.

	TAp63 (isoforms)		ΔNp63 (isoforms)		KO mice with epidermal phenotype
IKK α	all	↑	all	-	Yes
PERP	α and γ	↑	α and γ	↑	Yes
EGFR	γ	↓	α	↑	Yes
FGFR2	α	↑	α	↓	Yes
AP2- γ	α	↑	α	-	
K14	α	↑	α	↑	Yes
Envoplakin	γ	↑			Yes
p21	all	↑	α	↓	
Jagged-1 & 2	γ	↑			Yes
Hes-1	α	↓	α	↓	
Hey-1,2	α	↑	α	↑	
GATA-3	α		α		Yes
BPAG-1	β and γ	↑	β	↑	Yes
Ets-1	α	↑	α	-	no skin phenotype

(↑)=Upregulation by p63, (↓)=downregulation by p63 (-) = no effect

Figure 8: Selected p63 isoform targets with known impact on development in epidermal tissue, particularly the skin. Adapted from Candi et al., 2007

Given the variety of pathways that p63 is controlling, it is clear that p63 is a crucial factor for maintaining the correct cascade of events in a multitude of disciplines. As p63 exists as two different isoforms, it begs the question as to the role of the individual isoforms in orchestrating this vast network of processes.

1.7 Isoforms of p63

In the literature, there is a large amount of conflict between details of the temporal expression and importance of each of the isoforms at different stages of development. However, there is strong evidence to suggest that the TAp63 isoforms play key roles in the establishment of the germline and the ectodermal stages. Δ Np63 isoforms are predominantly found during the proliferative stages of development and are

accountable for approximately 99% of the p63 expression found in differentiated keratinocytes. However, recent studies have found that TAp63 isoforms are overexpressed in terminally differentiating keratinocytes (Candi et al., 2007)

The shorter, truncated version of p63, termed Δ Np63, is the most commonly discussed isoform in literature to date in terms of its role in epidermal development. This is due to the high level of expression of these isoforms making studies much easier to carry out. Most studies carried out in mice contend that Δ Np63 isoforms are highly expressed in the early stages of epidermal morphogenesis and continues to be expressed throughout the basal layer in addition to other epithelial tissues (Laurikkala et al., 2006; Romano et al., 2012). Research into the specific roles of Δ Np63 has found some of the key mechanisms with which these isoforms are associated. Protein analysis has demonstrated that Δ Np63 forms are down regulated in more differentiated layers of the epidermis.

In contrast to Δ Np63, TAp63 is noted to be weakly expressed in the skin. However, it has been shown to be expressed prior to Δ Np63. In overexpression studies of TAp63, it has been shown that when TAp63 was overexpressed the cells went into a hyperproliferative state meaning TAp63 was able to prevent the cells going into differentiation. This function was noted only for the TAp63 isoforms. The authors of this work speculate that TAp63 is the molecular switch, which is responsible for the pluristratification of the epidermis, and that Δ Np63 is the counteracting force, allowing cellular differentiation. (Koster et al., 2004) These findings are in contrast to other data,

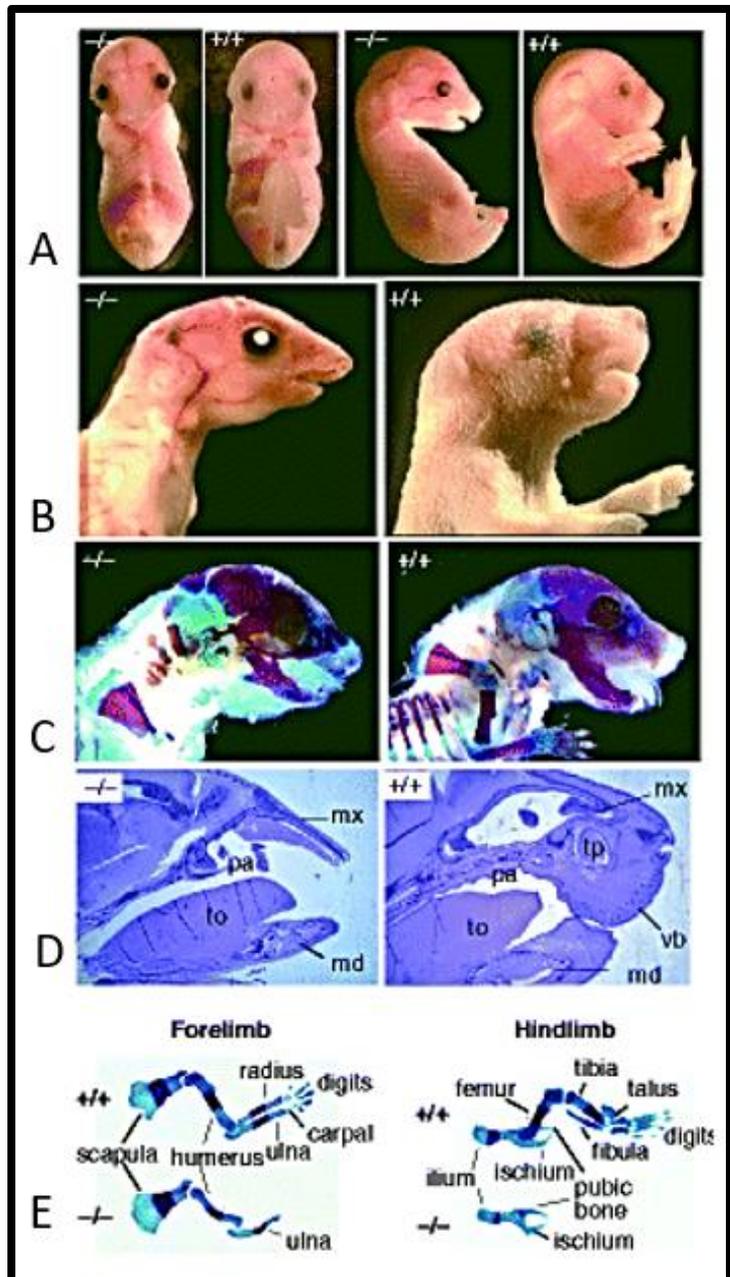


Figure 9: A) Front and sagittal views of mouse control (+/+) and p63 null (-/-) E17 mice embryos. B) P63 null mouse pups one-day postnatal day 1. p63 -/- lacking eyelids, footpads, lack whisker pads and have hypoplastic upper and lower jaw. C) Bone and cartilage staining D) Hematoxylin and eosin staining of sagittal sections revealing malformation of maxilla (mx), mandible (md), and secondary palate (pa) and complete lack of vibrissae (vb), tooth primordia (tp) and soft tissue structures in null mice. E) Bone and cartilage structures of fore and hind limbs of E15 p63 -/- mice compared to control littermates. Adapted from Yang et al., 1999.

which suggests that Δ Np63 is the only isoform expressed in epidermal development and that it is solely expressed in the basal layer, where cells are undifferentiated. It is rapidly degraded throughout differentiation (Pellegrini et al., 2001; Yang et al., 1998). The basis behind the contrasting theories is still not understood.

As a regulation dogma, the p63 system works through competition between the two isoforms (Candi et al., 2007). Δ Np63 has been linked to many regulatory roles, including many which repress transcription of cell cycle related genes such as cyclin B2, Cdc2 and topoisomerase II α (Testoni & Mantovani, 2006). Additionally, cross-talk between p63 and Notch signaling can allow the modulation of Notch-1 dependent transcription in the process of epidermal stratification and differentiation (Rangarajan et al., 2001).

1.8 Mutations in p63 and its Association to Disease

Due to its importance in multiple transcriptional regulation cascades, mutations in the *TP63* gene can lead to severe developmental conditions. Genetic ablation of p63 in mouse models have been shown to result in failure of the stratification of the epidermal tissue alongside other developmental abnormalities such as lack of epidermal appendage formation, limb malformation or complete lack of limb formation (Mills et al., 1999) (Figure 9). Given the severity of the phenotype when complete p63 knockout is carried out; it demonstrates a clear importance for p63 in correct development of limbs and epithelial tissues. Additionally, heterozygous mutation in the p63 gene is generally believed to play a dominant-negative effect on the wild type due to formations of wild type and mutant tetramers, hence reducing the amount of cellular functional p63 protein.

1.8.1 EEC and AEC

In humans, mutations in the *TP63* gene has severe effects also leading to many developmental conditions (Brunner et al., 2002). One p63-related disorder is ankyloblepharon-ectodermal dysplasia clefting (AEC, also known as Hay-Wells Syndrome) (Grange, 2013). This condition is a rare autosomal condition with fewer than 100 affected individuals mentioned in literature with the exact incidence or prevalence of the disorder unknown. The symptoms of this conditions present in different ways, even amongst family members. Many of the signs of AEC present at birth with many infants presenting with symptoms such as fused eyelids- ankyloblepharon filiforme adnatum. Another key sign is the erosion of the skin, which may be life threatening. If skin erosions occur on the scalp, this can lead to severe hair loss and formation of scar tissue. Throughout the patient's life, the skin erosions may recur even into adulthood. These erosions are an obvious sign and can be painful. Alongside the erosions, there may be other signs such as redness, waxiness or dry scaly patches with hyperkeratosis of the knees and elbows.

Furthermore, other areas other than the skin are affected, with many epidermal tissues forming incorrectly. This can include signs of hypodontia, chronic inflammation of the eyelids and other limb formation issues.

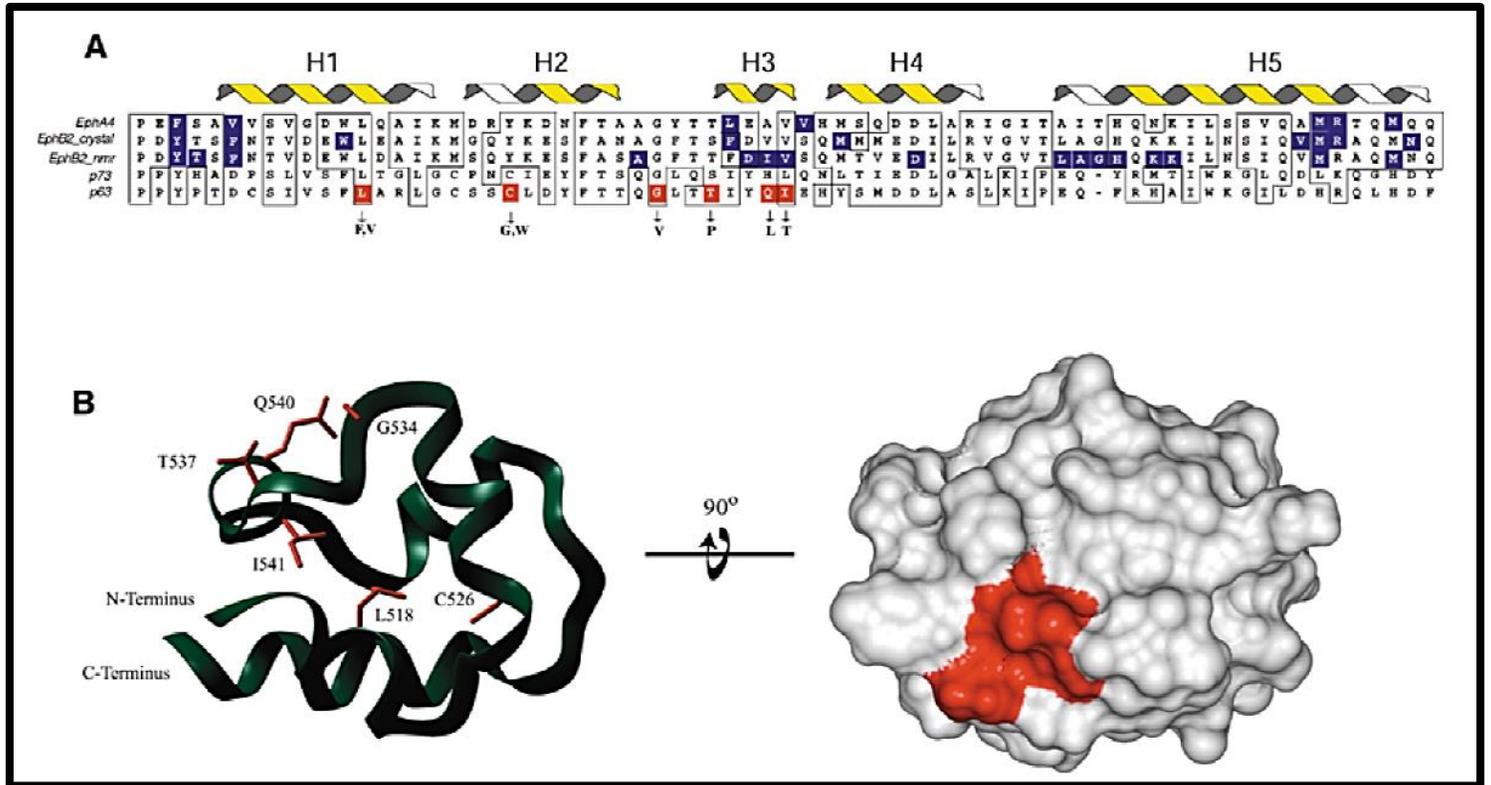


Figure 10: A) results from multiple sequence alignment of p63 SAM domain alongside p73 and Sterile alpha motif domains which have previously been solved. Highlighted in orange are the sites for missense mutation to occur, all of which are present in sufferers of AEC. B) Ribbon model shows amino acids that are mutated in AEC and their relative positions. Space fill model shows the clustering of three mutated amino acids on protein surface (glycine 534, threonine 537 and glutamine 540) (McGrath et al., 2001)

The disease is genetic and many studies have investigated the cause of it. Multiple sequence alignments have identified that mutation in the sterile alpha motif region of the p63 protein lead to the disease (McGrath et al., 2001). Single nucleotide polymorphisms in this region created missense mutations leading to the condition, as seen in Figure 10. Despite this work, a majority of AEC-related mutations occur in the C terminal of p63 (Rinne et al., 2006). More recently work carried out by Russo (2018) on 227 AEC patients has found that protein aggregation of p63 is an underlying factor for the skin fragility in AEC syndrome (Russo et al., 2018). This work identified many AEC-associated p63 mutations that lead to thermodynamically destabilized, misfolded and aggregated protein products. These disrupted protein structures were shown by the work to show a higher propensity to aggregate creating a dominant-negative effect on the wild type and that reversal of the aggregation restored normal p63 activation.

Another similar condition to AEC is EEC (ectrodactyly, ectodermal dysplasia and cleft lip palate). It again is autosomal dominantly inherited and affects the hair, nails, sweat glands and tooth formation (Rodini & Richieri-Costa, 1990). p63 mutations account for the vast majority, if not all of the cases of classical EEC. Heterozygous mutations in the *TP63* gene occur in the DNA-binding domain which is common in all p63 isoforms. The Arginine residues at five common positions are the most common abnormalities, accounting for approximately 75% of EEC cases (Brunner et al., 2002).

1.8.2 Psoriasis

Psoriasis is another skin condition of which p63 has been implicated. Psoriasis presents as chronic keratinization characterized by hyperkeratosis with inflammation and epidermal hyperplasia. The presence of hyperplasia in psoriatic epidermis are due to increased amounts of dividing keratinocytes (Weinstein et al., 1985). Despite this, the mechanisms underlying the epidermal remodeling associated with psoriasis are not fully understood. Studies have been carried out to investigate the role of p63 in psoriasis due to p63's importance in proliferating keratinocytes. Studies have used stainings to identify spatial expression of p63 isoforms in hyper-proliferative conditions (Shen et al., 2005) along with mRNA and protein analyses (Gu et al., 2006).

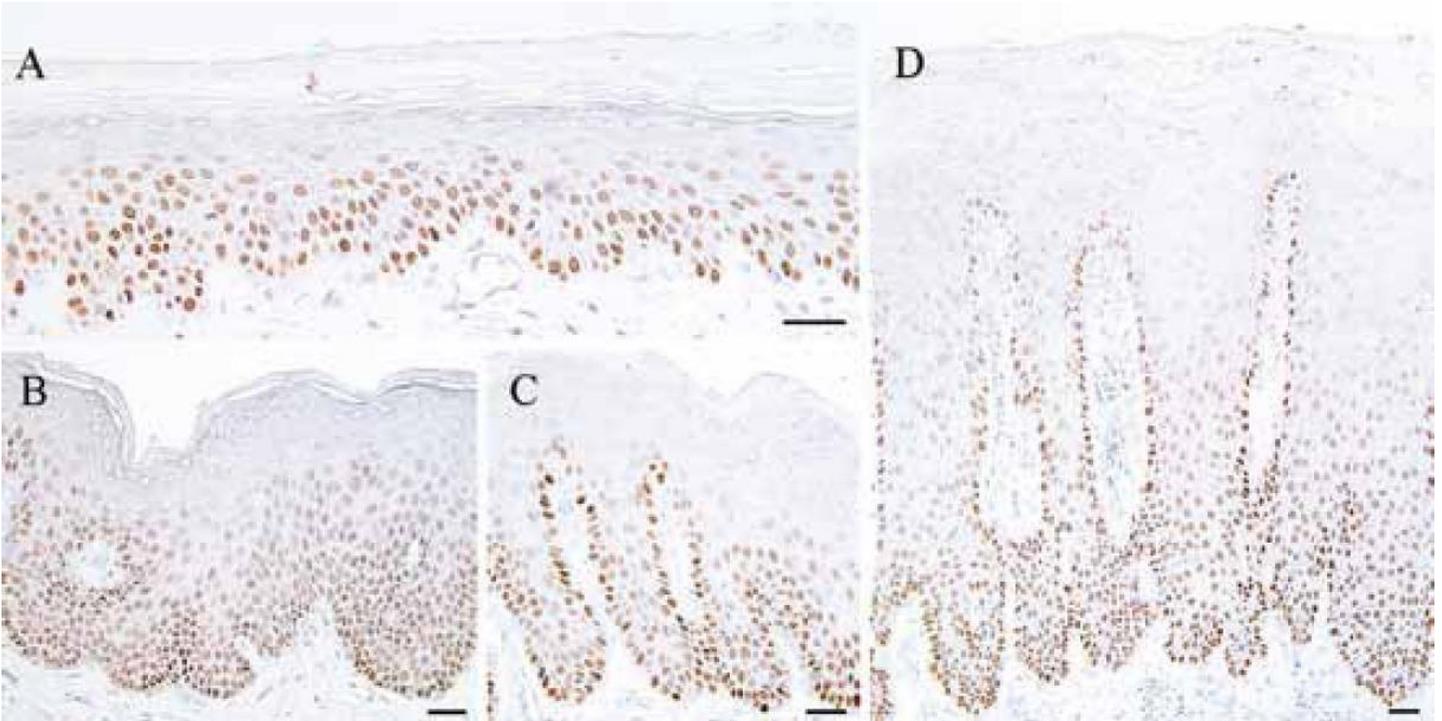


Figure 11: Expression of p63 in psoriatic skin. A) In early stages, p63 is expressed in the basal cells. B) During the thickening of the rete edges, strong expression of p63 is seen. C) Once rete edges have fully formed, p63 expression is seen localized in the protruding edges. D) In fully developed psoriasis, p63 is seen in the basal cell layer and the lower regions of the rete edges. Adapted from Shen et al., 2005.

High throughput transcriptomic analyses such as RNASeq in the study of diseases such as psoriasis allows comparison of large sample sizes to develop an understanding of disease mechanisms. Previously, microarray analysis of psoriatic samples revealed large numbers of differentially-expressed genes (Gudjonsson et al., 2010). RNASeq studies have taken the findings of microarray analysis further to identify unmapped transcripts and alternative splicing (Wang et al., 2009).

In psoriatic skin, p63 has been shown to be upregulated in particular in elongated rete edges. More specifically, Δ Np63 α is overexpressed and this upregulation leads to further upregulation of protein kinase genes leading to proliferation (Shen et al., 2005). In contrast, cyclins and other cell cycle-related genes have been found not to be Δ Np63 α regulated suggesting that p63 is not the only signal essential for causing the hyper-proliferative state in psoriasis (Meyerson et al., 1992; Okuda et al., 1994; Wu et al., 2003).

1.9 Genes of Interest

We wanted to look at the isoforms of p63 alongside a collection of other marker genes:

KLF4: Kruppel-like factor 4 (KLF4) is a transcription factor from the Kruppel family. The protein is a zinc finger protein which has been implicated in the normal development and function of the skin's barrier. KLF4 expression is usually found in well-differentiated, non-proliferating cells (Ghaleb et al., 2005). In literature, KLF4 and p63 have been linked by work using ChIP (chromatin immunoprecipitation reactions) (Testoni et al., 2006; Viganò et al., 2006). KLF4 will be used in this study due to the fact that work has uncovered that KLF4 is a transcriptional target of p63 (Cordani et al., 2010). Despite work identifying p63 as a transcriptional regulator of KLF4, it is not known as to which isoform is responsible.

FLG: Filaggrin is a structural protein encoded by the *FLG* gene and is associated with maintaining the barrier function of the epidermis. This makes it a strong marker for epidermal differentiation as its expression is expected during only the final stages of differentiation- making it a good marker for terminal differentiation. Filaggrin specifically interacts with keratins and other intermediate filaments. In addition to being a differentiation marker, *FLG* expression has been reported as being blocked by TA-containing isoforms of p63, more notably TAp63 α and TAp63 γ (King et al., 2006). Furthermore, the α -tail of the Δ Np63 α forms block profilaggrin expression, supporting the concept of p63 isoforms working in tandem during epidermal differentiation (Candi et al., 2007).

ETS-1: This protein is a member of the ETS family of transcription factors that are defined by their common DNA-binding domain that recognizes the consensus DNA sequence of GGAA/T. As a transcription factor, it acts to promote and repress gene expression. The genes associated are noted to be involved in stem cell development, cell senescence and death. It is normally expressed in the proliferative layer of the stratified epithelium (Nagarajan et al., 2010). *ETS-1* is an oncogene heavily linked to squamous cell carcinomas: tumors with aberrations in the normal epidermal differentiation pattern (Bhawan, 2007). It has also been noted in literature as being a transcriptional target for TAp63 isoforms in epidermal differentiation pathways and is a known regulator of *CHUK*, another gene marker studied in this work (Candi et al., 2007).

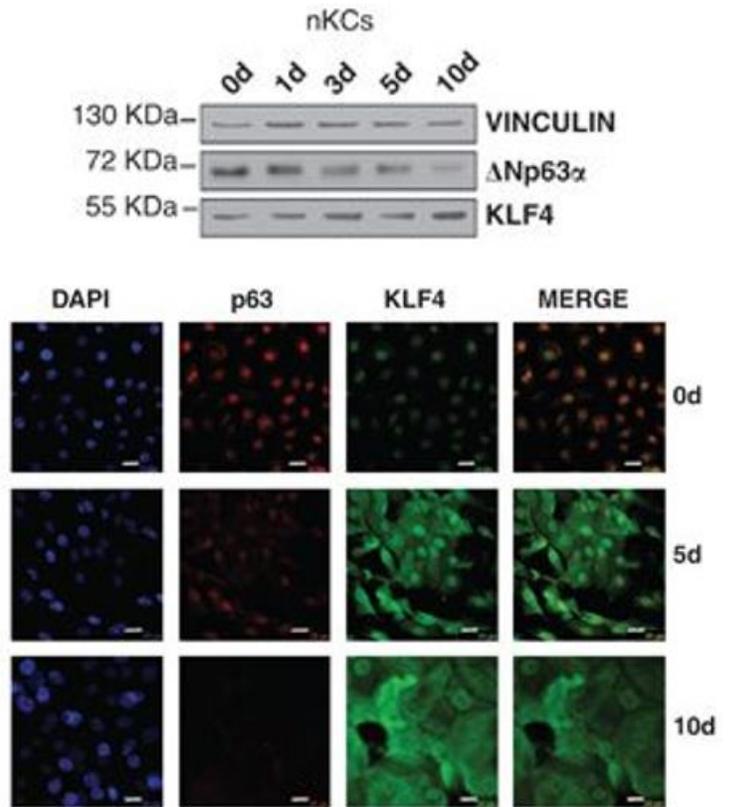


Figure 12: KLF4 expression increases during keratinocyte differentiation. Western Blot analysis of keratinocytes over a period of 10 days of differentiation. Immunofluorescence analysis of p63 and KLF4 expression. Adapted from (Cordani et al., 2010)

CHUK (IKK- α): CHUK is a serine kinase, which plays an essential role in the NF-Kappa-B pathways and can be activated by DNA damage, inflammatory cytokines or other cellular stresses. As a serine kinase, it phosphorylates inhibitors of the NF-Kappa-B pathway. These modifications allow the subsequent degradation of the proteasome allowing NF-Kappa-B to activate genes associated with aversion from apoptosis, growth control and some immune response applications. One transcription factor that has been demonstrated to regulate CHUK expression is ETS-1 during epidermal differentiation (Candi et al., 2007) (Figure 13).

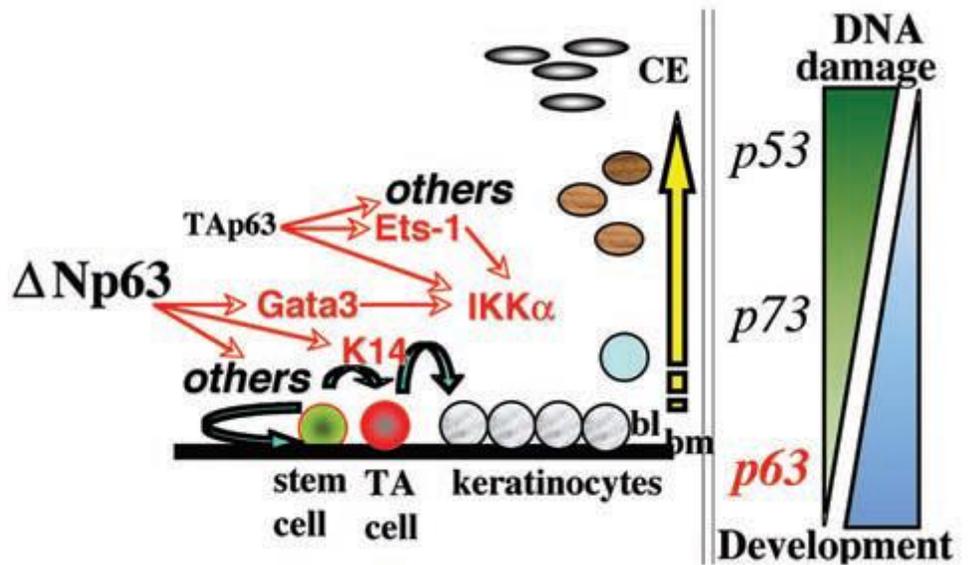


Figure 13: *Left Panel:* Speculated roles of p63 isoforms in epidermal differentiation pathways. $\Delta Np63$ is demonstrated as regulating CHUK (IKK- α) indirectly through Gata3. CHUK is shown to be regulated by ETS-1 also, which itself is regulated by TAp63 isoforms. *Right Panel:* P53 family members have varying roles from development (more predominantly p63) right through to DNA damage (predominantly p53). (Candi et al., 2007)

TGM1: This gene encodes the transglutaminase 1 enzyme. This enzyme is found in the epidermis and is involved with the cornified envelope formation. Here it forms strong cross-links between structural proteins which makes up the cornified envelope, providing stability and strength.

1.10 Aims and Objectives

The aims of this work are to investigate how the isoform specific expression of p63 influences the gene expression and regulation of multiple keratinocyte differentiation markers during differentiation. To achieve this, we want to investigate the marker expression in normal cases using monolayer culture of keratinocyte cell lines. This research includes culture of HaCaT cell lines alongside primary keratinocytes. The cells will be cultured for 13 days of differentiation with RNA extracted from cells at given time points. Gene expression analysis will be quantified via qRT-PCR using the TaqMan system. To further the investigation, we have decided to design and optimize siRNA-induced knockdown systems of the p63 isoforms. We want to quantify the gene expression of the isoforms and gene markers in basal keratinocytes with the knockdowns and then look into how the expression of these genes change during keratinocyte differentiation in vivo.

There is a clear need for research into the roles of p63 isoforms due to a high level of conflict present in current research (Billant et al., 2016; Candi, Rufini, et al., 2006; Zhao et al., 2015). The main purpose of this study was to develop a technique to allow analysis of genes in basal cells, differentiating

cells and cells at these stages with p63-isoform knockdown. By developing knockdown cells, the importance of individual isoforms can be examined in more detail, allowing downstream implications to also be investigated. The contrasting findings currently published is mainly due to the inability to differentiate between each isoform's activities. These roles are yet to be uncovered fully due to the limited technology and techniques allowing specific studies. Herein lies the issue that is preventing the full picture of p63 isoform activity in keratinocytes being discovered.

The expected conclusions from this research is that there is further evidence that p63 has a dual role in keratinocyte differentiation, which arises from the differential expression of the two main classes of isoforms. The structural differences between the two classes give rise to varying functionality and are associated to different networking systems. Previous studies have identified TAp63 as a component present in terminal differentiation and hence we expect expression to be higher at the late stages of differentiation in keratinocytes. Through generation of knock down lines, there is an expectation that expression of other markers associated with p63 will be disrupted and this may lead to further downstream effects on expression at the gene level.

2.0 Materials and Methods

2.1 Materials List

Reagents and Components:

Reagent	Company/Supplier	Order ID
500 ml Filter System	Corning	430758
Adenin	Sigma	A2786-5G
CaCl ₂	Sigma	C2661-500G
Cholera Toxin from <i>Vibrio cholera</i>	Sigma	10236276001
6 well plate	Corning	355467
Dimethylsulfoxid (DMSO) Rotipuran	CarlRoth	4720.4
Distilled water (DNase/RNase free)	Gibco	10977-035
DMEM	Gibco	11960-044
DMEM F12 (1:1) (1x)	Gibco	21331-020
DMEM Low Calcium	Gibco	21068028
Ethanol Absolute	Acros Organics	445740010
Fetal Bovine Serum (FBS)	Gibco	10270-106
Gentamicin (10mg/mL)	Sigma	G1272-100mL
Human Epidermal Growth Factor (EGF)	Sigma	E9644-.2mg
Hydrocortisone	Sigma	H0888-1G
Insulin from Bovine Pancreas	Sigma	I1882-100mg
Keratinocyte Basal Media (KBM)	Lonza	CC-3101
Gold Keratinocyte Growth Media (KGM Gold)	Lonza	Catalog #: 00192060
KGM Single Quot Kit	Lonza	CC-4131
L-Glutamine 200mM (100x)	Gibco	25030-123
Lipofectamine 2000	Thermo	10696153
OptiMEM Reduced Serum Media	Gibco	31985062
PBS pH 7.2 (1x)	Gibco	20012-019

Penicillin-Streptomycin (P/S)	Gibco	15140-122
ProFreeze NAO/CD Freeze (2x)	Lonza	12-769E
Stealth siRNA Assays*	Thermo Scientific	*Details in Table 7
Stealth Medium GC Negative Control	Thermo Scientific	10143902
TaqMan Fast Universal PCR Master Mix	Applied Biosystems	4364103
TaqMan Gene Expression Assays *	Applied Biosystems	*Details in Table 6
Trypsin EDTA 0.05% (1x)	Gibco	25300-096
Trypsin Inhibitor Soybean	Gibco	17075-029

Kits:

Kit	Compnay/ Supplier	Order ID
High Capacity RNA-to-cDNA Kit	Applied Biosystems	4387406
PureLink RNA Mini Kit	Ambion	12183018A

2.2 Probands and Ethical Approval

Ethical approval for all work carried out in these studies were approved by the University of Huddersfield Ethics Committee. Any primary cells used for the purpose of this research were collected with the correct ethical approval from the clinic or laboratory from which they were taken. The cell lines used in keratinocyte differentiation were obtained from voluntary persons undergoing plastic surgery procedures. In all cases, samples were anonymized and denoted an alphanumeric code. Any donor was given full written notice about the study and a consent form was completed. As cells were of human origin, the cells were stored and handled under the Human Tissue Act and all relevant measures were observed.

The cells denoted HKc17, were obtained from Genoskin. This company has its own ethics that states they use real human skin to create skin models and samples for research purposes. The skin samples are derived from excess skin that is left over following surgical intervention. Genoskin follow the Declaration of Helsinki which outlines the ethics involved. The proband in this case was a 37-year-old female, obtained from the abdomen. Cell lines denoted HKc13 and HKc15 were samples obtained from abdomen biopsies of separate donors. These samples were obtained from the Huddersfield Royal Infirmary but donor information was not disclosed. The HaCaT cells were donated as a gift from the German Cancer Centre.

2.3 Cell culture

All cell culture work was carried out in Class II laminar flow hoods that are suitable for BSL-1, 2 and 3 materials. All general laboratory protocols for safe practice and work were observed including use of protective equipment. All cultures were at 37°C and 5% CO₂.

2.3.1 HaCaT Cell Culture

HaCaT cells are immortalized human keratinocytes which have been used in many epidermal homeostasis studies. They have been evaluated as a model cell system to study normal and abnormal skin barrier function and gene expression work. These cells have been extensively used in the laboratory as a model system: mainly in comparative studies with human epidermal keratinocytes.

For the purpose of these experiments, cells were cultured *in vitro* in a 2D culture with low calcium media to maintain the cells at basal phenotype until ready for differentiating. Cells were from the laboratory stock: a gift from the German Cancer Research Center. Passage number 124 cells were plated in p10 (Falcon) dishes to allow growth and for cells to become confluent. Keratinocytes grown in low calcium concentration below 0.03mM proliferate but fail to differentiate. Above 0.13mM calcium cells begin to differentiate into the stratified layers. In the laboratory, these conditions were mimicked by adding a calcium stock to the calcium-free media.

Table 1: Media composition for calcium- free media for HaCaT cell culture

Media Component	Volume (250ml total volume)	Concentration
Calcium- free DMEM (GIBCO)	150ml	
Standard DMEM 1.8mM Ca²⁺ (GIBCO)	9ml	6%
Fetal Bovine Serum (GIBCO) Chelex100 Treated	54.5ml	10%
F12 Nutrient Mix (GIBCO)	25ml	0.3mM
Glutamine (GIBCO)	5ml	4mM
Pen/Strep (GIBCO)	2.5ml	100U/ml

Once cells had reached 80% coverage of the plate, they were split using standard laboratory protocols into smaller vessels (p60- supplied by Falcon). Cells were plated at a density of 0.2 million cells per plate. The media composition is shown in **Table 1**. All components were added straight to the media in the state supplied by the company with exception of the Fetal Bovine Serum (FBS), which was filtered using Chelex100 to remove calcium ions from the serum.

Once the cells in the p60 dishes were approximately 60-70% confluent the cells were introduced to the high calcium media. This media was prepared in the same fashion as the calcium-free media with the exception of calcium stock (Sigma Aldrich) added to a final concentration of 0.13mM (**Table 2**).

Table 2: Media composition for the differentiation of HaCaTs in vitro containing high calcium concentration.

Media Component	Volume (250ml total volume)	Concentration
Calcium- free DMEM (GIBCO)	150ml	
Standard DMEM 1.8mM Ca²⁺ (GIBCO)	9ml	6%
Fetal Bovine Serum (GIBCO) Chelex100 Treated	54.5ml	10%
F12 Nutrient Mix (GIBCO)	25ml	0.3mM
Glutamine (GIBCO)	5ml	4mM
Pen/Strep (GIBCO)	2.5ml	100U/ml
Calcium (CaCl₂ stock- SIGMA Aldrich)	205.2µl	0.13mM

As gene expression throughout differentiation was investigated, cells were harvested at pre-defined time points of every day from Day 0 to Day 7 and then Days 9, 11 and 13 were collected. This in total gave 11 time points from a two-week differentiation experiment. Day 0 samples were collected before the differentiation media was added to cells meaning these cells are still basal in phenotype. On each day of collection, the cells were washed with 1x PBS (GIBCO) to remove old media. Cells were then incubated for 10 minutes at 37°C, 5% CO₂ with 5x Trypsin EDTA (GIBCO) to dislodge cells from the plate. After the incubation with trypsin EDTA, 1x volume of serum-containing media was added to inactivate the trypsin activity. Gentle mixing and pipetting of the supernatant was used to collect any cells that were loosely attached to the surface. The total cell suspension was collected in a 15ml Falcon and centrifuged in a bench centrifuge for 4 minutes at 1000rpm. This process pelleted the cells, allowing the

removal of the supernatant. The pellets were stored at -20°C until required for downstream use. All this work was carried out using established protocols that the laboratory has optimized and tested previously.

2.3.2 Keratinocyte Cell Culture

Culture of normal human keratinocytes was carried out in a similar fashion to the HaCaT culture. These cells were again maintained in a basal phenotype until ready for differentiation. Two comparative lines were used for these gene expression studies:

- The first keratinocyte (HKLonza) line was a commercial line (Lonza). These cells are provided in a cryo-preserved state with each vial containing >500,000 cells. For this research, these cells in passage 4 were used.
- The second line of keratinocytes used for this work were extracted from a skin sample supplied by Genoskin (HKc17). The sample was obtained from an abdomen section from a 37-year-old female. The cells used from this sample were at passage 2.

Table 3: Media components for calcium-free KGM Gold media for human keratinocyte culture

Media Component (all supplied by Lonza)	Volume (500ml total volume, to manufacturer's recommendations. Reagent concentrations not disclosed)
KGM Gold Media	500ml
Hydrocortisone	0.5ml
Transferrin	0.5ml
Epinephrine	0.25ml
Gentamicin Sulphate Amphotericin 1000	0.25ml
Bovine Pituitary Extract	2.00ml
Human Epidermal Growth factor	0.5ml
Insulin	0.5ml

As the HKLonza were optimized for growth using KGM Gold™ Growth Media (catalogue number 00192152), both cell lines were cultured using this media. This media is recommended for culturing human adult keratinocytes. The media components are all supplied in bullet form for addition to the basic

KGM Gold™ media. Addition of all the aliquots supplied means the media is prepared. The aliquots are shown in **Table 3**.

For calcium-induced differentiation experiments in normal keratinocytes, HKc13 and HKc15 cells were used at low passage number. These cells were harvested on pre-determined days throughout a 21-day period of differentiation. These cells were cultured as described and induced into differentiation by addition of calcium to the media. These cells were grown in standard KGM media (Lonza) as there was no need to culture these in Gold due to that fact they had not been optimized to grow in this media.

As with the HaCaT culture, differentiation was induced by addition of calcium stock to the media. The final concentration of calcium in the media was again made up to 0.13mM. Again, similar to the HaCaT experiments, differentiation was induced and cells were harvested at defined time points. The cells were harvested in the same way as described for the HaCaT experiments with one change to the protocol. As keratinocytes are more sensitive than the immortalized HaCaTs, the trypsin EDTA activity was inactivated by a trypsin inhibitor (ThermoFisher) rather than through addition of one volume of serum-containing media. The days on which the cells were harvested at the same time points to allow for comparison between the cell lines. Once all cells from the differentiation were harvested, they were frozen at -20°C until the RNA from these cells could be extracted.

2.4 RNA Extraction and cDNA Synthesis

The total RNA was extracted from each of the cell pellets via the Ambion RNA Pure-link mini kit supplied by Life Technologies. This process involves a series of washes and spinning to isolate the RNA product before re-suspending in RNase-free water. The protocol supplied with the kit was observed throughout; with an additional DNase 1 treatment step added (**Appendix 7.6**). To begin, the cell pellets were washed with 0.6ml of Lysis buffer with 1% 2-mercaptoethanol to lyse the cell membranes. The same volume of freshly prepared 70% ethanol was added to the Falcon and the suspension was vortexed on a high setting until all precipitate was no longer visible. The liquid was passed through a spin cartridge containing a filter. Following each spin, the liquid was discarded and the filter kept. Following on, there were a series of washes with Wash Buffer 1. The samples were then exposed to a DNase 1 treatment where 3U/μL DNase 1 enzyme (Ambion) was added to the columns for 15 minutes at room temperature (**Appendix 7.6**). This step removed any unwanted DNA in the samples that may interfere with the RNA quality and quantification. The samples were then washed twice with Wash Buffer 2 and finally eluted into 50μl of RNase-free water. The samples were stored at -20°C for short-term storage.

The RNA quality and concentration was quantified on the Nanodrop 2000 (ThermoFisher) using standard settings for RNA analysis. Any RNA with low yield or quality were discounted from downstream work and cells were re-cultured to replace these poor yields. Purity readings of the 260/280 and 260/230 ratios were used to assess the quality of the RNA yields at this stage. For RNA A260/280 ratio reading of ~2.0 is considered “pure” RNA and the secondary ratio of 260/230 should have expected values of between 2.0-2.2 for “pure” RNA. All samples had to fulfill this standard before use in cDNA synthesis.

To create the cDNA from the RNA samples, reverse transcription was carried out using the cDNA Synthesis kit from Applied Biosystems. This kit contains the MultiScribe™ reverse transcriptase enzyme, which allows for downstream applications including qRT-PCR. This protocol requires 2μg of RNA for every 20ul reaction. The protocol was followed as supplied with the reaction components as shown in

Table 4. A control sample without reverse transcriptase enzyme was used. The reverse transcriptase reaction mix was prepared on ice. The reverse transcription reaction was carried out at 37°C for 60 minutes followed by 95°C for 5 minutes and then maintained at 4°C permanently. Once produced the cDNA was quantified on the Nanodrop 2000 spectrophotometer and stored at -20°C until required.

Table 4: Components in the reverse transcription reaction mix to create cDNA from RNA

Component	Volume per Reaction (µl)- All volumes as per instruction from the manufacturer	
	+RT	-RT
2x RT Buffer	10.0	10.0
20x RT Enzyme Mix (containing MultiScribe™ reverse transcriptase)	1.0	-
Nuclease-free Water	Quantity sufficient to 20µl	Quantity sufficient to 20µl
RNA Sample	Up to 9µl (2ng)	Up to 9µl (2ng)
Total per Reaction	20.0	20.0

2.5 Real-time PCR

Real time PCR was our chosen method for assessing the gene expression throughout differentiation. The system which was used for the purpose of this worked was the TaqMan system.

For the purpose of this research, TaqMan assays were ordered through ThermoFisher Scientific. Each assay was a predesigned assay that was recommended for this research type. All assays were tested in house either by the author. Previously in the laboratory, a different TAp63 assay (Hs00186613) was tested but the assay did not bind specifically and failed to amplify. Hs00978349 replaced this assay.

Table 5: TaqMan assays used for the purpose of this research. All probes labelled with FAM.

Gene	Product Number	Function
GAPDH	Hs04420631	Housekeeping gene (Barber et al., 2005)
RPL13A	Hs04194366	Housekeeping gene (Mane et al., 2008)
TAp63	Hs00978349	P63 isoform, transcription factor
Δ Np63	Hs00978339	P63 isoform, transcription factor
FLG	Hs00856927	Terminal differentiation marker, structural protein
KLF4	Hs00358836	Transcription factor, associated with terminal differentiation
TGM1	Hs01070310	Enzyme involved with cornification process
ETS-1	Hs00428293	Transcription factor, regulated by p63
CHUK	Hs00989497	Serine kinase, regulated by ETS-1

For the purpose of these experiments, the same label (FAM) was attached to each of the probes and all experiments involved only one gene of interest at a time. Benefits occur when a different label is assigned to the housekeeping gene than the gene of interest and ran in the same experiment. This was not employed here due to the small scale at which TAp63 expression has been reported in comparison

to the housekeeping gene and other genes of interest (Candi et al., 2007). This may have skewed the data obtained from this work.

qRT-PCR is a technique which assesses relative gene expression as all quantification is relative to expression of a housekeeping gene; in this case *GAPDH* and *RPL13A* were used. *GAPDH* is a commonly used housekeeping gene used in cellular gene expression studies, as it is a ubiquitously found compound essential for step 6 of glycolysis to take place. Expression of *GAPDH* is thought to be relatively constant in cell types meaning normalization around *GAPDH* for other genes can give a relative expression reading (Barber et al., 2005). *RPL13A* is another housekeeping gene which is a ribosomal protein (Mane et al., 2008) and was used in this study as a second housekeeping to allow comparison between the relative expression data. Raw data from the qRT-PCR is manipulated to allow relative expression of each gene to be calculated.

The components of the TaqMan reaction were all supplied by Applied Biosystems and utilised in 10µl reactions: 0.5µl 20xTaqMan Assay, 5µl 2x Real Time Buffer, ~400ng cDNA Template, RNase Free water.

The raw data given out of a qRT-PCR experiment is the number of cycles of PCR needed in order to reach a threshold of fluorescence given off by the probes. These values are called Ct values and it is the raw amount of cycles needed. In each experiment, *GAPDH* and *RPL13A* were used as housekeeping genes for normalizing results. Each experimental run included triplicate technical repeats of each gene in each condition to allow for mean averages and standard deviations to be calculated. In addition, each experimental condition and gene was tested in triplicate in three biological replicates to increase reliability of the data. The mean Ct values from each of the experiments were then converted to Δ Ct values through normalization of the expression data to the housekeeping gene. The data was then assessed in comparison from basal and to the day of differentiation that the sample was taken. The values obtained from this assessment are termed $\Delta\Delta$ Ct values. This value was obtained by subtracting the Δ Ct value from the day of differentiation from the value for day 0. A transformation using the equation $2^{-\Delta\Delta Ct}$ allowed a fold increase of expression to be calculated. All data therefore was relative to housekeeping expression and a fold increase of expression based on basal cell expression. All standard deviations are calculated within each triplicate repeat and have been displayed as the error bars in the data.

2.6 siRNA Knockdowns

In this project, we wanted to explore how knockdown of the p63 isoforms would affect gene expression of the other isoforms as well as marker genes. To do this we needed to develop a knockdown system that targeted each isoform specifically. As there was a need for this high specificity, design of the system required serious considerations on which technique would be most appropriate. The method we settled on was the transient knockdown system utilizing siRNAs. Once a system type was determined, the specific type of siRNA knockdown had to be finalized. The final system was the Stealth RNAi knockdown designed and supplied by Thermo Fisher scientific.

The design of specific siRNAs which targeted a single isoform with no cross reactivity presented a number of problems due to the high sequence similarity between the isoforms. As the TA isoforms contained a transactivation region which the Δ Np63 isoforms do not possess, we chose to target this area for an assay for TAp63 isoforms. This transactivation domain is at the 5' end of the protein and is

encoded by the promoter at the most 5' end in the *TP63* gene. At the cDNA level, this region spans 214 nucleotides that is identical between α , β and γ forms of TAp63. Despite this region allowing for discrimination between TA and ΔN isoforms, it did not allow for specificity between α , β or γ isoforms.

This led to examining the full alignments of the transcripts to identify regions of specificity between the isoforms. Another issue that arose from examining the alignments was that sequence identity between $\Delta Np63\alpha$ and TAp63 α was very high outside of the transactivation domain. This was the same for the β and γ isoforms. This again presented problems for specific design of the siRNA. The solution that was decided on was to design a number of siRNAs that identified collections of isoforms and compare expression when these knockdowns were applied.

The first siRNA assay that was decided upon was a general p63 knockdown. This assay was a pre-designed Silencer Select assay from Ambion (Cat# 4392420, Assay ID s229400). This general knockdown for p63 has been described in literature as a successful assay for general p63 knockdown. The second assay that was decided on was a Silencer Stealth assay based around the transactivation domain found in the TAp63 isoforms. This assay was specific for all TAp63 isoforms and was a 21nt sequence which identified from nucleotide 49 of the target sequence. The final assay was designed to knockdown all β isoforms including TAp63 β and $\Delta Np63\beta$ isoforms. This assay was designed around a region specific to the β isoforms. This region spans 21nt from nucleotide position 1745 of the target sequence (**Table 9 and Figure 14**).

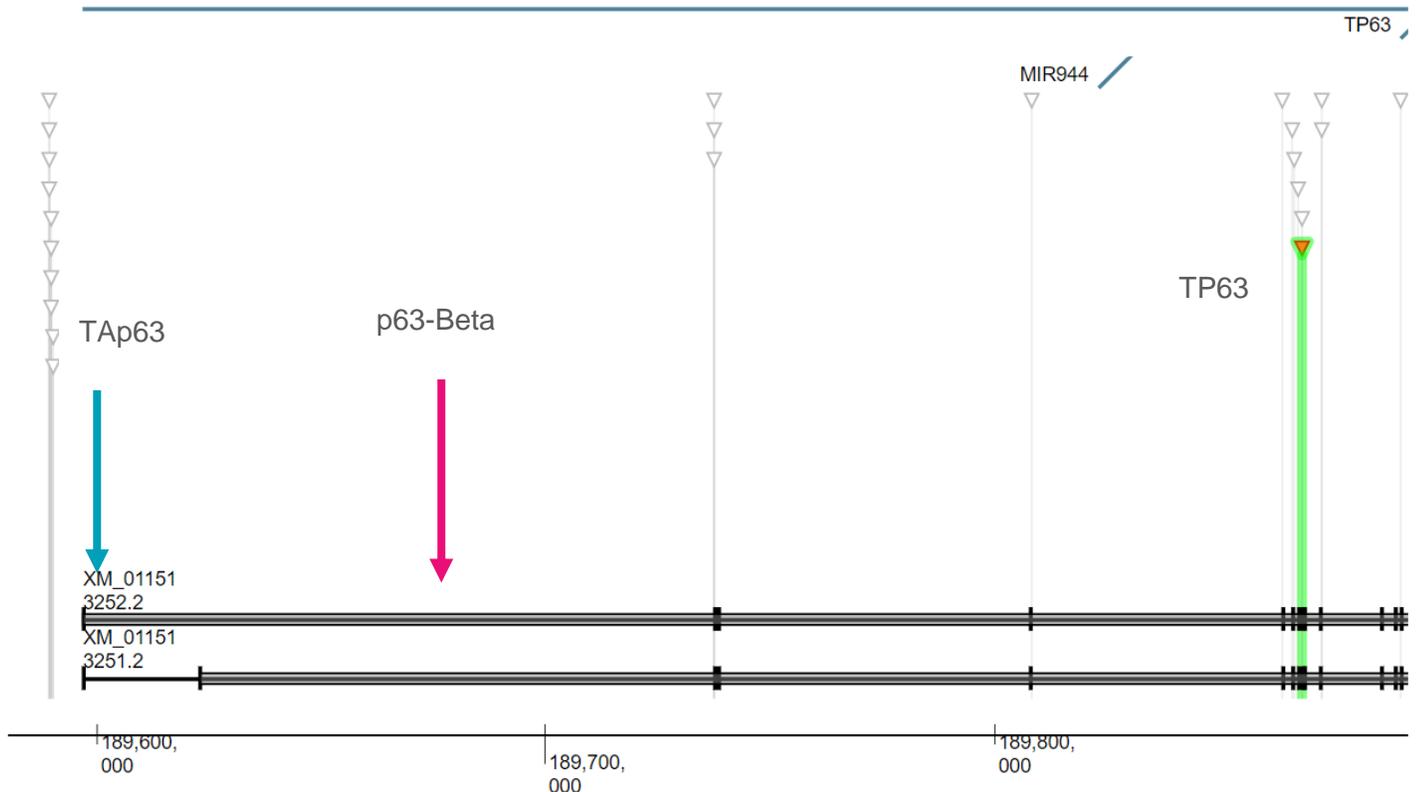


Figure 14: Approximate location of siRNA assay targets. Blue arrow denotes TAp63 specific, pink arrow denotes p63-Beta assay and green arrow denotes design to knockdown all six of the main p63 isoforms. Figure displays exon structure of TP63.

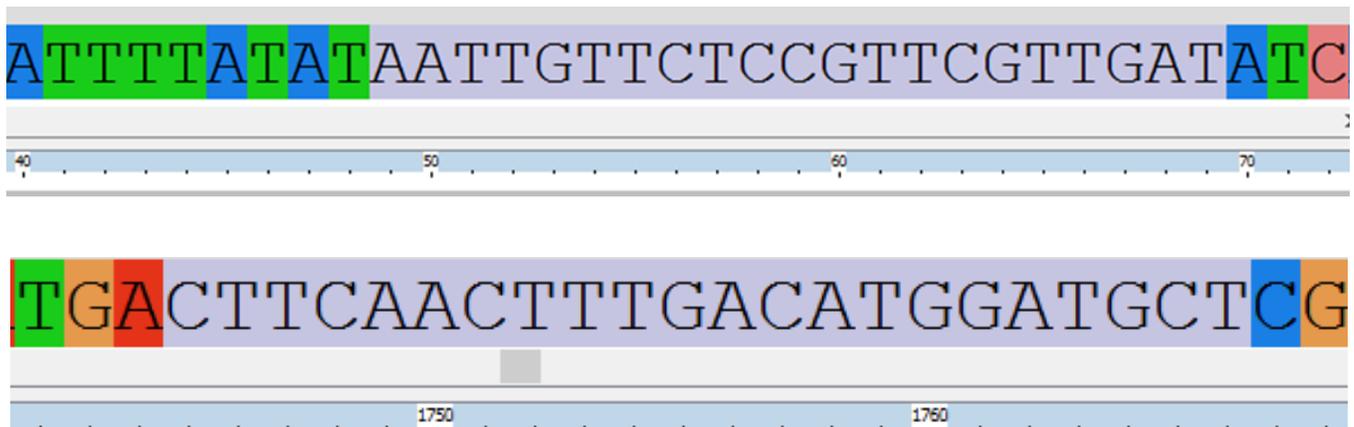


Figure 15: Target sequences for the siRNA assay, which were self-designed. Top panel is region targeted in the TAp63 assay. The bottom panel is the region targeted in the p63-Beta isoforms. Purple highlighting represents target region.

As the TAp63 and Beta assays were highly specific, manual design had to be carried out, observing design requirements for optimal knockdown. These points included a 21-nucleotide sequence that started at two A residues where possible. Another requirement for optimal design involves a 3' overhang that contains UU dinucleotide overhangs. It is recommended that G residues are avoided due to the likelihood of cleavage of the siRNA by RNase at single stranded G residues. Finally, an efficient siRNA knockdown system involves a 21-nucleotide sequence with 30-50% GC content. Avoiding stretches of more than 4Ts or As in the target sequence further increase specificity. All these requirements were first determined in 2001 and have been further verified and used throughout siRNA design from then on (Elbashir et al., 2001).

Once received, the powdered siRNAs were suspended in 1000µl RNase-free water and tested to optimize working concentrations to allow for optimal transfection.

2.6.1 siRNA Testing

To optimize the working concentrations of the three siRNA assays an experiment was designed to test three concentrations of each of the assays. The working concentrations for testing were 10pmol/µl, 20pmol/µl and 30pmol/µl siRNA. To test the siRNA assays, Human keratinocytes from Lonza at passage 3 were used. The cells were cultured in KGM Gold without Gentamicin (as standard siRNA media is antibiotic free). The protocol for transfection using Stealth Select siRNAs suggest Lipofectamine 2000 (ThermoFisher) as a transfection reagent. The protocol for transfecting these cells was followed as supplied by ThermoFisher with all volumes adjusted for culture of cells p60 cell culture plates.

Table 6: siRNA sequences used for knockdown of TP63, TAp63 and Beta isoforms

siRNA Name	Total p63	TAp63 isoforms	Beta isoforms
Sequence Format	siRNA sequence	siRNA sequence	siRNA sequence
Sense (5'-3')	GGAUGAAGAUAGCAUC AGAtt	UUGUUCUCCGUUCGUU GAUaU	GUCAGGAUCUGGCAAG UCUga
Antisense (5'-3')	UCUGAUGCUAUCUUCA UCCgc	AUCAACGAACGGAGAAC AAUU	AGACUUGCCAGAUCU GACaa
GC Content	38% Sense/ 48% Antisense	38% Sense/ 38% Antisense	52% Sense/ 48% Antisense
Modification	Silencer Select	Silencer Select	Silencer Select
Pre-designed / Custom	Pre-designed	Custom	Custom

Cells were cultured one day before transfection cells were plated in growth media without antibiotics such that the cells would be 30-50% confluent by the time of transfection. This confluency is suggested to avoid cell overgrowth. For transfection, an oligomer-Lipofectamine 2000 complex was made by diluting the siRNA in Opti-MEM Reduced Serum media (Applied Biosystems) and Lipofectamine 2000 in Opti-MEM media. These two suspensions were combined to create the oligomer-Lipofectamine 2000 complex. This complex was added to the cells and were incubated for 24 hours in a CO₂ incubator at 37°C (**Appendix 7.9**). After 24 hours the cells were washed with 1x PBS and incubated with 5x trypsin EDTA for 5 minutes to detach the cells from the plates. The cells were pelleted and stored at -20°C. RNA was extracted from these cells and cDNA was made as described previously. Gene expression analysis was carried out using qRT-PCR using procedure as described above. This analysis involved looking at expression of both p63 isoforms and ETS-1 as another marker to assess the percentage efficiency of each concentration. In addition, a Stealth RNAi negative control of scrambled siRNA sequences with medium GC content was used (Thermo Scientific, Catalog. No 12935300).

2.6.2 Isoform Knockdowns in Comparative Cell Lines

Once working concentrations for each of the siRNA assays were determined, an experiment to test how the knockdowns would affect differentiation were devised using normal human keratinocytes. Two cell lines were used; the Lonza keratinocytes at passage 4 and the HKc17 cells at passage 2. Four conditions were set up to test each of the assays along with negative control cells, using the medium GC content control. The cells were plated at 0.1 million cells per p60 plate and cultured for 24 hours until transfection day. These cells were cultured in KGM Gold without antibiotic. The period of differentiation for this test was carried out over 12 days, with cells harvested on days 0, 4, 8 and 12. On transfection day, cells were transfected as previously described in 2.2.1 and incubated with the transfection complexes for 24 hours. After 24 hours, according to the protocol, the transfection media can be changed. At this point, high calcium differentiation media, as described in 2.1.1, was added to the cells to begin differentiation. One p60 plate of each cell type and each assay were harvested using standard protocol. This process was repeated on days 4, 8 and 12 with all pellets stored at -20°C until they were used for RNA extraction.

3.0 Results

3.1 RNA Purification

Before cDNA was made, the quality of RNA extracted from the cell pellets were assessed using the NanoDrop 2000. This assessed RNA quality along with the concentrations extracted. Any impure or low quality RNA was not included in experiments downstream. **Table 7** demonstrates an example of the data obtained from the Nanodrop 2000 for HaCaT cells.

Table 7: Example Nanodrop 2000 reading for RNA quality extracted from HaCaT cells

Day of Differentiation	RNA concentration (ng/μl)	260/280	260/230
0	564.4	1.99	2.13
1	659.0	2.05	2.04
2	903.3	2.04	2.04
3	843.0	2.02	1.97
4	998.4	2.03	2.20
5	710.8	2.02	2.07
6	1081.6	2.01	2.12
7	760.5	2.01	2.14
9	872.8	2.03	2.06
11	730.9	1.99	2.19
13	516.5	1.96	1.97

3.2 HaCaT differentiation

The first way in which cell differentiation was tracked was with photographs of the cells taken when cells were harvested for RNA extraction. These photographs allowed assessment of the cell state, with morphological changes suggesting differentiation had occurred. All photographs were taken using an EVOS XL Core Imaging System (ThermoFisher) at 20x magnification. At day 0, before differentiation, the cells appear in a classical basal keratinocyte state, they are cobblestone like with bright, defined edges. The cells appear rounded and full. As differentiation progresses, the cells begin to lose the bright edges and start to flatten. At terminal differentiation, the cells become completely flattened with no clear, defined edges. The cells also seem to encroach into each other with large pockets of highly differentiated cells.

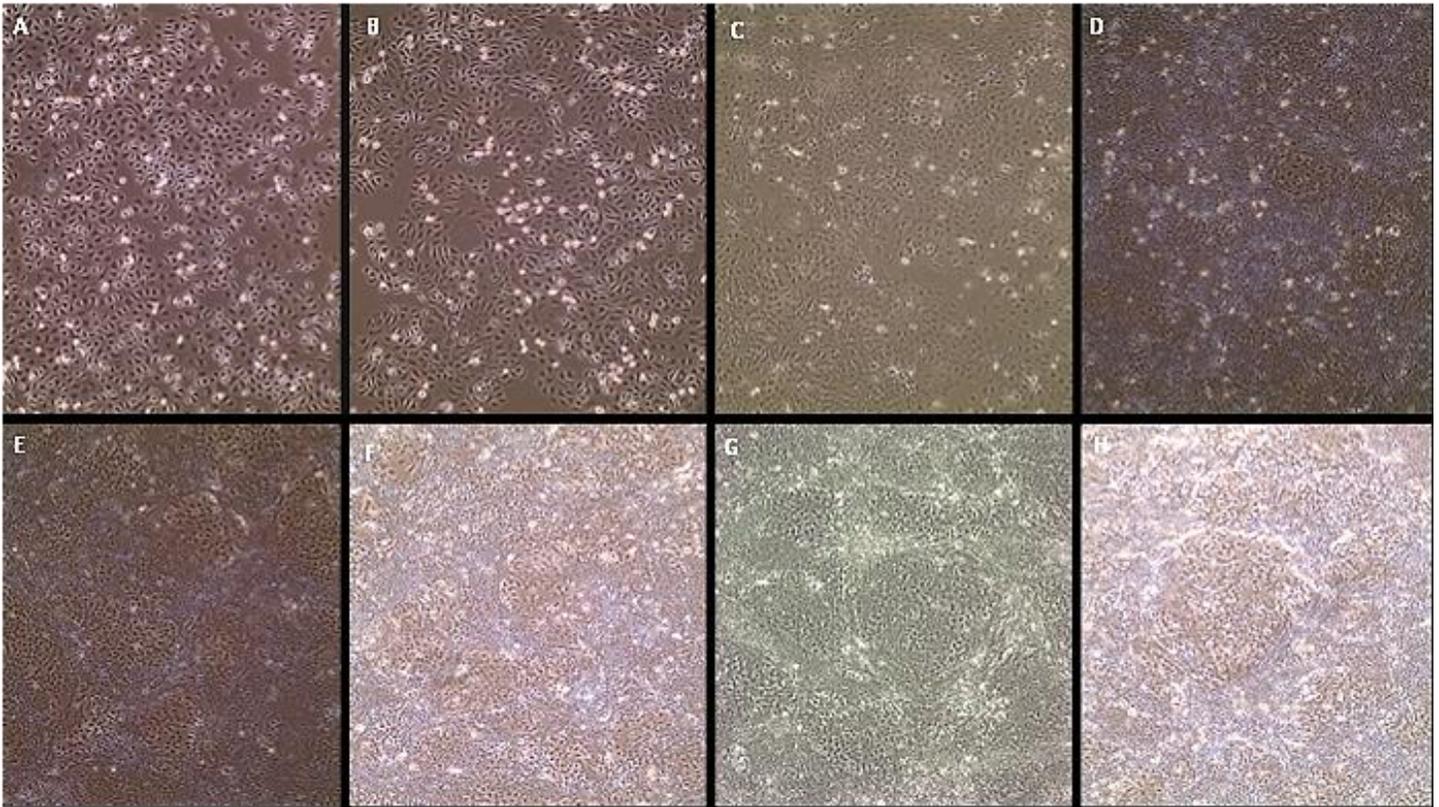


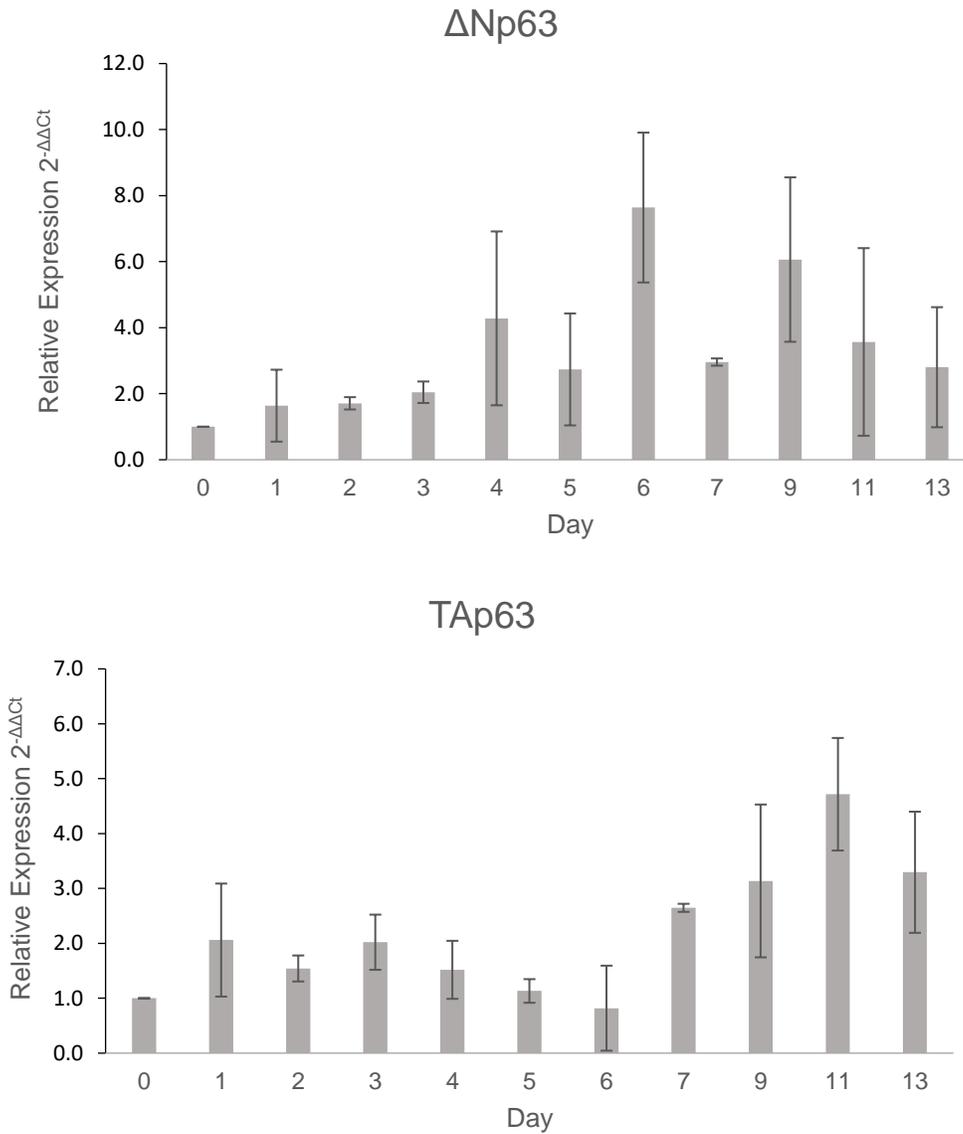
Figure 16: Photographs taken of HaCaT cells taken at time points during calcium-induce differentiation. Top Left: Day 0, Top Centre Left: Day 1, Top Centre Right: Day 2, Top Right: Day 4, Bottom Left: Day 7, Bottom Left Centre: Day 9, Bottom Right Centre: Day 11 and Bottom Right: Day 13.

The differentiation process for these HaCaT cells was carried out over two weeks with cells collected on days 0,1,2,3,4,5,6,7,9,11 and 13. The genes that were assessed were both p63 isoforms, KLF4, FLG and ETS-1. The housekeeping genes used were both GAPDH and RPL13A. All experiments used TaqMan qRT-PCR and were completed in triplicate on three separate occasions.

The relative expression data obtained from the real time PCR experiments give some indication as to the mirrored nature of the expression of each isoform in differentiation. Looking at the expression

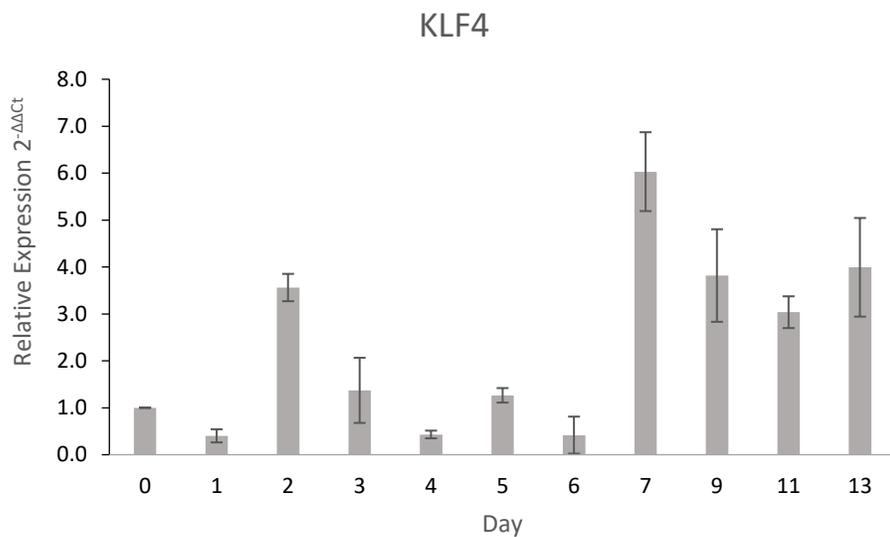
of Δ Np63 isoforms (**Figure 17, Panel A**), it can be seen that this isoform is not particularly over expressed in the early days of differentiation. However, as differentiation reaches around Day 4 expression starts to increase.

This increasing trend continues through days five and six whereupon expression peaks about half way through the 2-week experiment. Beyond Day 6, expression of Δ Np63 isoforms decreases steadily, continually reducing all the way through to Day 13 when cells were no longer harvested.



In contrast, the TAp63 expression (**Figure 17, Panel B**) pattern looks different to that of the Δ Np63 expression pattern. Like the Δ Np63 expression in the early days of differentiation, TAp63 expression is relatively unchanged through to Day 6 of differentiation. At Day 7 of differentiation, expression of the TAp63 isoforms begins to increase, and keeps increasing through to Day 11 of differentiation. This

Figure 17: HaCaT differentiation results. A) Δ Np63 expression shows a steady increase in relative expression up until Day 6. Beyond Day 6 expression seems to decrease towards terminal differentiation at Day 13. B) TAp63 expression appears to be low in the early days of differentiation up until around Day 7 when expression increases and continues to increase towards the final days of differentiation. GAPDH used as housekeeping in this case. Error bars represent Standard deviation. This data was published in Barragán Vázquez I, Lima Cunha D, Hockney S, Tang KY, Thomforde Kitson S, Eckl KM, Hennies HC, *The role of p63 isoforms in the epidermal development as replicated in cellular models for normal human skin and genetic skin diseases*. Presented at the 67th Annual Meeting of the American Society of Human Genetics, Orlando, Florida, USA, 2017 and presented at the BSID Annual Conference 2018, London, United Kingdom

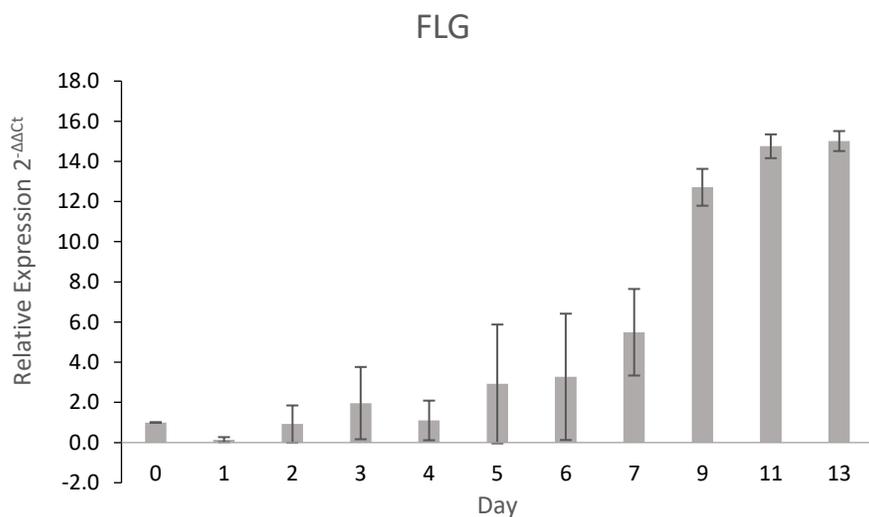
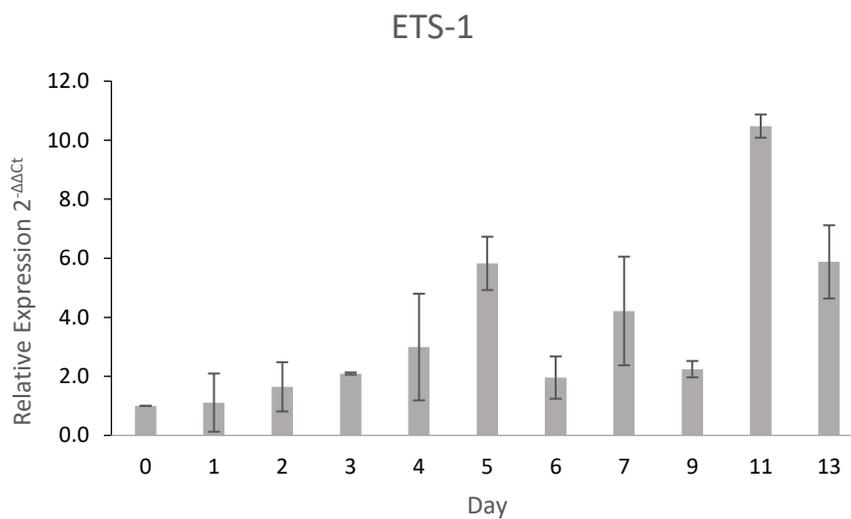


peak towards the latter stages of keratinocyte differentiation suggests a role for TAp63 isoforms in regulating the final steps of differentiation. These results come with restraints when assessing them, as again these data are relative to GAPDH and are from an immortalized cell line in HaCaTs. Comparison between the expression patterns of the two p63 isoforms is seen in **Figure 19**. Around Day 5 or 6 in each of the expression profiles there appears to be a switch in expression whereby TAp63 expression levels begin to increase and $\Delta Np63$ expression decrease.

As for the other markers assessed in the HaCaT cell line differentiation, they followed other expression patterns.

The transcription factor KLF4 has previously been implicated in regulating terminal differentiation (Segre et al., 1999). In our experiments in HaCaTs, expression of KLF4 did not show a particularly clear expression pattern with fluctuating expression in the

Figure 18: Real time PCR results from the marker genes assessed during HaCaT differentiation. A) KLF4 expression has less of a clearly defined pattern of expression than the other markers however, expression peaks around days 7-9. B) Expression of ETS-1 shows a gradual increase in expression throughout the differentiation process with peak expression being towards terminal differentiation. C) Filaggrin is a terminal differentiation marker and this expression profile matches that of a terminal differentiation marker with peak expression being in the later days. Error bars represent standard deviations.



first week of differentiation. However, beyond Day 6, a large peak of expression is seen and a relatively high expression level is maintained from then to the final days tested, with a small decrease after Day 7. This supports the idea of KLF4 being involved in terminal differentiation.

Another transcription factor ETS-1 showed a steady increase as differentiation progressed with a small decrease between days 6-9 but the general increasing trend continues right up until Day11. This transcription factor has been identified as p63-regulated and has been implicated alongside CHUK as being involved of progressing differentiation in keratinocytes (Candi et al., 2007).

Finally, the terminal differentiation marker FLG expression was as expected (Zeitvogel et al., 2017), increasing steadily throughout differentiation with peaks occurring at days 11 and 13. At the early stages, the expression levels are low, around the baseline relative expression. It is only around Days 5, 6 and 7 where an increase in expression can be observed, peaking at approximately 15 times higher expression than Day 0.

These expression profiles matched what was hypothesized given what was previously reported into the function of each of these markers. To reinforce this data, we differentiated normal keratinocytes in order to have a comparative line.

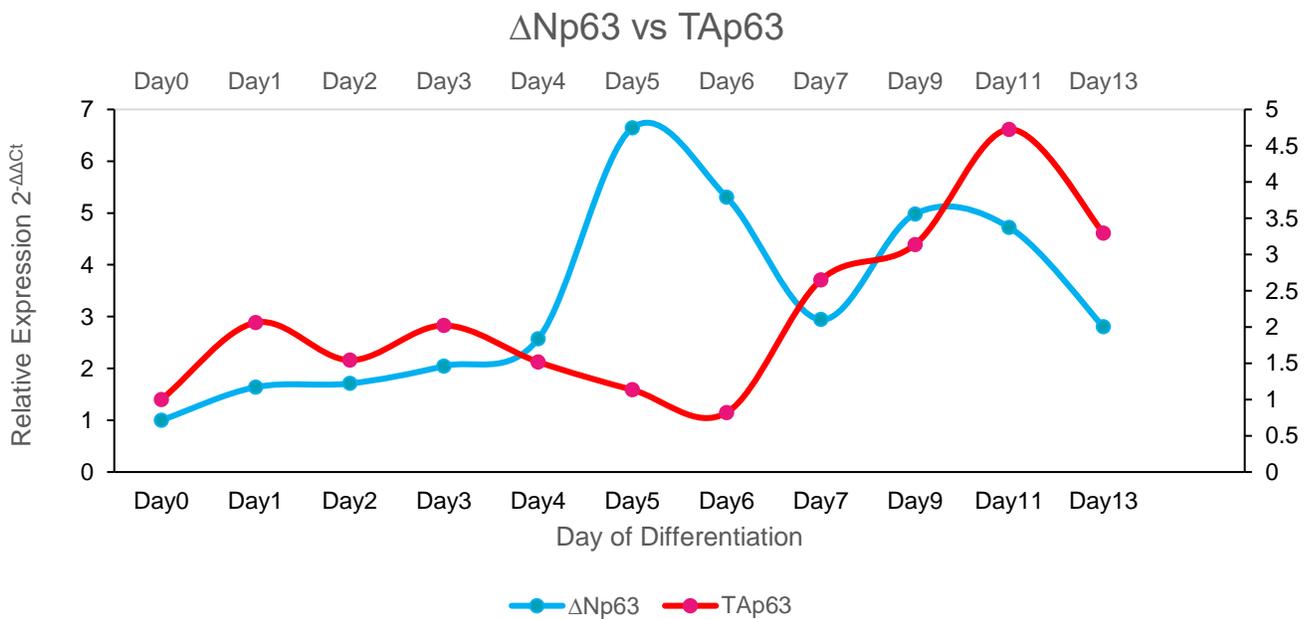
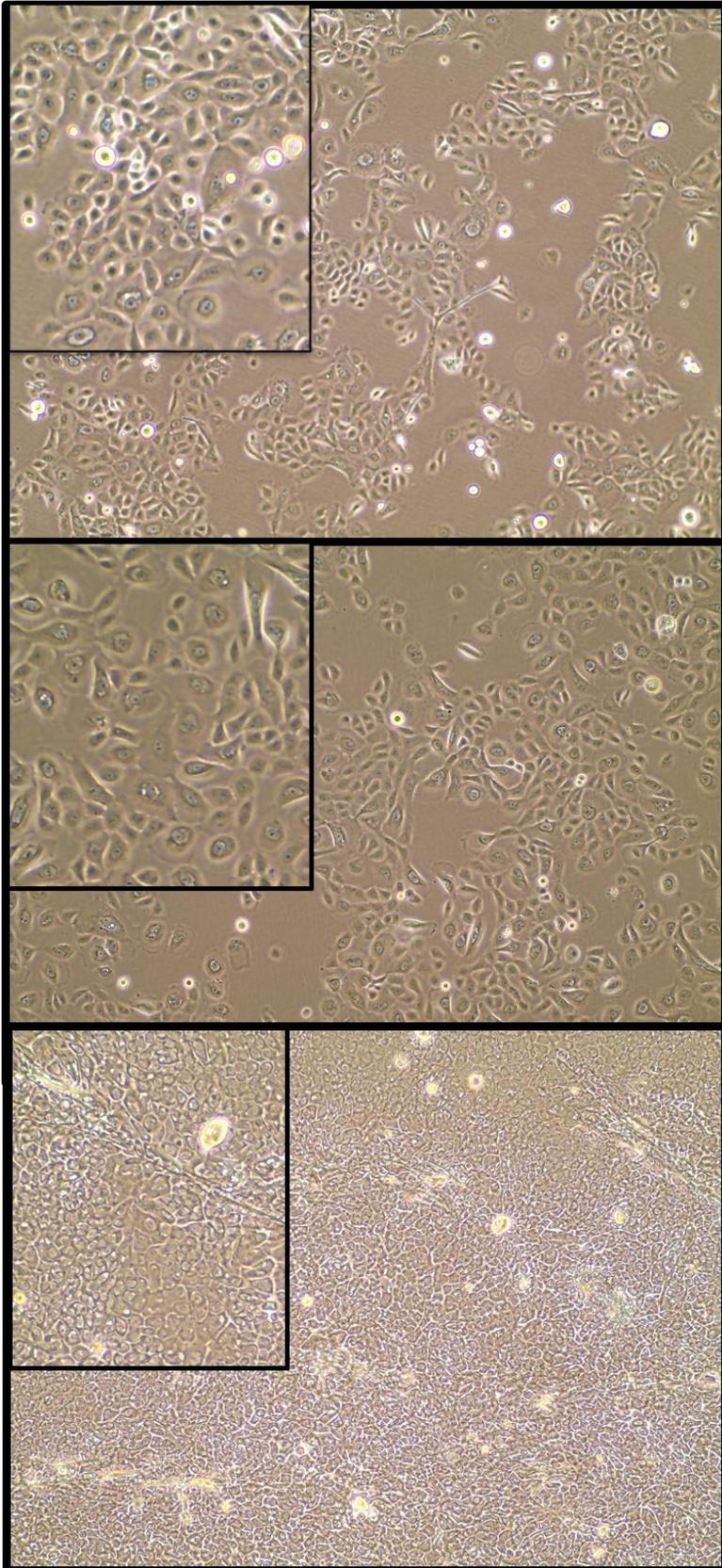


Figure 19: Expression for both p63 isoforms during calcium-induced differentiation in HaCaT cells. Expression of both isoforms is low at the early stages of differentiation with ΔNp63 expression peaking early at Days 5-6. TAp63 expression has a converse pattern peaking later at Day 11 after ΔNp63 expression begins to decrease.

3.3 HK Differentiation



Experimentally the differentiation of the normal human keratinocytes was similar to that of the HaCaTs with only the culture media being different. The cells used in this work were low passage normal human keratinocytes denoted as HKc13 and HKc15. The cells grew well with basal cells exhibiting the typical morphological features such as bright, defined edges and full appearance. The morphology changed through differentiation, similar to how the HaCaTs changed with cells flattening out with less defined edges. For the purpose of this analysis, we looked at expression of the p63 isoforms, FLG, KLF4 and TGM1. The results shown are from experiments where GAPDH was used as the housekeeping.

Figure 20: Photographs of Lonza keratinocytes throughout differentiation. Top Panel- basal cells, before addition of calcium to media. Middle Panel- cells after 4 days of differentiation. Bottom Panel- cells after 10 days of differentiation.

When the p63 isoforms were examined through qRT-PCR, we observed a similar pattern of expression for Δ Np63 isoforms in each cell line. The amount of increase in expression relative to Day 0 cells was small compared to TAp63. This was consistently seen in both HKc13 (**Figure 21, Panel A**) and HKc15 cells (**Figure 21, Panel B**). In both cell lines, peak expression for Δ Np63 was observed between

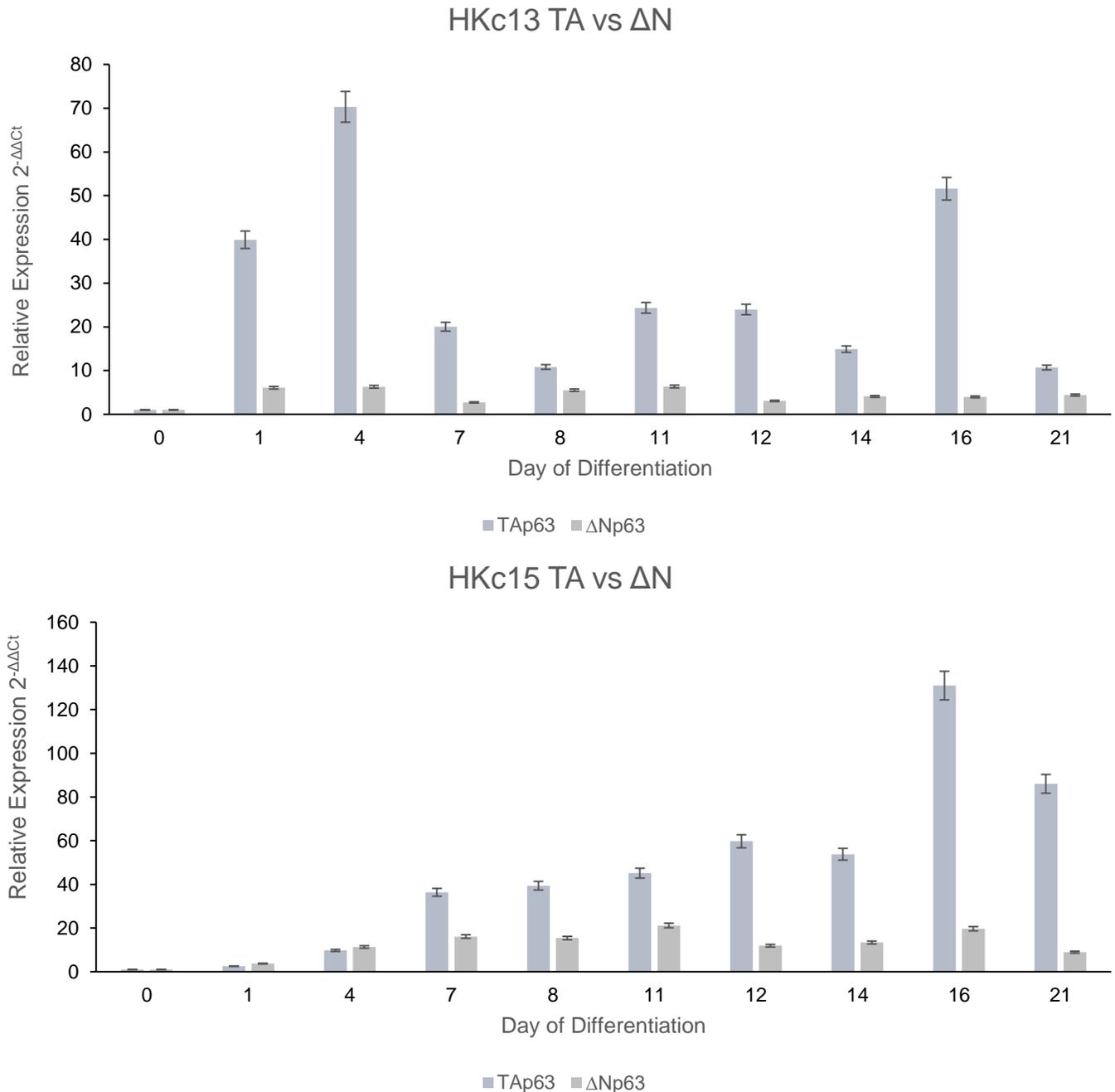
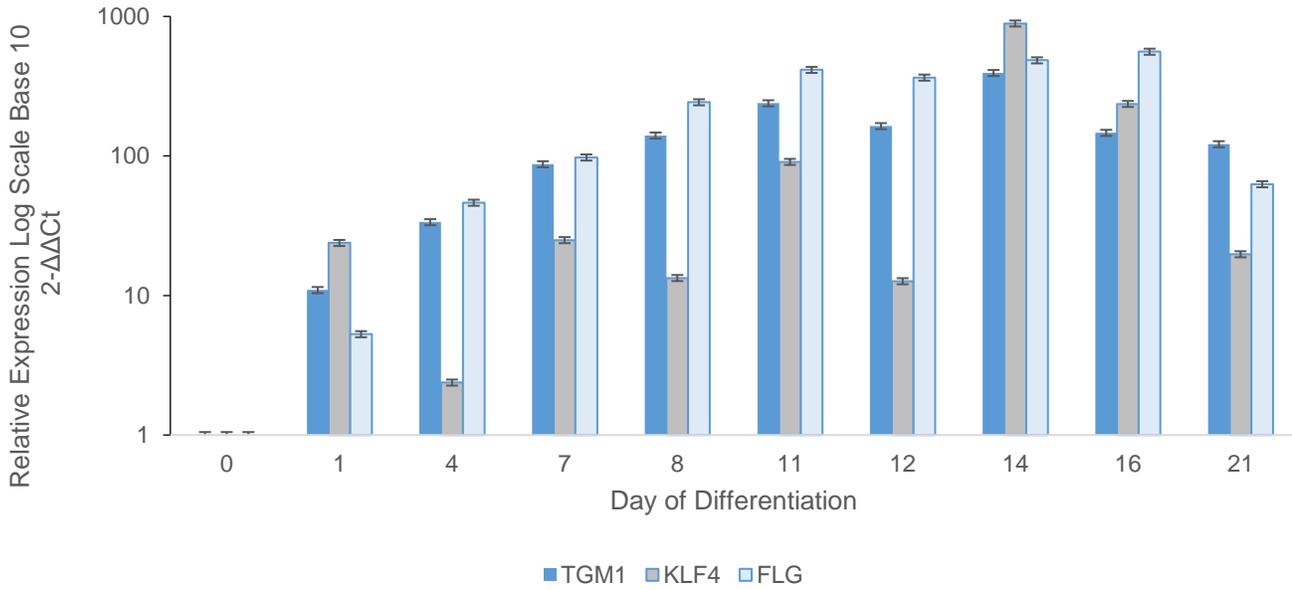


Figure 21: Real time PCR data from normal human keratinocytes HKc13 and HKc15. Differences in pattern of expression for TAp63 was observed between the two cell lines with Δ Np63 exhibiting small but consistent changes in expression. Error bars represent standard error.

Days 8 and 11. With this being said, the relative fold change in expression was low in comparison to the TAp63 isoforms, with expression change remaining below 20-fold. As for TAp63, there was some conflict in the results obtained with inconsistencies between the expression profiles between cell lines. HKc13 cells exhibited a strong peak of expression in the early stages of differentiation between Days 1 and 4. Beyond these days, there was a lower fold increase on Day 0 expression before a peak was seen at Day 16. In contrast, the HKc15 cells showed a steady increase in expression of TAp63 throughout differentiation, peaking around Day 16. This peak is consistent with the peak seen in the HKc13 cells.

TGM1 as a terminal differentiation marker would be expected to have peak expression towards the later days of differentiation. This pattern was seen in the HKc13 cells (**Figure 22, Panel A**) and to some extent, the HKc15 cells (**Figure 22, Panel B**). The clear curve of increasing magnitude of expression in HKc13 cells fits with TGM1's role in terminal differentiation. The pattern seen in HKc15 cells is more flattened with expression staying reasonably consistent throughout differentiation. Like TGM1, we expected FLG expression to peak late also. Again, in HKc13 cells we saw this curve of steadily increasing expression, with peaks beyond Day 11 through to Day 16. Similarly, to the TGM1 expression, FLG expression in the HKc15 cells was flattened however, a significant peak around Day 11 was observed. As for KLF4, the expression profiles are not as smooth with peaks and troughs of expression throughout each cell line. However, in both lines the highest fold increase was observed towards terminal differentiation (**Figure 22**).

HKc13 TGM1, KLF4 and FLG



HKc15 TGM1, KLF4 and FLG

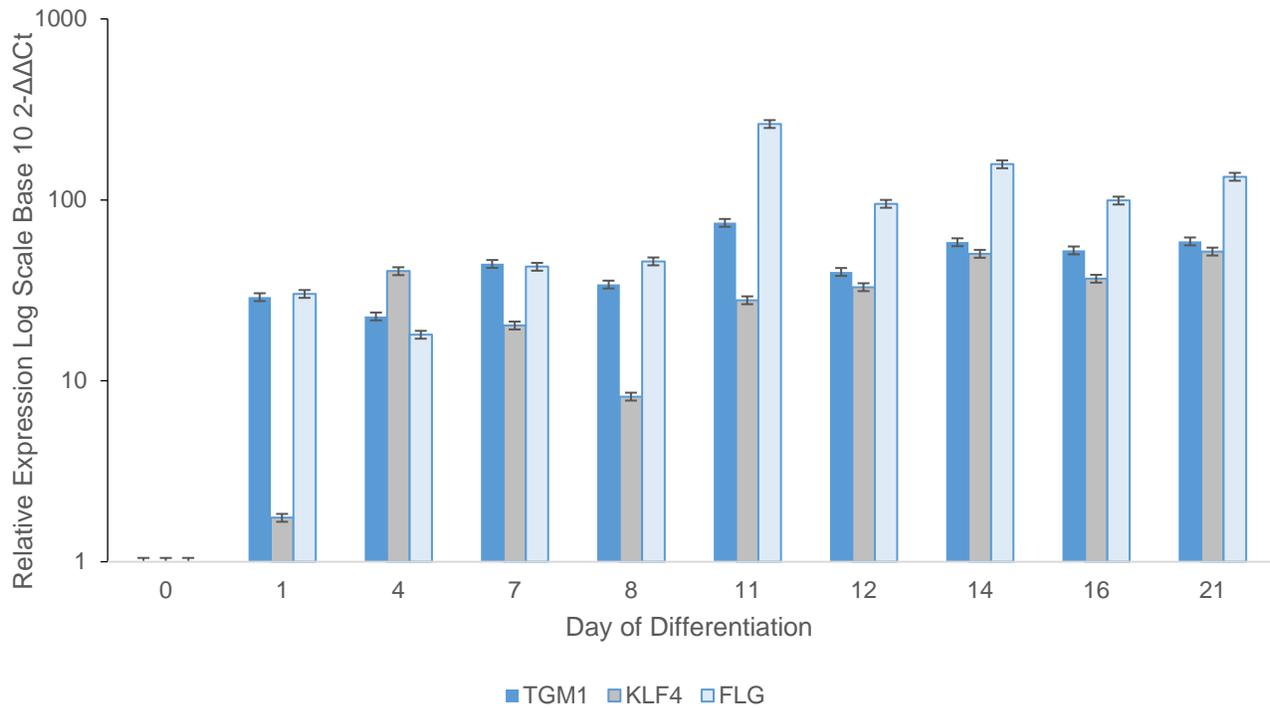


Figure 22: Relative expression of TGM1, KLF4 and FLG- three marker genes used for study of the differentiation. TGM1 and FLG are terminal differentiation markers while KLF4 is a transcription factor involved with regulating steps leading to terminal differentiation. Error bars represent standard error.

3.4 Knockdown of p63 isoforms test results

Given the data obtained from the experiments with HaCaTs and human keratinocytes, we wanted to induce p63 knockdowns using the systems described previously on basal cells. The siRNA assays needed to be tested to calculate optimal working concentrations. To evaluate each assay, three concentrations of siRNA were tested- 10pmol/ μ l, 20pmol/ μ l and 30pmol/ μ l. For each of these concentrations, both the p63 isoforms' expression was tested, using GAPDH as the housekeeping. These experiments were used to calculate percentage knockdown for each of the siRNA assays. The negative control in this case appeared to be approximately 2.5 times more expressed than the normal HKs. These cells were included as it is a representation of the experimental process without the siRNA assays. This point was used as a comparison for expression as it takes into account the experimental process' impact on the cells and their respective expression as a result of this treatment. Normal cells, which have not been exposed to Lipofectamine- driven transfection methods, may express differently to those that have.

As seen in **Figure 23**, the varying amounts of each assay had different effects on isoform expression in basal keratinocytes. When cells were exposed to the total p63 knockdown assay, small decreases in isoform expression occurred. At 10pmol/ μ l siRNA, expression of Δ Np63 reduced a small amount compared to the normal Human keratinocytes, but a large amount in comparison to the negative

siRNA Assay Assessment

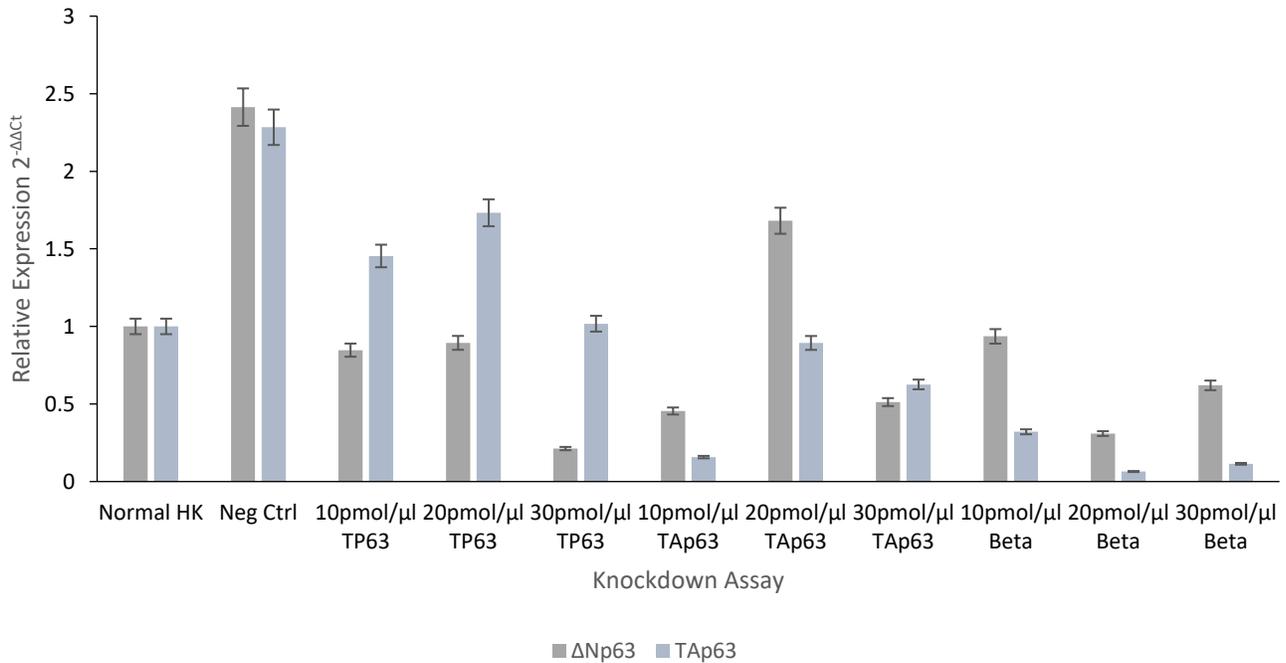


Figure 23: Relative expression of p63 isoforms under the respective knockdown assays. When total p63 was knocked down, expression of Δ Np63 was decreased when 30pmol/ μ l siRNA was added, TAp63 expression increased with a small margin at lower concentrations. When TAp63 was knocked down, levels of TAp63 expression did decrease at 10pmol/ μ l RNA and 30pmol/ μ l siRNA. Δ Np63 expression increased when siRNA concentration was at 20pmol/ μ l. When p63-Beta was knocked down, the expression of both isoform, types decreased at all siRNA concentrations. Medium GC content negative siRNA control used as negative control. Error bars represent standard error.

control cells. At 20pmol/μl siRNA, the change in ΔNp63 expression is very similar, however at the higher concentration of 30pmol/μl siRNA, a very large decrease in expression is observed from the negative control cells. This decrease of approximately two fold suggests this concentration was the most effective at knocking down total p63. This is supported by a large decrease in expression of TAp63 occurring when 30pmol/μl siRNA was used. Small decreases on the negative control cells was observed when 10pmol/μl and 20pmol/μl siRNA was used in this context.

When cells were exposed to the TAp63 knockdown, expression patterns of each isoform was different. At 10pmol/μl siRNA, there were decreases in both the expression of ΔNp63 and TAp63. At 20pmol/μl siRNA, a different pattern is exhibited with very limited decreases in the expression of ΔNp63 and a lesser decrease in the TAp63 compared to 10pmol/μl siRNA. At 30pmol/μl siRNA, again a decrease in both the isoforms' expression is seen. The largest decrease was when only 10pmol/μl siRNA was used.

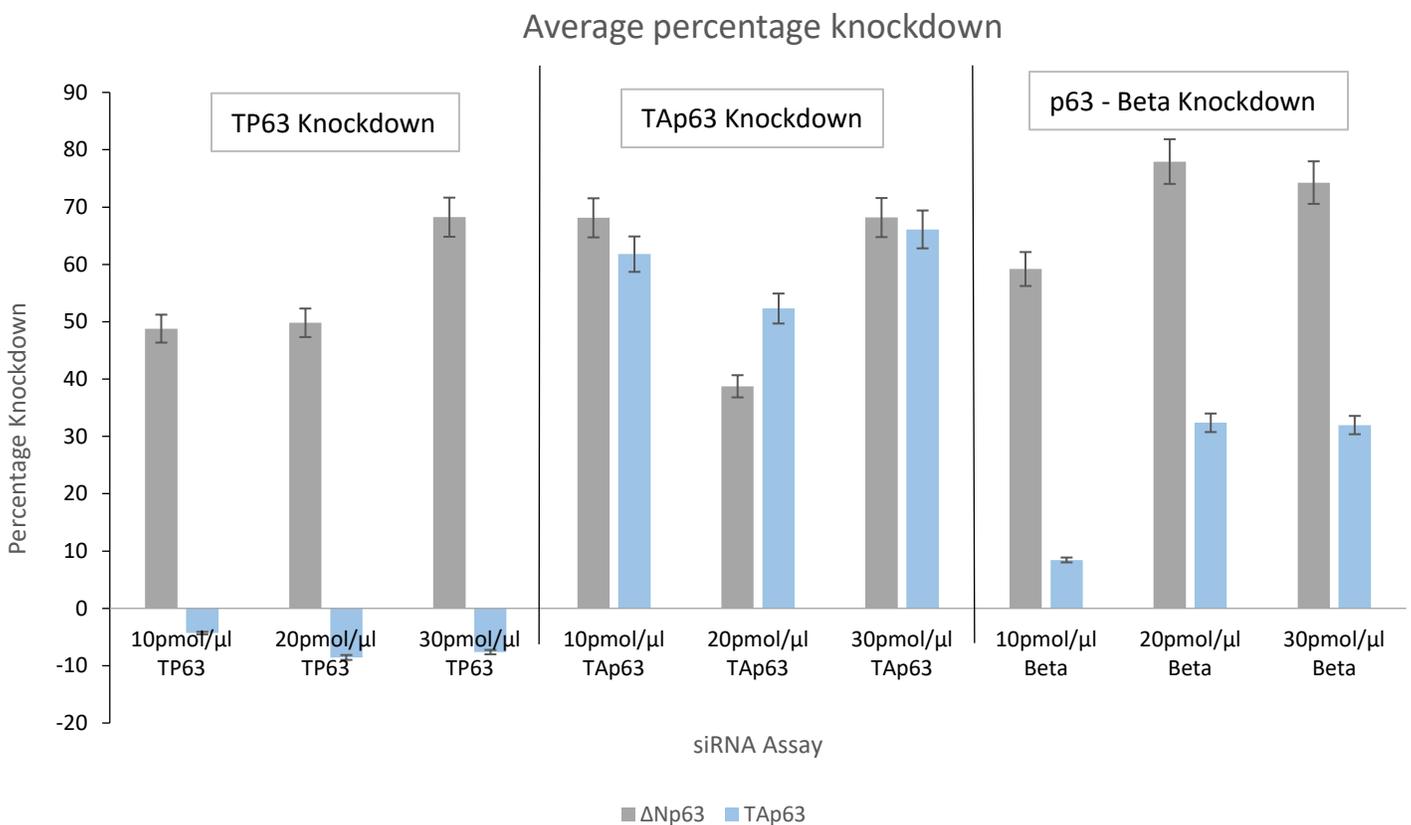


Figure 24: Percentage knockdown for each of the three assays at three concentrations. TP63 assay- knocked down ΔNp63 isoforms at an efficiency between 50-60% whereas TAp63 isoforms appeared to have a small increase in expression. TAp63 assay- 60% knockdown for ΔNp63 when 10 and 30pmol/μl siRNA was used. TAp63 expression had approximately 60% knockdown. Beta knockdown- percentage knockdown of ΔNp63 isoforms was between 55% and 75% dependent on concentration of assay used. TAp63 knockdown was less efficient achieving a maximum of 30% knockdown. Error bars represent standard error.

Finally, when the beta isoforms were targeted for knockdown, there was a large decrease in isoform expression when all concentrations of siRNA were used. The optimal concentration for

knockdown when analyzing the different concentrations appeared to be when 20pmol/ μ l siRNA was used. As the Beta isoforms appear in both a Δ Np63 form and the TAp63 form, as expected the expression of both these isoforms were affected.

As all of these tests used relative expression compared to GAPDH and negative control cells, another level of assessment was employed to determine the most affective concentration of assays. This was achieved by calculating the percentage knockdown in each situation (**Figure 24**) relative to the negative control line expression.

The efficiency of each of the assays at each of the different concentrations varied considerably. When cells were exposed to the TP63 assay and analysis focused on Δ Np63, 50 and 20pmol/ μ l siRNA gave percentage knockdown of just under 50%. When the concentration of siRNA was increased to 30pmol/ μ l, the efficiency of the knockdown increased to 70% knockdown. This is the most efficient of the three concentrations in this case. As for analysis of the TAp63 isoforms, the assay appears to cause a small increase in expression. This increase is less than 10% in all concentrations. This may suggest a problem with the specificity of the assay in that it only targets Δ Np63 isoforms or that there was a methodical problem with the experimental process.

When examining the efficiency of the TAp63 knockdown, there is variation between the different concentrations of the assays. At 10pmol/ μ l, the efficiency of the knockdown for Δ Np63 was approximately 65%. There was a similar efficiency when 30pmol/ μ l siRNA was used with 35-40% efficiency when 20pmol/ μ l was used. This presents an issue over specificity as the TAp63 assay should have no effect on the expression of Δ Np63. As for TAp63 knockdown efficiency, at 10pmol/ μ l there is 60% knockdown, 20pmol/ μ l there is 50% knockdown and at 110pmol/ μ l, there is 65% knockdown. Much like the TP63 assay, the highest concentration of siRNA assay was the most efficient in regards the TAp63 isoforms.

The efficiency of the p63-Beta assay had a large contrast between the two p63 isoforms. The efficiency of the Δ Np63 knockdown ranged from 55% (10pmol/ μ l) to 75% (20pmol/ μ l). These efficiencies are high in regards to Δ Np63 suggesting that Beta isoforms account for a large proportion of the population of Δ Np63 in basal cells. In contrast, the efficiencies for TAp63 with this assay was much lower. At 10pmol/ μ l siRNA, the efficiency of the knockdown was below 10%. When 100 and 30pmol/ μ l siRNA was used the efficiency increased but still was only at 30%. This may suggest that TAp63- β isoforms account for a much smaller proportion of TAp63 isoforms in basal cells. Alternatively, this may suggest that the assay is not specific enough to target TAp63- β .

This test raised some questions about the effectiveness of each of the assays on each of the isoforms. In the majority of cases there was a percentage knockdown, however these percentages were low compared to the manufacturers' claim of between 60-80% knockdown efficiency. As two of the assays (TAp63 and p63-Beta) were self-designed, it may suggest sequences were not as efficient as they could be. To validate if these percentage efficiencies were correct, another test was designed to use all three assays at the most efficient concentrations. This test would include analysis of basal cells but also differentiated cells to assess if differentiated cells allow for knockdowns that are more efficient.

3.5 HK Lonza vs HKc17

To carry out this work, two normal keratinocyte cell lines were chosen to allow for comparative analysis. In addition, as the Lonza cells were commercially sourced and produced, use of a cell line

extracted from a skin sample carried out in the lab was used to see if there was variation in expression. The morphology of the cells during this experiment appeared to be not as expected, with the cell shape being flat and looking dead. With the RNA extraction, this was confirmed as the cells not being viable for use in qRT-PCR due to poor RNA quality. With that being said, the cells obtained on Day 0 had high quality RNA from the extraction so these were used for qRT-PCR analysis.

Table 8: Example of RNA extraction data from HKLonza and HKc17 after knock down and differentiation

Cells	RNA Concentration (ng/ μ)	A260/280	A260/230
HKLonza Day 4 TAp63 KD	1.8	2.52	0.24
HKLonza Day4 Neg Ctl	5.1	1.70	0.30
HKLonza Day 4 TP63 KD	8.9	1.74	0.16
HKLonza Day 4 Beta KD	1.2	2.34	0.33
HKc17 Day 4 TAp63 KD	15.2	2.08	2.27
HKc17 Day 4 Neg Ctl	5.4	1.79	0.16
HKc17 Day 4 TP63 KD	4.6	1.58	0.37
HKc17 Day 4 Beta KD	9.9	2.64	0.30

With Day 0 cells from both cell lines and with each knockdown assay, basal (pre-differentiated) cell analysis could be carried out. This gave a comparison between two keratinocyte lines and between each assay. The fact that the non-differentiated cells had high quality RNA product, it suggests that differentiation may have disrupted the cell growth once p63 knockdowns had been introduced. Time restraints prevented repeating this process but this is the next step to optimize and investigate in order to allow for a full differentiation timespan with each of the knockdowns incorporated. The RNA extraction from these cell pellets produced limited to no RNA, and any which was obtained was low quality. This meant that the only viable RNA from this experiment was that obtained from the basal cells. These cells included the knockdowns but were not differentiated.

For the purpose of analyzing expression in basal cells when the knockdowns were in place, qRT-PCR was carried out using assays designed for each isoform and ETS-1 as an independent marker. Like other knockdown experiments, a medium GC negative control was used but in this case was used to normalize the relative expression (**Figure 25**).

When Δ Np63 was examined in HKc17 (**Figure 25, Panel A**) it appeared that its expression was very little when the total siRNA assay was added with a slight increase in expression when Beta isoforms were knocked down with no significant change when TAp63 knockdown was completed. In The HK Lonza cells (**Figure 25, Panel D**) a small decrease was seen when either total p63 or Beta isoforms were knockdown however, there was an overexpression of Δ Np63 when TAp63 was knocked down. When TAp63 expression was examined in HKc17 cells (**Figure 25, Panel B**) there was no expression when the total p63 siRNA was added however there were increases in expression when the assay designed to knock down TAp63 was involved. A large increase in expression of TAp63 was observed when Beta isoforms were knocked down. In Lonza keratinocytes (**Figure 25, Panel E**) TAp63 expression increased when total p63 and Beta isoforms were knocked down with little-to-no change when the TAp63 knockdown was introduced. ETS-1 expression in HKc17 (**Figure 25, Panel C**) and HK Lonza (**Figure 25, Panel F**) followed a similar pattern with small increases in expression when any of the knockdowns were introduced with Beta isoform knockdown resulting in the highest level of ETS-1 expression.

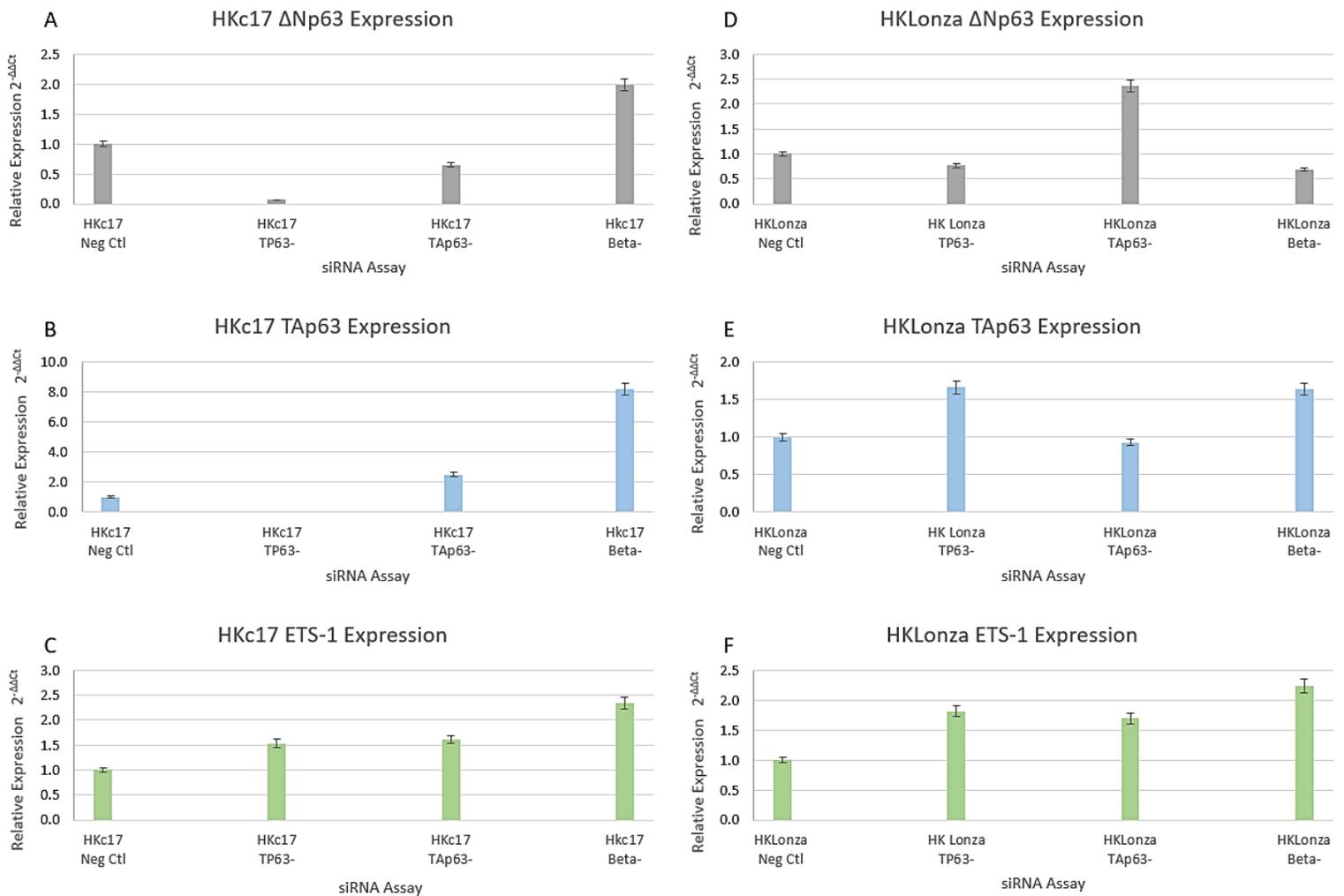


Figure 25: qRT-PCR results from basal (non-differentiated) cells with knockdowns present in two cell lines- HKc17 and HK Lonza, examining expression of the two p63 isoforms and ETS-1. Error bars represent standard error.

Comparison between the two cell lines presented some interesting findings with different levels of expression of each of the p63 isoforms depending on which cell line the knockdowns were carried out. When Δ Np63 isoforms were examined using qRT-PCR in HKc17 cells, there were significantly lower levels of Δ Np63 when total p63 was targeted with the siRNA. When the assay designed to target TAp63 isoforms was used in these cells a small decrease in Δ Np63 was observed. Finally, when p63- Beta isoforms were targeted for knockdown, an increase in Δ Np63 expression was seen. In comparison, when Δ Np63 was examined using HK Lonza cells as the model system, a different profile of expression was seen. When these cells were exposed to the total p63 knockdown siRNA assay, there was a small decrease in expression of Δ Np63 compared to the negative control cells. When the TAp63 knockdown was introduced, Δ Np63 expression had a large increase compared to the control cells suggesting knockdown of the TAp63 isoforms allowed Δ Np63 isoforms to take over in basal cells at least. Much like when the total p63 assay was introduced into these cells, when the Beta isoform knockdown was introduced a small decrease in Δ Np63 was observed.

As for TAp63 expression in the HKc17 cells, there was a small issue surrounding data collection when the total p63 siRNA was added to the cells as two out three triplicate repeats resulted in an undetermined result. This may be due to an experimental error as qRT-PCR is a very sensitive technique or the total p63 knockdown may have disrupted the RNA of the TAp63 isoforms in some way. As for the other two assays, increases in expression of TAp63 were seen with a small increase when TAp63 knockdown was introduced and a much larger increase seen when Beta isoforms were targeted. With an increase in expression in TAp63, when a knockdown specifically designed to knockdown these isoforms was introduced, suggests a non-specific interaction between the siRNA assay and the target. The expression of TAp63 in HK Lonza cells increased significantly when total p63 was knocked down, with a similar level of expression when the Beta isoforms were targeted. As for TAp63 expression, when the siRNA designed to knock TAp63 isoforms down was introduced, showed little to no change on the negative control cells, again suggesting the knockdown assay was not specific.

When ETS-1 was examined in each of these cell lines exposed to the various knockdowns (HKc17 and HKLonza), a similar pattern of expression change was observed with small increases in expression in all cases. The largest increase in expression of ETS-1 was seen when the Beta isoforms of p63 were targeted for knockdown. This may suggest the Beta isoforms of p63 have a repressive role over ETS-1 expression, which when removed allows for higher levels of expression. These genes were selected as the cells investigated were basal cells and we were not assessing differentiation at this point. To continue this work differentiating the cells and assessing differentiation markers would provide deeper understanding.

4.0 Discussion

The transcription factor p63 is a master regulator of a number of key processes involved in the correct development of the epidermal layers. The gene encodes multiple isoforms each of which play different roles in these events that determine the epidermis formation (Soares & Zhou, 2018). Information regarding the particular roles of each of these isoforms has been only partly investigated with a lot more research into this area required. The difficulty with working at an isoform level is the sequence homology between each of the species creating issues with siRNA or even other systems. Other systems which could be used for knock out or knock in experiments is CRISPR- Cas9 or shRNAs, however currently there are no commercially available guide strand RNAs available with specificity required to differentiate between each isoform. In addition, there are currently limited options when looking at the protein expression level with only a handful of commercially available antibodies specific for TAp63 or Δ Np63 and none that identify particular isoforms. Antibodies for pan p63, TAp63-specific and Δ Np63-specific have been acquired and are in the early stages of being optimised. Again, we were unable to use α , β or γ - specific TaqMan probes as these have not been designed and are difficult to design due to high sequence homology amongst the isoforms that would lead to non-specific effects. Working with assays targeting TAp63 or Δ Np63 isoforms is enough to identify trends in expression of these isoforms but for a detailed interrogation of α , β or γ forms, individually either more specific assays or alternative methods are required.

When designing siRNA assays for the experiments described, there were a number of issues encountered in order to design a specific assay. The sequence homology between the isoforms is high and regions that are specific to the TAp63 isoforms are identical. The transactivation domain present in all TA isoforms has an identical sequence which makes it ideal for differentiating between TAp63 isoforms and Δ Np63 isoforms but cannot be used for specific targets within the TAp63 subclass (α , β and γ). To further complicate matters, the regions present which differentiates the TAp63- γ from TAp63- α and β is identical in sequence to the region that differentiates Δ Np63- γ from Δ Np63- α and β . The same principle applies to the regions which define the α and β isoforms. This resulted in not being able to develop siRNA assays that particularly targeted one of the six main isoforms of p63. To circumvent this issue, we chose to look specifically at different assay types and then collate the information obtained to elucidate what is happening to gene expression when cells are exposed to different p63 knockdowns. The first assay for total p63 would give a complete coverage of p63 knockdown which would allow generalizations over p63's influence on gene marker expression. By using a general knockdown, all six of the main isoforms are targeted via a conserved region that is common in all isoforms (**Figure 23**).

When the HKc17 and HKLonza cells were cultured for the purpose of testing these assays, there was irregular growth and morphology observed. The RNA quality obtained from these cell samples was low in concentration and low in quality. However, we obtained high quality RNA from the basal cells and these were used for comparative analysis. The results, which we saw from this experiment, may be down to the fact that p63 activity was removed from these cells. On the molecular level, p63 knockdown can genes that arrest the cell cycle such as *p21*. This halt on the cell cycle may explain as to why the cells we cultured appeared to be not growing. Additionally, knockdown of p63 can also downregulate genes associated with positively regulating proliferation, whilst upregulating genes associated with negative regulation of proliferation. Finally, genes which are important for epidermal differentiation, including *Perp* and *K14* are downregulated when p63 is knocked down (Romano et al., 2007). Another explanation that

may describe how the cells we collected were not viable, may be due to p63's role in regulating the glycolytic metabolism cascades within keratinocytes. Hexokinase 2 and 6-phospho-2-kinase/fructose-2,6-bisphosphate 3 (Hamanaka & Mutlu, 2017; Viticchiè et al., 2015) are p63 regulated and removal of p63 sees decreased expression of these proteins. The removal of the normal p63 activity in these cases may suggest as to why the cells failed to be viable beyond the first day of differentiation. The proliferative potential of the cells prior to introduction of the differentiation agents may have led the cells out of the cell cycle and towards cell death. Once the differentiation media was added, 24 hours later, the cells may have already lost the capability to differentiate either through loss of expression of *Perp* and *K14* or through dysregulation of the glycolytic pathways.

4.1 TAp63

Beyond the general knockdown, a more specific approach, which we decided upon, was to look at detail into the TAp63 isoforms. We chose TAp63 as this isoform has been rarely talked about previously in the literature in the context of keratinocyte differentiation. Δ Np63 is widely reported as the most populous isoform in keratinocytes throughout the epidermis, accounting for 99% of total p63 presence (Candi et al., 2007). With that being said, TAp63 accounting for 1% of p63 population suggests there is a role for this isoform. To support this notion that there is some small level of TAp63 present in differentiating keratinocytes, the experimental data obtained from our experiments in differentiating HaCaT cells reinforced this. As shown in **Figure 25**, we observed a peak in TAp63 expression on Day 11 of differentiation. At this stage, the cells are in the later stages of differentiation. Before Day 11, the expression of TAp63 is low, with it staying low right up until after Day 6. At this point, the expression levels climb to its peak at Day 11 before dropping slightly. The increase in expression of TAp63 suggests this is the period in the differentiation process when it is important. As a transcription factor, TAp63 must be regulating some of the steps involved in either the structural changes or morphological changes associated with terminal differentiation. Furthermore, the expression pattern of the Δ Np63 appears to mirror that of the TAp63. When TAp63 expression is at its lowest between Days 4 and 6, expression of Δ Np63 peaks at this time. This peak is after low levels of expression in the very early stages of differentiation and beyond the peak, levels drop as the cells progress towards terminal differentiation.

The expression profiles between the two normal human keratinocyte cell lines for Δ Np63 were consistent; however, there were variations when TAp63 was examined. In Hkc13 cells, a large peak was seen in the early stages of differentiation that is not consistent to data we have from HaCaT cells and HKc15 cells. This may have appeared due to experimental error. Discounting this peak, there was an expected profile of expression with peak expression occurring on the days when terminal differentiation is expected. This again provides weight to the idea of TAp63 playing a role in regulating terminal differentiation.

In literature, there has been suggestions that there are dominant negative relationships between the p63 isoforms much like the dominant- negative relationships between the rest of the p53 family (Billant et al., 2016). This notion was arrived upon by the observation that since Δ Np63 lacked a transactivation domain; it acted as a dominant negative towards TAp63. More recently, this theory has been changed significantly with discovery of an additional transactivation domain at the C-terminal end of the Δ Np63 isoforms (Duijff et al., 2002; Marcel et al., 2012). With this being said, the mirrored expression of the isoforms during differentiation, may be due to more dominant isoforms downregulating expression of less dominant forms. As differentiation progresses, the dominance of each isoform may flip, allowing for the

characteristics of the expression patterns seen. Transcription networks are complex and it is very unlikely that only the interplay between the isoforms is responsible for the changing expression in differentiating cells. Vast networks of transcriptional control allow for the correct progression through differentiation with many markers involved including the likes of KLF4 and ETS-1.

Interestingly, some research into TAp63 expression in keratinocyte differentiation has been carried out recently with findings being conflicting not only to data obtained in our study but with other studies. When TAp63 α is expressed and Δ Np63 is not, induction of Keratin 5 and 14 occurs. These keratins are allowing stratification of keratinocytes. TAp63 null mice have demonstrated, interestingly, that this isoform is dispensable for the development of the epidermis (Su et al., 2009). Candi et al (2007) suggests that TA isoforms play a role in the terminal differentiation steps in mice epidermis and TAp63 protein has been found in the mature epidermis- despite Δ Np63 being the main isoform found. This paper also mentions a study in which TAp63 was observed to induce pluristratification in single layered lung epithelium and to block skin differentiation (Koster et al., 2007). These findings suggest a role for TAp63 in early stages of differentiation however; we are only seeing peak expression towards the later stages of differentiation. Conversely, in the study carried out by Candi et al (2007), they conclude that TAp63 expression is found later in the differentiation process, playing a role in the steps preceding the formation of the cornified envelope, regulating ETS-1, CHUK and other genes involved. These findings support the data obtained in our work, with peak TAp63 appearing at the latter stages instead of early in the process.

To investigate TAp63's role further in terminal differentiation, development of knock out skin models could be developed without the active TAp63 isoforms. The barrier function of the skin models produced could provide some insight into if the expression of the proteins associated with barrier function are disrupted due to TAp63 activity being removed or reduced. Additionally, comparison between mRNA data and protein data for TAp63 expression in the later days of differentiation could reinforce roles for these isoforms.

Another study carried out recently identified no role for TAp63 in the whole process of keratinocyte differentiation and this was verified through mRNA and protein expression work (Kouwenhoven et al., 2015). This highlights the difficulty of analyzing p63 at an isoform level. Due to the high level of homology and the limited specific technologies to study at the isoform level, it complicates analysis and evaluation of work. A clear need for design of specific antibodies and primers to allow for study at this level is of high importance due to the role of p63 in not only development but also disease and cancer. TAp63 isoforms only account for approximately 1% of total p63 expression in keratinocytes creating further complications as Δ Np63 expression may cloud TAp63 patterns due to the sheer scale difference between the two.

TAp63 regulation is another area that is interesting to look into as the regulators of TAp63 can allow for predictions on its roles. The NF- κ B transcription factor mediates a multitude of cellular processes including growth, differentiation and apoptosis. TAp63 has been demonstrated to be a transcriptional target for the NF- κ B complex through co-transfection methods (Wu et al., 2010). The conclusion drawn from this research was that TAp63 negatively regulates cellular growth and activation of NF- κ B can arrest cell growth by mediation through TAp63, however the role of NF- κ B in epidermal differentiation, if there is any, is still unknown.

A study in 2008 identified that in p53-mutated HaCaT cell lines TAp63- α was induced with the upregulation of GDF15 (Growth differentiation factor 15) (Ichikawa et al., 2008). They demonstrated

through siRNA knockdown of endogenous TAp63 that there was a reduction in the expression of GDF15. GDF15's role is not entirely certain however, it appears to play a role in controlling inflammatory pathways along with roles in apoptosis and cellular growth. It has also been implicated as a prognostic protein in cancer (Wallentin et al., 2013). This study identified TAp63- α as being present in earlier stages of differentiation with upregulation appearing as soon as 12 hours after differentiation was induced. They noted an increase in the beta isoform but a downregulation of the gamma form. They verified this finding through protein assays, identifying TAp63- α as being readily found in proliferating HaCaT cells but was induced once differentiation stimuli were added. They showed in a time-dependent manner GDF15 was expressed in concordance to the upregulation of TAp63- α . A siRNA knockdown of TAp63 demonstrated a marked reduction in GDF15 suggesting a relationship between the two.

Not only does this highlight the complexity of the transcriptional networks involving p63, it demonstrates a role for TAp63 in earlier stages of differentiation opening up a new aspect of TAp63 activity that has rarely been investigated. It is possible that there are significant roles for TAp63 throughout the differentiation process, and not only in the early stages of epidermal commitment or terminal differentiation that have been previously reported.

With this being said, the preliminary results obtained in our study suggest a role for TAp63 in terminal differentiation that needs to be further investigated. To verify the activity of TAp63 isoforms at this stage, the knockdown procedure needs to be optimized and increased in specificity to allow analysis of downstream marker expression. This may mean a permanent knockdown system, instead of the transient knockdown siRNAs, with options including CRISPR or shRNA systems. This would allow for a single knockout line of cells to use for gene expression or protein expression analysis. Beyond this, 3D skin model culture could be used to assess the structural organization and morphology when TAp63 is not present.

4.2 Δ Np63

As for Δ Np63, analysis is much easier to assess due to the scale of expression of the isoforms in differentiation. This is reflected in the quantity of literature surrounding the role of Δ Np63 in keratinocytes. Δ Np63- α is an isoform that is strongly expressed at the basal layer where the epithelial cells with the highest clonogenic and proliferative capacity reside. Previous research has given indications that these forms are required to maintain the proliferative potential (Truong et al., 2006). The majority of current work agrees that Δ N forms are the major functional isoform in epithelial tissues. Phenotypes in Δ Np63 knockout mice support this notion as they exhibit similar defects that mice lacking all p63 isoforms show (Soares & Zhou, 2018).

We observed that the peak of Δ Np63 expression in HaCaT cells was around Day 5 of differentiation, supporting the idea that the Δ N isoforms are key for maintaining the cells in their basal state but also for regulating the progression through the early stages of differentiation. We also observed that when the general siRNA assay for total p63 was introduced into the HKc17 cells and qRT-PCR targeted Δ Np63, that there was a large decrease in expression of Δ Np63. This may suggest that the majority of p63 found in these basal cells was Δ Np63. The decrease observed in the Lonza cells was smaller but there was still a decrease. Again, in basal cells, we saw a percentage knockdown efficiency between 50-70% for Δ Np63. This was dependent on siRNA concentration when total p63 was knocked

down (**Figure 21**). This again supports that in basal cells the majority of the p63 population is made up of Δ Np63.

When Δ Np63 was examined upon introduction of TAp63 knockdown, it was noted that in HK Lonza cells Δ Np63 expression increased. This again supports the idea of each of the isoforms being controlled somewhat by each other. When TAp63 isoforms are removed, it appears that Δ Np63 expression is allowed to increase due to the TAp63 being removed. As previously described, TAp63 is a pluripotency marker and blocks differentiation, so when it is removed, Δ Np63 isoforms become more prevalent allowing cells to be prepared to differentiate. This pattern was not seen in the HKc17 line. This means that too much cannot be derived from what was observed in the HK Lonza, but provides an area to investigate further.

When normal keratinocytes without knockdowns were analyzed through differentiation we observed interesting profiles which match the HaCaT data. When we focused on Δ Np63 expression, we noted that the fold changes of expression remained constant throughout. This was observed in both the HKc13 and 15 cells. Although the fold increases were small compared to the likes of TAp63, the peak level of expression was seen around Day 7 of differentiation. The fact that the fold increase was as small as it was for Δ Np63, it suggests the level of Δ Np63 remains consistent throughout all stages of differentiation. With small changes in expression on Day 0- a pre-differentiation state- suggestions are that Δ Np63 has a role to play in basal cells and differentiating cells. This is consistent with suggestions made previously that Δ Np63 accounts for the majority of p63 isoforms present in differentiating keratinocytes.

Commonly talked about in the context of keratinocyte differentiation, the role of Δ Np63 isoforms appear to be more concerned with maintaining the proliferative potential of cells rather than allowing them to proceed towards terminal differentiation. TAp63 may be the agent that prevents the Δ Np63 isoforms from maintaining the natural proliferation pattern and interacts with the Δ Np63 isoforms to release cells into the differentiation pathway. The processes by which epidermal proliferation are reasonably well studied; however, there is still lack of understanding as to how Δ Np63 induces epidermal differentiation. A possible role for this regulation is through microRNAs such as miR-944, a Δ Np63 intronic microRNA, which are generated from the transcription units of their host so in theory should be expressed at the same time. At the basal layer, the proliferative potential of cells is governed by Δ Np63. ERK signaling pushes cells towards proliferative capacity; however, when cells migrate into the spinous layer of the epidermis the expression of Δ Np63 is reduced. Here the miR-944 acts on cells to induce differentiation. It works to reduce ERK signaling and upregulates expression of markers such as p53. By promoting the expression of p53, cells are encouraged towards differentiating ([Kim et al., 2015](#)).

Δ Np63 has also been associated with regulating upstream of GATA3, and CHUK. By regulating these markers typically associated with terminal differentiation, Δ Np63 is directly involved in this process. We see peak expression in differentiating keratinocytes occur around Day7 of differentiation. With a peak expression at this time the sequence of events leading to upregulation of transcription factors needed for terminal differentiation is started.

4.3 ETS-1

ETS-1 was chosen as a marker to investigate as it has been previously identified as being regulated by one of the p63 isoforms in keratinocytes- TAp63. This marker is much like p63 in that it acts as a transcription factor, regulating the steps for correct cellular differentiation. ETS-1 has also been previously implicated as a transcription factor that regulates CHUK further down the cascade. Through microarray analysis, Δ Np63 has been identified as being a regulator of CHUK however it only interacts indirectly through GATA-3 regulation (Candi et al., 2006). In comparison, TAp63 regulates ETS-1 in a much more direct manner that in turn directly regulates CHUK. In our panel of gene markers, we already had structural markers such as FLG and a transcription factor in KLF4, but we wanted two markers that not only were regulated by p63 isoforms, but also regulate each other. In ETS-1 and CHUK, we have two markers that have been distinctly linked previously.

During the HaCaT differentiation experiments, ETS-1 expression had a slowly climbing level of expression throughout differentiation. This expression peaked at a similar time to TAp63 which falls in line with previous studies which suggests that TAp63 directly regulates ETS-1 in keratinocyte differentiation (Candi et al., 2007). When the two keratinocyte cell lines (Hkc17 and HK Lonza) were exposed to each of the three knockdown assays, they exhibited very similar expression patterns of ETS-1. Unlike the data obtained for TAp63 and Δ Np63 that varied somewhat between the two cell lines, there was a striking similarity when ETS-1 was interrogated. With that being said however, when either cell line had TAp63 knocked down, there was an increase, albeit small, in expression of ETS-1. This may be due to the assay specificity for TAp63 isoforms not being as desired, which has been previously explored. Alternatively, knock down of TAp63 may allow other transcription factors, be it Δ Np63 or others to take over the regulation of ETS-1.

In the literature ETS-1 has been described in the context of expression in keratinocytes previously, however there is conflict here also. ETS-1 is an oncogene that is a downstream effector of the Ras-MAPK pathway. However, in normal tissues, it has been described as only present in the basal layers of the epidermis (Nagarajan et al., 2010). We observed expression in differentiated HaCaT cells which is in line with the relationship between ETS-1 and TAp63 described by Candi et al (2007) in terminal differentiation but is in contrast to the findings by Nagarajan et al., 2010. Nagarajan et al., 2010 goes on to suggest that ectopic expression of ETS-1 in the differentiated layers of the epidermis leads to developmental defects and hinders the formation of the barrier. We studied ETS-1 in a 2D culture format that would not allow determination of the effect of ETS-1 expression in the differentiated keratinocytes and on the barrier function, however we have seen, at least in this cell line, that ETS-1 is present when differentiation is in progress. Furthermore, we saw peak expression around Day 11 of differentiation that is far from the basal expression described previously.

Additionally, through the two siRNA-treated cell lines we have reinforced the idea of ETS-1 being present in basal cells. Both these lines exhibited similar expression profiles for ETS-1 in each of the knockdown conditions. We have demonstrated, at least at the mRNA level, there is expression of ETS-1 throughout differentiation with data for basal cells (HKc13 and 15) and differentiated cells (HaCaT).

4.4 FLG

During the calcium-induced differentiation of the HaCaT cells, the expression of FLG followed the profile as expected. FLG acts as a structural protein that is involved in establishment of the structure of the cornified layers and is hence seen in terminally differentiated keratinocytes. The expressed pattern we observed matched the expectation that peak expression would be towards terminal differentiation, with a slowly increasing level throughout. In the HKc13 and HKc15 cells, we observed similar profiles, again with peak expression at the later days. FLG has not been implicated directly as being regulated by either of the p63 isoforms and this could be assessed in the future by assessing FLG expression when p63 was knocked down. Once the knockdowns are established and fully optimized then this can take place.

As previously mentioned in the context of ETS-1 and CHUK, GATA 3 is involved with regulating FLG in terminal differentiation. This transcription factor is expressed in epidermal keratinocytes and is heavily associated with establishment of the epidermal barrier. Additionally, in hyperproliferative conditions such as psoriasis, GATA3 expression is decreased in the stratum granulosum and stratum spinosum (Zeitvogel et al., 2017). As FLG is directly influenced by GATA3, FLG is linked to the network of transcription that regulates CHUK and other markers. This network is controlled upstream by the p63 isoforms.

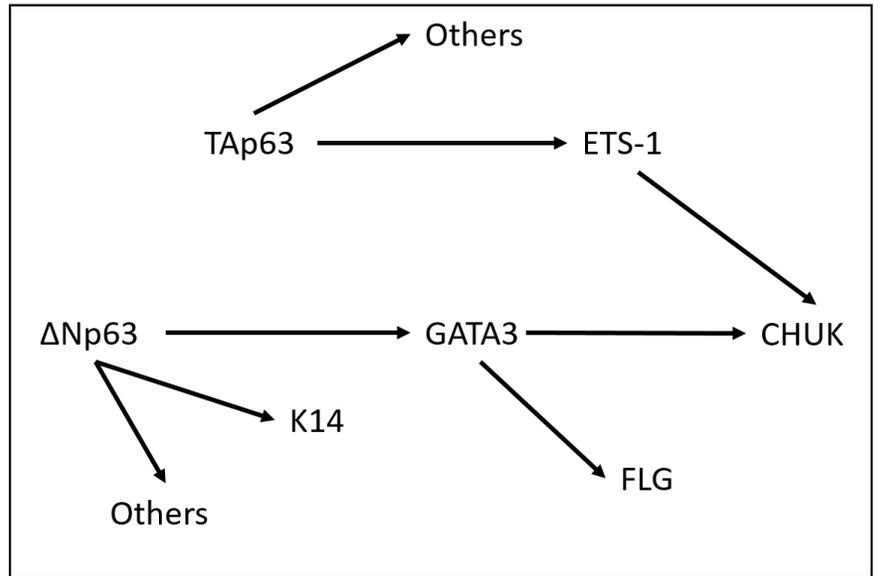


Figure 26: Predicted network controlled by p63 isoforms in terminal differentiation. CHUK can be regulated indirectly by either p63 isoform. GATA3 is crucial for allowing the expression of FLG and the formation of the epidermal barrier

4.5 KLF4

KLF4 gene expression is different, as seen in the HaCaT differentiation. This transcription factor has previously been linked to p63 in that there is a level of indirect regulation of KLF4 by p63 (Cordani et al., 2010). This indirect regulation is mediated through p63- regulated expression of ZNF750 which in turn allows upregulation of KLF4. Upregulation of KLF4 leads to activation of other terminal differentiation genes (Figure 27) (Sen et al., 2012). ZNF750 is crucial for terminal differentiation in keratinocytes as it turns on the terminal differentiation sequence, including KLF4.

The expression pattern observed in HaCaT cells was similar to what was expected for a transcription factor involved in orchestrating terminal differentiation. There is little expression of KLF4 in the early stages of differentiation with peak expression being around Day 7 with expression continuing to be high for a few days before declining slowly towards terminal differentiation. This fits with the role of KLF4. As a transcription factor, the upregulation of KLF4 needs to occur earlier than terminal differentiation in order for it carry out its role as a transcription factor. KLF4 regulates the expression of structural proteins that allow for the morphological changes that are identified as terminally differentiated keratinocytes. The peak expression of KLF4 occurring before peak expression of structural proteins make sense given their individual roles.

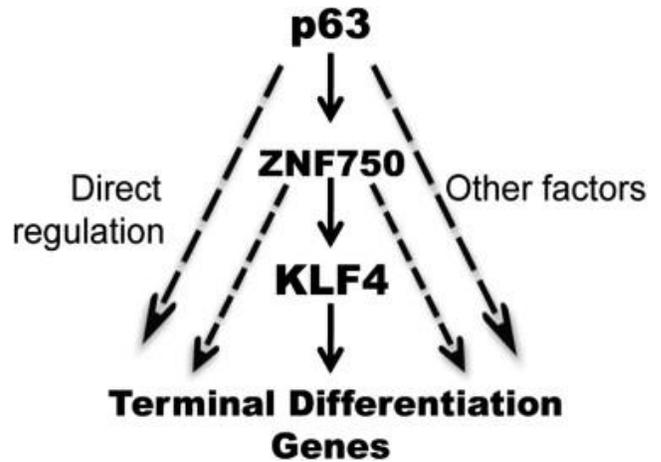


Figure 27: Indirect regulation of KLF4 by p63 through upregulation of ZNF750. *Sen et al., 2012*

An interesting area to study in the future would be to assess ZNF750 expression and KLF4 expression when various p63 isoforms are knocked down. In addition, a knockdown of ZNF750 to examine the expression of KLF4 without ZNF750 could create significant evidence of this cascade of regulation in terminal differentiation.

KLF4 is highly conserved through evolution, demonstrating its importance as a transcription factor associated with development, proliferation and differentiation (Ghaleb & Yang, 2017). It has come to the forefront of research in recent years due to its role in inducing pluripotent stem cells. One of the key features of KLF4 is its role in suppressing apoptosis and hence promoting survival. It has been shown to suppress the p53- dependent apoptotic pathways. KLF4 is highly expressed in the epidermal layers of the skin and mouse models with null KLF4 exhibited a loss of barrier function (Segre et al., 1999). The role of KLF4 was identified as its ectopic expression accelerates the barrier formation (Jaubert et al., 2003).

The data we obtained supports a correlation between the theory of KLF4 accelerating the formation of the external cornified barrier. We saw in HaCaT cells that the expression of KLF4 was highest at the later stages of differentiation, but before the peak expression of structural proteins such as FLG. This is a correlation between the expression analyses but needs to be investigated further to identify any specific causality. This would suggest the structural proteins are the ones being regulated by KLF4 activity. As a transcription factor, the orchestration of these steps to upregulate structural proteins needs to be tightly regulated. A significant upregulation of KLF4 and other transcription factors allows for the normal progression and formation of the barrier of the epidermis, allowing for one of the key features of the skin.

4.6 TGM1

The work carried out on TGM1, this was a continuation on work previous to this study where we observed expression of TGM1 in late stages of differentiation (data not shown) in HaCaT cells. To further

the understanding and assess the viability of HaCaT cells as an expression system for differentiation experiments we monitored changes in TGM1 expression in differentiating HKc13 and HKc15 cells. The data collected regarding TGM1 expression matched what we saw in HaCaT cells with peak expression being towards the later days of differentiation. This also falls in line with TGM1 function as an enzyme associated with the formation of the cornified layer in the epidermis. The series of events that creates the cornified barrier occurs later in the differentiation process.

TGM1 was investigated only briefly due to the limited links described between TGM1 and p63 previously. TGM1 is a hugely important marker for terminal differentiation and is of interest concerning downstream regulation from p63. TGM1 is a candidate gene that is associated with disease such as congenital ichthyosis and may be implicated in other diseases of the barrier function. With that said, there is little evidence in the literature of p63 being involved directly with TGM1 regulation. This would be something that would be of interest to investigate further with the implications for disease.

4.7 Disease

Diseases associated with p63 mutation are rare but severe in their phenotype, with a large range of developmental defects in epidermal appendages. All forms of ectodermal dysplasia are at a prevalence of 1 in 100,000 newborn babies in the USA. These conditions are classically autosomal dominant in inheritance meaning only one allele is needed to give a disease genotype. As p63 is so critical for the correct formation of the epidermis, and other developmental processes, understanding the exact processes that it controls is critical for understanding modes of tackling these diseases. It is complicated by the existence of multiple isoforms and the roles they each play in the complex pathways.

With work similar to this study, there is data emerging that allows the generation of networks and pathways that can be verified in the lab. By creating the networks, we can identify areas that are affected by mutated p63 and develop therapeutic approaches to help patients. Additionally, hyperproliferative conditions such as psoriasis have been linked to p63 mutation. These conditions are more common than the p63-related dysplasia. Restoration of p63 in null animal models has seen a return to wild type so using this theory, gene replacement approaches may be an avenue for treatment for these types of conditions.

Currently there is no available cure for p63-associated disorders. The only available treatments are surgery or cosmetic corrections. The rapidly developing field of human disease modelling has allowed steps to be made in this area. APR-246/PRIMA-1^{MET} is a small compound that has been discovered in the context of cancers, and interacts with the mutant core domain of p53. This compound is in phase I/II of clinical trials for treatment of hematological malignancies ([Lehmann et al., 2012](#)). Given that p63 is from the p53 family, there is significant sequence homology between the two DNA-binding domains potentially meaning APR-246 can be used a therapy for p63-related disorders to restore mutant p63 conformation ([Shen et al., 2013](#)).

Another strategy, which is being explored, is the use of siRNAs as a therapeutic in treating these diseases. Studies focusing on the R304W mutation in EEC rescued defects when a panel of siRNAs targeting this mutation were introduced. Furthermore, the siRNA approach also rescued differentiation capacity of the cells ([Barbaro et al., 2016](#); [Novelli et al., 2016](#)).

4.8 Regulation

Direct regulation of many genes by p63 is a contributing factor making the picture so complicated in respect to unravelling the full networks that it controls. Additionally, the roles of each individual isoform and how epidermal formation and barrier function is affected by temporal and spatial expression of p63 isoforms is of key importance.

The control through p63 of many target genes is not the only method of transcriptional control. Via higher control, p63 can play a role in epigenetic regulation also. Several studies have demonstrated that p63 operates via several independent mechanisms. p63 has been shown to be able to recruit factors such as HDAC1/2 to control repression of cell cycle arrest genes. Additionally, many chromatin factors are p63 target genes. Recently p63 has been shown to be involved in regulating nuclear envelope proteins. In p63 knock out mice, these genes were downregulated suggesting a direct interaction between p63 and those genes.

New evidence has suggested a role for p63 in regulating enhancers. In cells where p63 is not expressed, genomic regions of p63-bound enhancers are inaccessible. The studies also suggest that p63 and its related co-regulators have a pioneer effect in epidermal development. The pioneer factors are described as the first factors that interact with the chromatin in its compacted state and makes it more accessible down the line. This modulation allows for binding of other transcription factors, creating an accepting epigenetic environment (Soares & Zhou, 2018).

These examples of extra levels of control from p63 further highlight its importance in development and disease. The regulation of critical events in a cell's lifetime result from tightly controlled expression of various factors and an unbalance or dysregulation of these events present significant problems. These cases also create a curiosity to the scale of regulation that p63 has, further pushing the level of knowledge required to understand the full picture.

Dysregulation of p63 targets have been studied more in the context of cancers instead of in epidermal development. Studies looking at oral development and associated disease and cancers have described a dominant negative affect for mutated p63, whereby the mutated form of the protein can compromise the functionality of the normal wild type allele. Further studies looking at head and neck carcinomas have identified the nature of these dominant negative mutants and how they may disrupt wild type p63 activity. There is suggestion in the context of cancers, that mutant p63 isoforms can antagonize the regular action of p53, either directly or indirectly. Analysis through Western blots showed that when p63 isoforms ($\Delta Np63$) were over expressed in cancer, p53 levels were lacking. Conversely, when p63 expression was low, p53 was high. In cancer p63 isoforms, particularly $\Delta Np63$ is overexpressed and hence the dysregulation of *TP63* at the promoter responsible for $\Delta Np63$ isoforms leads to low levels of p53. Reduction of p53 activity removes this critical tumor suppressor gene and therefore cancers may form (Sniezek et al., 2004). As p53 and p63 have such high sequence, homology it is reasonable to expect similar dominant negative effects between isoforms.

This is in the context of cancer but demonstrates that changes in the normal expression of p63 isoforms can lead to catastrophic events in the cell, whether this is through direct dysregulation of target genes or via indirect effects.

The *TP63* gene is also tightly regulated for proper transcription of the correct isoforms to occur. The gene itself is regulated by Notch signaling pathways that are responsible for control of epidermal differentiation. Notch signaling has been shown to repress expression of p63 in keratinocytes. This pathway is subject to self-feedback loops through Δ Np63 as this isoform can activate Notch associated genes. Interferon Regulatory Factor 6 (IRF6), along with p63 mutations, are associated with craniofacial malformations such as cleft lip palate. Δ Np63 and IRF6 are closely associated in that IRF6 is activated by Δ Np63 and IRF6, surprisingly, causes proteasomal degradation of Δ Np63 (Moretti et al., 2010). Notch has also been described as a member of the p63/ IRF6 axis in keratinocytes suggesting a tightly organized network to allow for epidermal development. Therefore, if not only p63 is dysregulated but also either of the other members in this network are, there could be drastic knock on effects downstream in the network, possibly leading to further dysregulation of other genes and potentially disease.

Other signaling networks including Hedgehog signaling, Wnt signaling and EGFR pathways have all been implicated in being closely involved with regulation of p63 isoforms in the epidermis. This begins to show the scale of the network in which p63 is involved; meaning not only is mutation in p63 dangerous but mutation and dysregulation of the regulatory members involved with p63 can lead to further issues.

5.0 Perspectives

5.1 Future Work

To further this work a number of different angles of investigation can be used. Given more time, the first place to start would be to investigate the design of the siRNA assays. These assays appeared to show knockdown patterns which were not expected. Repeating these experiments again with the same cell lines would allow for a much clearer idea as to if what we were seeing was true. If it is true, then potentially redesigning the assays or switching methodological approach completely could be considered. As for systems to change to would be shRNA and CRISPR. Both systems create permanent knockdowns, which is different to siRNAs that have a transient effect. By switching technique however, the specificity problem remains, as currently these systems are not designed with specific p63 isoforms in mind. This would need to be explored in more detail before determining the viability of each method.

Another way of progressing this work would be to examine the protein expression side of p63 and use isoform specific antibodies to look at spatial expression in cell culture and 3D skin models. We have acquired specific antibodies for TAp63 and Δ Np63 as well as a pan p63 antibody for this purpose. We hope to optimise these antibodies and use them for staining purposes and Western analysis. This approach could further be stretched when the knockdown approach was established to include skin models that contain certain p63 isoform knockdowns. This would allow assessment of morphological features and be used for sectioning and antibody staining in these models. To further the validity of this research, the same experiments need to be competed in comparative cell lines from different donors. Cell lines, which are matched on age, sex and body area extracted from, should be used to identify if these patterns are the same. As information regarding the donors were limited in this case due to the nature of the collection, it was difficult to match cells in this way.

The method that we want to progress with is to use RNASeq analysis to determine gene expression of a whole host of markers from keratinocytes undergoing differentiation. By using a highly specific and sensitive technique, it would allow detections of potentially new markers that could be used for study in this context whereas qRT-PCR allows for highly specific analysis of known genes. This technique provides information on the whole transcriptome in a given cell sample using high throughput technologies. Analysis using qRT-PCR would allow for comparison between different cell lines such as p63-isoform knockdown lines compared to normal wild type cells. RNASeq is more desirable for this type of analysis over the likes of microarrays as it allows for a higher magnitude of data produced ([Zhao et al., 2014](#)).

Using the mRNA level as a method of study has merit in that it allows gene expression to be studied and this can provide information into the changes that occur during differentiation. However, this analysis needs to be reinforced by protein analysis, as protein expression can provide a bigger insight into the changes. Protein expression can have a higher impact on differentiation and cellular phenotype so must be considered before conclusions are made. Coupled together, both gene expression and protein expression provide more of a perspective into the networks involved.

As the network of transcriptional control in keratinocyte differentiation is complex, it would be beneficial to identify and investigate other markers involved in this process. By identifying multiple members of these cascades, the whole picture of the regulatory network can begin to be hypothesised.

This will have impact on disease applications as different markers can be investigated as candidate genes for various developmental and epidermal conditions. By examining the normal processes, comparison to patient skin can be used to identify genes involved. This will have impact not only when looking at p63-related diseases such as AEC and EEC, but also hyperproliferative conditions such as psoriasis and even eczema.

In the literature, there is still conflict into the specific roles of p63 isoforms with debate over not only specific roles but also the temporal expression of the isoforms. It is widely accepted that Δ Np63 is the most prominent isoform in differentiating keratinocytes and has roles in maintaining the basal layer whilst also allowing for proliferative potential in the keratinocytes. Recently with more developed technologies, there has been progress in identifying specific roles for the p63 isoforms. The generation of the Δ Np63 knockout mouse has shed light into the importance of these isoforms for development. Null mice show poorly developed epithelia, including the epidermis. This demonstrates that this particular isoform is responsible for epithelia development and maintenance of epithelial stem cells (Romano et al., 2012). As for TAp63-null animals, the phenotypes included premature ageing, alteration to glucose and lipid metabolism and eventually premature death (Su et al., 2012; Su et al., 2009).

Generation of these models shows progress in the area, allowing for more specific experimentation regarding individual isoforms. These models will be crucial for progressing the understanding of the interplay between p63 isoforms and other genes in a live model. Obviously, there are limitations of mice models such as the ethical issues surrounding animal models. Furthermore, the skin of the mouse differs from human skin due to higher numbers of follicles in mouse. This may affect any staining experiments. This leads to generation of knock out cell lines that can be used on large scale without the issues of ethical confrontation or other issues arising from mouse models. These cell model-based approaches are beneficial as cell lines can be generated with isoform knockdowns that can be stored and reproduced on a commercial scale, allowing for a larger amount of research into roles of p63.

Production of these lines would vastly improve the speed at which gene expression studies related to p63 isoforms could be produced. This could have benefits in the pharmaceutical or gene therapy markets as currently patients with p63-related conditions have limited treatments. In mouse models, reintroduction of p63 in null subjects showed a level of restoration of the wild-type phenotype that could be an avenue for investigation through gene therapy applications.

5.2 Conclusion

As a preliminary study into the role of p63 isoforms in keratinocyte differentiation, we have managed to identify the normal expression patterns of both Δ Np63 and TAp63 expression in differentiating keratinocytes. Alongside the p63 isoforms, we have also assessed expression of other differentiation markers including FLG, ETS-1 and KLF4. We have identified that during normal differentiation, the expression of the isoforms appears to have mirrored profiles with Δ Np63 expression peaking early and decreasing towards terminal differentiation with TAp63 expression climbing at this time. Peak TAp63 expression was observed around Day11 of differentiation suggesting a role for these isoforms in regulating the final steps. There has been recent work that has highlighted this expression of TAp63 isoforms and speculation into their roles are at an early stage. By removing TAp63 expression through transient knockdowns in basal cells, we have demonstrated a system that can effectively reduce TAp63 expression; however, we have experienced some cross reactivity with the Δ Np63 isoforms. By

furthering the system through optimization and developing the methods, we hope to knock down TAp63 isoforms solely and investigate the effects that has on terminal differentiation. With so much data present on Δ Np63 isoforms in this context, there is a need for a shift in focus to TAp63 isoforms in order to understand fully its role here.

Despite the difficulties found in this study there are exciting possibilities that have emerged for further work. Through the literature, we can gather more information for target genes to assess alongside p63 to elucidate the networks in which p63 isoforms are involved. Along with the siRNA knockdown, verification through alternative methods can provide evidence for the regulatory role of each of the isoforms. The aims of this study were to develop siRNA knockout systems that are isoform specific and allow for study of individual isoforms in differentiating keratinocytes. The use of siRNAs has some downfalls in that they are transient and have limited lifespans but this technology is the most feasible at this current time. There need to be reconsiderations in our case for the specific sequences and regions used, but this technology allows for the study of knockdown keratinocytes.

There is a need for the technology to develop in order to create isoform-specific systems that reduces the cross-reactivity and non-specific binding of primers currently. This lack of specific technologies mean alternative measures need to be taken, as with this study. Inconsistencies in the findings of literature could be caused by the lack of very specific systems.

The area of p63 in the context of keratinocyte differentiation is an exciting one as with the discovery of the individual isoforms creates a huge amount that is needed to be understood not only to map the relevant networks but also to help combat rare epidermal disease.

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7.0 Appendices

7.1 Ethical Approval

THE UNIVERSITY OF HUDDERSFIELD
School of Applied Sciences

Form 2: Ethical Review Application

Please complete and return via email to J.Ledgard@hud.ac.uk along with the required documents (shown below).

SECTION A: TO BE COMPLETED BY THE APPLICANT

Before completing this section please refer to the School Research Ethics web pages which can be found at at <https://www.hud.ac.uk/sas/research/researchgovernanceandethics/> Applicants should consult the appropriate ethical guidelines.

Please ensure that the statements in Section C are completed by the applicant (and project supervisors for PGR, PGT, and UG students) prior to submission.

Project Title	The importance of the transcription factor p63 in epidermal development and disease
Applicant	Sean Hockney
Supervisor (where applicable)	Dr Hans Hennies
Project start date	02/10/2017
Project end date	17/09/2018
Department	School of Applied Sciences

SECTION B: PROJECT OUTLINE (TO BE COMPLETED IN FULL BY THE APPLICANT)

Issue	Please provide sufficient detail to allow appropriate consideration of any ethical issues. Forms with insufficient detail will need to be resubmitted.
Aims and objectives of the study. Please state the aims and objectives of the study.	To investigate the role of the transcription factor p63 (with particular focus on the Tap63 isoform) and its role in differentiation process of keratinocyte differentiation using HaCat cell lines and primary human keratinocytes as models. To explore the expression patterns of epidermal markers in the epidermis through histological analysis
Brief overview of experimental design The experimental design only needs to be explained in sufficient detail to explain the research methods that will be used during the study.	The project will involve cell culture based approaches in order to culture primary human keratinocytes and HaCaT cell lines which will allow RNA extraction and quantification of expression levels of marker genes. Histological staining techniques on embedded sections using antibodies for various epidermal markers.
Does your study require any permissions for study such as NHS Research Ethics Committee and R&D approval? If so, please give details of current status of any applications made or permissions granted	Samples of primary keratinocytes were obtained after approval from the IRB at the Medical University of Innsbruck. The IRB is acknowledged by the Medical Association and is equivalent to NHS Research Ethics Committees.
Participants Please outline who will participate in your research. Might any of the participants be considered 'vulnerable' (e.g. children)	Voluntary adult persons undergoing plastic surgery.

<p>Access to participants Please give details about how participants will be identified and contacted.</p>	<p>Potential participants were informed of the study by specialised physicians at the Department of Plastic Surgery, University Hospital Innsbruck.</p>
<p>How will your data be recorded and stored?</p>	<p>Samples are anonymised with an alphanumeric code. Experimental results will be stored in a lab work book which will be held at the University at all times.</p>
<p>Informed consent. Please outline how you will obtain informed consent.</p>	<p>Participants were given written information about the study and then asked to sign a consent form.</p>
<p>Confidentiality Please outline the level of confidentiality you will offer respondents and how this will be respected. You should also state who will have access to the data and how it will be stored (this information should be included on your information sheet).</p>	<p>Only study physicians at the institution where the sample was taken have access to confidential information, which is stored on a secure network</p>
<p>Anonymity If you offer your participants anonymity, please indicate how this will be achieved.</p>	<p>Samples are anonymised with an alphanumeric code that does not contain any information about the proband.</p>
<p>Harm Please outline your assessment of the extent to which your research might induce psychological stress, anxiety, cause harm or negative consequences for the participants (beyond the risks encountered in normal life). If more than minimal risk, you should outline what support there will be for participants. If you believe that that there is minimal likely harm, please articulate why you believe this to be so.</p>	<p>Only residual tissues from surgical procedures indicated for unrelated reasons are used.</p>
<p>Does the project involve the use of any human tissue? If so, please provide detail on the nature of the samples and any actions you are taking to ensure compliance with the Human Tissue Act.</p>	<p>Cell lines from human keratinocyte origin. Both model cell line, HaCaTs, will be used alongside primary human keratinocyte cell lines. Frozen and embedded sections of skin will be used for histological analysis</p>
<p>Does the project involve the use of Genetically Modified Organisms (GMOs)? Please provide details explain how you are minimising any ethical issues associated with researching and producing GMOs (NB this committee does not consider H&S matters pertaining to the generation and use of GMOs).</p>	<p>N/A</p>

<p>Does the project include any security sensitive information? Please explain how processing of all security sensitive information will be in full compliance with the "Oversight of security sensitive research material in UK universities: guidance (October 2012)" (Universities UK, recommended by the Association of Chief Police Officers)</p> <p>Security sensitive materials are confirmed as research:</p> <ul style="list-style-type: none"> • Commissioned by the military • Commissioned under an EU security call • Involves the acquisition of security clearance • Concerns terrorist or extreme groups 	<p>N/A</p>
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Retrospective applications. If your application for Ethics approval is retrospective, please explain why this has arisen.

SECTION C – SUMMARY OF ETHICAL ISSUES (TO BE COMPLETED BY THE APPLICANT)

Please give a summary of the ethical issues arising from your research and any action that will be taken to address the issue(s).

The project will involve use of cell lines and tissue samples obtained from human origin. These samples will be used to assess gene expression patterns through molecular and histological based experiments. Correct documentation for collection of cells is kept by the dermatologist who took sample however lab possesses relevant ethics and approval forms for the use of these cells in research.

SECTION D – ADDITIONAL DOCUMENTS CHECKLIST (TO BE COMPLETED BY THE APPLICANT)

Please supply copies of all relevant supporting documentation electronically. If this is not available electronically, please provide explanation and supply hard copy.

I have included the following documents

- | | | |
|--------------------|------------------------------|---|
| Information sheet | Yes <input type="checkbox"/> | Not applicable <input type="checkbox"/> |
| Consent form | Yes <input type="checkbox"/> | Not applicable <input type="checkbox"/> |
| Letters | Yes <input type="checkbox"/> | Not applicable <input type="checkbox"/> |
| Questionnaire | Yes <input type="checkbox"/> | Not applicable <input type="checkbox"/> |
| Interview schedule | Yes <input type="checkbox"/> | Not applicable <input type="checkbox"/> |

SECTION E – STATEMENT BY APPLICANT

I confirm that the information I have given in this form on ethical issues is correct.

Applicant name/signature: Sean Hockney



Date: 02/10/2017

Affirmation by Supervisor (where applicable)

I can confirm that, to the best of my understanding, the information presented by the applicant is correct and appropriate to allow an informed judgement on whether further ethical approval is required

Supervisor name/signature:

Dr Hans Henriches 

Date: 9/10/2017

All documentation must be submitted electronically to J.Ledgard@hud.ac.uk
If you have any queries relating to the completion or consideration of this form, please do not hesitate to contact J.Ledgard@hud.ac.uk (administrator), r.m.phillips@hud.ac.uk (School Research Integrity Champion) or g.a.williams@hud.ac.uk (Chair of SRIEC)

7.2 Project Plan

RESEARCH PLAN

Student Name	Sean Hockney
Student number	U1350528
Research Degree	MRes Biological Sciences
School	School of Applied Sciences
Main Supervisor	Dr Hans Hennies
Date of enrolment (DD/MM/YYYY)	18/09/2017
Mode of Attendance	<input checked="" type="checkbox"/> Full time <input type="checkbox"/> Part time
Planned completion date	17/09/2018

Is this proposal for practice based research? (This can include an artefact, design, performance, composition etc.)	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
Title of proposed research project	
<p>PLEASE ATTACH A RESEARCH PROPOSAL, this should normally include:</p> <ul style="list-style-type: none"> • Title of the project • Aims of the project • Background context of the research topic • Review of existing research literature, demonstrating the original contribution this project will make • Research questions or objectives • Methodology and project design • Project plan with timeline, including progression milestones (may be in e.g. Gantt chart format) • List of references 	
<p>PLEASE ATTACH COMPLETED SKILLS AUDIT</p>	
<p>Ethics</p> <p>1. What ethical principles/codes of practice will guide the research?</p> <p>Work involves culture of primary keratinocytes from voluntary probands. Samples are obtained from adults undergoing plastic surgery. Samples of primary keratinocytes were obtained after approval from the IRB at the Medical University of Innsbruck. The IRB is acknowledged by the Medical Association and is equivalent to NHS Research Ethics Committees. Cells are stored under regulations of Human Tissue Act.</p> <p>2. Are any particular ethical issues likely to arise in this research, and how will you address them?</p> <p>Research involves use of primary cell lines which may be manipulated to knock down specific genes. These cells will only be used in culture in vitro. Ethical approval forms attached.</p>	

Health and safety

1. Have any health and safety issues arisen, or might arise, and how will you address them?

It is recommended that all student and staff working with primary cells to have vaccination against Hepatitis B. Student has received full course of inoculation against Hep b.

Skills Audit (Please attach the completed Skills Audit)

Have you identified areas of training and development areas that have been identified by your Main Supervisor as being required for consideration of progression? (please list)

Introduction to Histology equipment in preparation for any work that involves these techniques.
Induction for use of any specialist equipment as and when needed.

Resources

Please describe any resources/facilities required (including whether these are readily available)

Cell culture facilities- Available
RNA extraction kit- available
cDNA synthesis- equipment and reagents available
TaqMan RT-PCR- available
Histology facilities- available
Immunohistochemistry-/ immunocytochemistry- available

Notes for the Applicant

- I wish to apply to my supervisory team for approval of my research programme on the basis of the information given in this application.
- I confirm that the particulars given are correct.
- I understand that, except with specific permission, I may not, during the period of my enrolment, be a candidate for another award.
- I understand that, except with the specific permission, I must prepare and defend my thesis in English.
- I agree to address any Skills Audit requirements that are necessary for consideration for progression.

SIGNATURES

Student: Sean Hockney	Main supervisor: Dr Hans Hennies
Signature: 	Signature: 
Date: 14/11/17.	Date: 14/11/17

7.3 Research proposal

Research proposal: The Importance of Transcription Factor p63 in Epidermal Development and Disease- focus on TAp63 Isoforms.

Background: The epidermis is the outermost layer of the skin- the largest organ in the human body. The epidermis provides protection for the internal environment of the body. The epidermis is divided into layers which play key roles in maintaining a barrier to pathogens, whilst also regulating the movement of desired molecules into the body; and unwanted out of the body (*Zhao et al., 2015*).

Keratinocytes are the most abundant cells in the epidermis. These cells produce proteins called keratins which play crucial roles in the maintenance of this layer. Regulating the transcription of the genes for these keratins are a group of transcription factors. One of the key transcription factors found in keratinocytes is p63, a protein encoded by the *TP63* gene. This protein is from the p53 family and is known to regulate thousands of genes, many of which are key to epidermal development and differentiation of keratinocytes. As a result, when mutation in this gene occurs it leads to developmental conditions which can have aggressive phenotypes.

The *TP63* gene contains two distinct promoters which gives rise to two forms of the p63 protein- the TAp63 forms and the Δ Np63 forms of which the Δ Np63 form lacks a transactivation domain at the 5' end. Within these two groups, alternative splicing of the *TP63* gene gives rise to a total of 6 common isoforms (TAp63 α , β and γ / Δ Np63 α β and γ) (*Candi, Rufini, et al., 2006*). Of these isoforms lots of work has been done previously to uncover the function of the Δ Np63 forms as these are most abundant during the differentiation pathways within keratinocytes. In addition, relative expression of Δ Np63 forms is higher than that of TAp63. Despite this, recent work has suggested that TAp63 may actually be over-expressed in the later days of differentiation, during the formation of the cornified layer (*Koh et al., 2015*).

Very little research has distinctly focused on the TAp63 isoforms and their transcriptional target. Despite this, recent work has suggested that TAp63 may actually be over expressed in the later days of differentiation, during the formation of the cornified layer. This means there is gap in the knowledge about this group which may have crucial roles in orchestrating the final steps during keratinocyte differentiation. Moreover, there is still conflict within the community as to whether TAp63 forms and Δ Np63 work cooperatively or in an antagonistic fashion during the differentiation pathway.

Given the limited knowledge on this group of isoforms, it is possible that they may have implications in disease associated with the skin cornification process or terminal differentiation. Loss of function, or limited function of these transcription factors may cause the cascade of events which lead to terminal differentiation not occurring or being less efficient.

In recent literature, some transcriptional targets for the TAp63 group of isoforms have begun to be uncovered through a variety of techniques such as microarrays and over expression of the isoforms *in vitro* (Koh *et al.*, 2015; Truong *et al.*, 2006). Despite this, many targets are still not known. These identified targets can be used as markers for assessment of the knockdown of TAp63. If when TAp63 is knocked down the marker is not expressed, or is expressed less, then TAp63 may be the transcriptional controller associated with that marker.

Aims: This project aims to continue work that has been started by the group into the key roles of the transcription factor p63 and most notably the function and transcriptional targets of the TAp63 group of isoforms. This work will look into the temporal expression patterns of the p63 isoforms during the process of keratinocyte differentiation whilst comparing to morphological changes which occur during this process. In addition, histological staining of differentiation markers will allow spatial expression to be analysed. This work will include work with primary keratinocytes obtained from patient samples, whilst also using control lines such as immortalised HaCaT cells as comparison.

Furthermore, this work will look to determine the transcriptional targets of the TAp63 group of isoforms that are crucial during keratinocyte differentiation. This work may be achieved via use of siRNA to knockdown the TAp63 gene. Alongside monitoring the effects on potential TAp63 targets during knockdown, this can be compared to the morphology of the cells to assess if the cornified layer actually forms when TAp63 is knocked down.

Methodology: Initial work with this project will involve a cell culture-based approach to culture the cell lines. The cell lines to be used will include immortalised keratinocytes (HaCaT) and primary keratinocytes from patient samples. The cells are to be maintained in a basal phenotype and then encouraged to begin the process of differentiation by addition of calcium to the media. Cells will be harvested on pre-defined time points to allow RNA to be extracted. Photographs will be taken to assess morphological changes. This RNA will be reverse transcribed into cDNA which will then be analysed via qRT-PCR using TaqMan assays to determine relative expression.

Beyond this, depending on results obtained, knockdown of the TAp63 gene via siRNA will be carried out. Markers which are transcriptional targets for the TAp63 isoforms will be used to assess the effect of this knockdown during the process of differentiation.

Also, histological work on embedded or frozen sections will be carried out to allow the marrying of spatial expression to temporal expression to provide a clear overview of the expression and hence the role of the TAp63 isoforms.

7.4 COSHH

7.4.1 Cell counting using a haemocytometer

Assessment Carried Out by:							REF.NO,	
Purpose of Assessment	Teaching <input type="checkbox"/>	Projects <input checked="" type="checkbox"/>	Research Activity <input type="checkbox"/>	Technical Prep <input type="checkbox"/>				
Module Name (where applicable)	Research Project SHB4001						Module No.	
Supervisor Name (where applicable)	Dr Hans Hennies						Extension No.	
Activity Assessed:	Counting cells							
Those at risk from this activity:	Academic Staff <input type="checkbox"/>	Technicians <input type="checkbox"/>	Researchers <input type="checkbox"/>	Students <input checked="" type="checkbox"/>	Visitors <input type="checkbox"/>			

Substance, mixture, organism etc	Signal Word	Hazard Code and Phrases	Route of Entry	Risk 1
Nitrotetrazolium Blue chloride	Warning	H302: Harmful if swallowed H371: May cause damage to organs	Ingestion Inhalation	
Factors affecting risk e.g state, quantity, handling etc	Precautionary Phrases		Controls	Risk 2
Acute Toxicity, Oral (Category 4), H302, Specific target organ toxicity- single exposure	P260: Do not breathe dust/fume/gas/mist/vapours/spray P308: IF exposed or concerned: P311: Call a POISON CENTER or doctor/physician		Surveillance	

7.4.2 General cell culture

Assessment Carried Out by:							REF.NO,	
Purpose of Assessment	Teaching <input type="checkbox"/>	Projects <input checked="" type="checkbox"/>	Research Activity <input type="checkbox"/>	Technical Prep <input type="checkbox"/>				
Module Name (where applicable)	Research Project SHB4001						Module No.	
Supervisor Name (where applicable)	Dr Hans Hennies						Extension No.	
Activity Assessed:	Cell Culture with HaCaT/ keratinocyte cell line							
Those at risk from this activity:	Academic Staff <input type="checkbox"/>	Technicians <input type="checkbox"/>	Researchers <input type="checkbox"/>	Students <input checked="" type="checkbox"/>	Visitors <input type="checkbox"/>			

Substance, mixture, organism etc	Signal Word	Hazard Code and Phrases	Route of Entry	Risk 1
DMEM Media (no Gln, no Ca2+)	Warning	H303: May be harmful if swallowed H313: May be harmful in contact with skin H333: May be harmful if inhaled	Ingestion Skin Contact Inhalation	Low
Factors affecting risk e.g state, quantity, handling etc	Precautionary Phrases		Controls	Risk 2

	<p>P302+352: IF ON SKIN: Wash with soap and water</p> <p>P305+351+338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing</p> <p>P304+341: IF INHALED: If breathing is difficult, remove victim to fresh air and keep at rest in a position comfortable for breathing</p>	<p>Lab Coat, Spectacles and Gloves</p> <p>Spectacles</p>	<p>Low</p>
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Substance, mixture, organism etc	Signal Word	Hazard Code and Phrases	Route of Entry	Risk 1
Foetal Bovine Serum	Warning	<p>H303: May be harmful if swallowed</p> <p>H333: May be harmful if inhaled</p> <p>H313: May be harmful in contact with skin</p>	<p>Ingestion</p> <p>Inhalation</p> <p>Skin Contact</p>	Low
Factors affecting risk e.g state, quantity, handling etc	Precautionary Phrases		Controls	Risk 2
The product does not contain any substances at their given concentrations are harmful to health	<p>P302+352: IF ON SKIN: Wash with soap and water</p> <p>P304+341: IF INHALED: If breathing is difficult, remove victim to fresh air and keep at rest in a position comfortable for breathing</p> <p>P305+351+338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing</p>		<p>Lab Coat, Spectacles and Gloves</p> <p>Spectacles</p>	

Substance, mixture, organism etc	Signal Word	Hazard Code and Phrases	Route of Entry	Risk 1
Chelex 100 sodium form	Warning	<p>H303: May be harmful if swallowed</p> <p>H313: May be harmful in contact with skin</p> <p>H333: May be harmful if inhaled</p>	<p>Ingestion</p> <p>Skin Contact</p> <p>Inhalation</p>	Low
Factors affecting risk e.g state, quantity, handling etc	Precautionary Phrases		Controls	Risk 2
Not a hazardous substance or mixture according to regulation (EC) No. 1272/2008. Not classified as dangerous according to directive 67/548/EEC.	<p>P302+352: IF ON SKIN: Wash with soap and water</p> <p>P304+341: IF INHALED: If breathing is difficult, remove victim to fresh air and keep at rest in a position comfortable for breathing</p> <p>P305+351+338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing</p>		<p>Lab Coat, Spectacles and Gloves</p> <p>Monitoring</p> <p>Lab Coat and Spectacles</p>	

SIZES and VOLUMES

TYPE of plate/flask	SIZE of plate/flask	Volume [MEDIUM]	Volume [TRYPsin]	Volume [WASH / PBS]
6 well plate	*10 cm ²	*2 ml	*1 ml	*2 ml
12 well plate	*3.8 cm ²	*1.5 ml	*0.5 ml	*1 ml
24 well plate	*2.0 cm ²	*0.5 ml	*0.1 ml	*0.5 ml
T25	25 cm ²	7-10 ml	3 ml	4 ml
T75	75 cm ²	18-25 ml	4-5 ml	5 ml
T225	225 cm ²	45-50 ml	10 ml	15-25 ml
p6	28 cm ²	3-4 ml	2 ml	2 ml
p10	58 cm ²	10-15 ml	5-7 ml	5-10 ml

* sizes and volumes for ONE well [not the whole plate]

7.6 RNA Extraction Protocol- Applied Biosystems

QUICK REFERENCE

PureLink® RNA Mini Kit

Cat. nos. 12183018A, 12183025

Rev: 21 May 2012

Pub. Part no. 100003505 Pub. No. MAN000272

Directions for purifying RNA from animal and plant cells using the PureLink® RNA Mini Kit are described below.

For detailed instructions, and protocols for isolating RNA from tissue, blood, bacteria, yeast liquid samples, and on-column DNase treatment, download the manual from www.lifetechnologies.com or by contacting Technical Support.

General Guidelines

- Follow proper aseptic RNA handling techniques to prevent RNase contamination of reagents and RNA samples.
- Keep freshly harvested samples on ice and quickly proceed to Lysis and Homogenization, or freeze samples immediately after collection in liquid nitrogen or on dry ice and keep at -80°C for later use.
- Do not exceed the RNA binding capacity of the spin cartridge by adding samples containing more than 1 mg of total RNA.



- Both Lysis Buffer and Wash Buffer I contain guanidine isothiocyanate. Do not add bleach or acidic solutions directly to solutions or sample preparation waste containing guanidinium isothiocyanate, a reactive compound and toxic gases are generated.
- Solutions containing ethanol are considered flammable. Use appropriate precautions when using this chemical.

Required Materials

- 96–100% ethanol
- 2-mercaptoethanol
- 70% ethanol (in RNase-Free Water)
- 1.5 mL RNase-free microcentrifuge tubes
- Homogenizer, RNase-free syringe (1 mL) with 18–21-gauge needle or Rotor-stator homogenizer
- Microcentrifuge capable of centrifuging $12,000 \times g$
- PBS (for samples with $>10^7$ cells)
- 15 mL RNase-free tubes (for samples with $>10^7$ cells), RNase-free pipet tips

Buffer Preparation

- When using Wash Buffer II for the first time, add 60 mL 96–100% ethanol (Cat. no. 12183018A) or 300 mL 96–100% ethanol (Cat. no. 12183025). Mark the label to indicate that ethanol is already added.
- Prepare fresh Lysis Buffer containing 1% 2-mercaptoethanol. Add 10 μL 2-mercaptoethanol for every 1 mL Lysis Buffer (see page 2).

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Lysis and Homogenization

Page 2

Required Volume of Lysis Buffer

Cell Number	Lysis Buffer Required for Each Sample
$\leq 1 \times 10^6$	0.3 mL (0.6 mL if using a rotor-stator for lysis/homogenization)
1×10^6 – 5×10^6	0.6 mL
5×10^6 – 5×10^7	0.6 mL per 5×10^6 cells (e.g., use 1.2 mL for 1×10^7 cells)

$\leq 5 \times 10^6$ Suspension Cells

- Transfer the cells to an RNase-free tube and centrifuge at $2,000 \times g$ for 5 min at 4°C to pellet. Discard the growth medium.
- Add 0.3 or 0.6 mL Lysis Buffer with 2-mercaptoethanol to the sample (see table above for volume).
- Vortex until the cell pellet is dispersed and the cells appear lysed.
- Proceed to **Homogenization** below.

$\leq 5 \times 10^6$ Monolayer Cells

- Remove the growth medium from the cells, then add 0.3 or 0.6 mL Lysis Buffer with 2-mercaptoethanol (see table above for volume).
- Vortex until the cell pellet is dispersed and the cells appear lysed.
- Proceed to **Homogenization** below.

5×10^6 – 5×10^7 Suspension Cells

- Transfer cells to a 15-mL tube and centrifuge at $2,000 \times g$ for 5 min at 4°C . Discard the supernatant.
- Add 0.6 mL Lysis Buffer with 2-mercaptoethanol (see table above for volume).
- Vortex until the cell pellet is dispersed and the cells appear lysed.
- Homogenize at room temperature with a rotor-stator homogenizer (see **Homogenization** below).

Frozen Cell Pellets

- Transfer cells to a 15-mL tube and add 0.6 mL Lysis Buffer with 2-mercaptoethanol (see table above for volume).
- Vortex until the cell pellet is dispersed and the cells appear lysed.
- Homogenize at room temperature with a rotor-stator homogenizer (see **Homogenization** below).

Homogenization

- Proceed with **one** of the following homogenization options at room temperature:
 - Transfer the lysate into a clean homogenization tube, and perform manual homogenization. Centrifuge the homogenate at $12,000 \times g$ for 2 minutes.
 - Pass the lysate 5–10 times through an 18- to 21-gauge syringe needle.
 - Transfer the lysate into a clean tube, and homogenize using a rotor-stator homogenizer at maximum speed for ≥ 45 s. Centrifuge the homogenate at $26,000 \times g$ for 5 minutes, then transfer the supernatant to a clean RNase-free tube.
- Proceed to **RNA Purification**, next page.

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RNA Purification

Binding, Washing, and Elution of RNA

1. Add one volume 70% ethanol to each volume of cell homogenate.
2. Vortex to mix thoroughly and to disperse any visible precipitate that may form after adding ethanol.
3. Transfer up to 700 μL of the sample (including any remaining precipitate) to the spin cartridge (with the collection tube).
4. Centrifuge at $12,000 \times g$ for 15 seconds at room temperature. Discard the flow-through, and reinsert the spin cartridge into the same collection tube.
5. Repeat Steps 3–4 until the entire sample has been processed.
6. Add 700 μL Wash Buffer I to the spin cartridge.
7. Centrifuge at $12,000 \times g$ for 15 seconds at room temperature. Discard the flow-through and the collection tube. Place the spin cartridge into a new collection tube.
8. Add 500 μL Wash Buffer II with ethanol to the spin cartridge.
9. Centrifuge at $12,000 \times g$ for 15 seconds at room temperature. Discard the flow-through.
10. Repeat Steps 8–9 once.
11. Centrifuge the spin cartridge at $12,000 \times g$ for 1–2 minutes to dry the membrane with bound RNA. Discard the collection tube and insert the spin cartridge into a recovery tube.
12. Add 30–100 μL RNase-free water to the center of the spin cartridge.
13. Incubate at room temperature for 1 minute.
14. Centrifuge the spin cartridge for 2 minutes at $\geq 12,000 \times g$ at room temperature to elute the RNA from the membrane into the recovery tube. **Note:** If the expected RNA yield is $>100 \mu\text{g}$, perform 3 sequential elutions of 100 μL each. Collect the eluates in a single tube.
15. Store your purified RNA or proceed to downstream application.

RNA Storage

Store the purified RNA on ice for immediate use. For long-term storage, keep the purified RNA at -80°C . Perform DNase I treatment after purification (refer to the PureLink® RNA Mini Kit manual) to assure highly pure RNA without genomic DNA contamination. Determine the quality and quantity of your RNA by UV absorbance at 260 nm.



Troubleshooting

Page 4

Observation	Cause	Solution
Low RNA yield	Incomplete lysis and homogenization	Use the appropriate method for lysate preparation based on amount of starting material (see page 2). Decrease the amount of starting material used. Cut tissue samples into smaller pieces and ensure the tissue is completely immersed in the Lysis Buffer to achieve optimal lysis.
	Poor quality of starting material	The yield and quality of RNA isolated depends on the type and age of the starting material. Use fresh sample and process immediately after collection or freeze the sample at -80°C or in liquid nitrogen immediately after harvesting.
	Clogged RNA Spin Cartridge	Clear the homogenate and remove particulate or viscous material by centrifugation. Use only the supernatant for subsequent loading onto the spin cartridge.
	Ethanol not added to Wash Buffer II	Add ethanol to Wash Buffer II before use (see page 1).
RNA degraded	Incorrect elution conditions	Add RNase-Free Water (30–100 μL) and incubate for 1 min before centrifugation. To recover more RNA, be sure to use up to 3 sequential elutions of 100 μL each (3 \times 100 μL) Elution Buffer (refer to protocol on page 3).
	RNA contaminated with RNase	Use proper aseptic RNA handling techniques. Use RNase-free plasticware, and wear disposable gloves. Remove RNase contamination from work surfaces and non-disposable items with RNase AWAY® Reagent (Cat. no. 10328-011).
Inhibition of downstream enzymatic reactions	Presence of ethanol in purified RNA	Traces of ethanol from Wash Buffer II can inhibit downstream enzymatic reactions. Discard Wash Buffer II flow through. Place the spin cartridge into the recovery tube and centrifuge at $12,000 \times g$ for 1–2 min to completely dry the cartridge.
	Presence of salt in purified RNA	Use Wash Buffers in the correct order. Always wash with Wash Buffer I followed by Wash Buffer II.
Low A_{260}/A_{280} ratio	Sample was diluted in water	Use 10 mM Tris-HCl (pH 7.5) to dilute sample for OD measurements.

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www.lifetechnologies.com



Appendix

On-column PureLink® DNase Treatment Protocol

Introduction

This section provides a protocol for removing DNA from your sample using PureLink® DNase (page 67), while the RNA is bound on the Spin Cartridge. This protocol includes the PureLink® DNase treatment, followed by steps to complete the washing and elution of your RNA (refer to your sample-specific protocol for the appropriate step at which to perform this on-column DNase treatment).

Note: Alternatively, you may perform a DNase I (page 67) digestion of the RNA sample *after* purification (page 65). However, this may result in reduced RNA yield.

PureLink® DNase

PureLink® DNase is optimized for use with the PureLink® RNA Mini Kit. It is designed to be used specifically for on-column digestion of DNA during critical RNA purification procedures for downstream application procedures that require DNA-free total RNA.

Resuspending PureLink® DNase

Resuspend the PureLink® DNase by dissolving the lyophilized DNase in 550 µL RNase-Free Water (supplied with PureLink® DNase).

Store at 4°C for short-term storage. For long-term storage, prepare aliquots of the DNase and store at -20°C. Avoid repeat freezing and thawing. Thawed DNase stocks may be stored at 4°C for up to six weeks.

Preparing PureLink® DNase

Before beginning, prepare PureLink® DNase for on-column treatment, add the following components (supplied with PureLink® DNase) to a clean, RNase-free microcentrifuge tube. Prepare 80 µL per sample.

<u>Component</u>	<u>Volume</u>
10X DNase I Reaction Buffer	8 µL
Resuspended DNase (~3U/µL)	10 µL
<u>RNase Free Water</u>	<u>62 µL</u>
Final Volume	80 µL

Continued on next page

On-column PureLink® DNase Treatment Protocol, Continued

On-column PureLink® DNase Treatment and Purification

Use the After binding your RNA to the membrane of the Spin Cartridge, perform this on-column PureLink® DNase treatment to purify DNA-free total RNA (refer to your sample-specific protocol for the appropriate step). Continue this protocol to complete the washing and elution steps of your RNA.

Note: There is a wash step after binding and prior to the addition of PureLink™ DNase.

1. Add 350 µL Wash Buffer I to the Spin Cartridge containing the bound RNA (see sample-specific protocol). Centrifuge at 12,000 × g for 15 seconds at room temperature. **Discard** the flow-through **and** the Collection Tube. Insert the Spin Cartridge into a **new** Collection Tube.
2. Add 80 µL PureLink® DNase mixture (prepared as described on previous page) directly onto the surface of the Spin Cartridge membrane.
3. Incubate at room temperature for 15 minutes.
4. Add 350 µL Wash Buffer I to the Spin Cartridge. Centrifuge at 12,000 × g for 15 seconds at room temperature. **Discard** flow-through **and** the Collection Tube and insert the Spin Cartridge into a **new** Collection Tube.
5. Add 500 µL Wash Buffer II with ethanol (page 11) to the Spin Cartridge.
6. Centrifuge at 12,000 × g for 15 seconds at room temperature. **Discard** flow-through and reinsert the Spin Cartridge into the same Collection Tube.
7. Repeat Steps 5–6, **once**.

Continued on next page

On-column PureLink[®] DNase Treatment Protocol, Continued

On-column PureLink[®] DNase Treatment, continued

8. Centrifuge the Spin Cartridge at 12,000 × g for 1 minute to dry the membrane with bound RNA. **Discard** Collection Tube and insert the Spin Cartridge into a Recovery Tube.
 9. Add 30 µL–100 µL RNase-Free Water to the center of the Spin Cartridge.
 10. Incubate at room temperature for 1 minute.
 11. Centrifuge Spin Cartridge and Recovery Tube for 1 minute at ≥12,000 × g at room temperature.
 12. Store your purified RNA (see page 4), or proceed to **Analyzing RNA Yield and Quality** (page 53).
-

7.7 cDNA Synthesis Protocol: Applied Biosystems

Procedure																						
STEP	ACTION																					
1	Input Amount of Total RNA	Use up to 2 µg of total RNA per 20 µL reaction.																				
2	Preparing the RT reaction	<p>Prepare the RT reaction mix using the kit components before preparing the reaction plate.</p> <p>To prepare the RT reaction mix (per 20 µL reaction):</p> <ol style="list-style-type: none"> Allow the kit components to thaw on ice. Referring to the following table, calculate the volume of components needed to prepare the required number of reactions. <table border="1" data-bbox="727 590 1243 827"> <thead> <tr> <th rowspan="2">Component</th> <th colspan="2">Volume/Reaction (µL)</th> </tr> <tr> <th>+RT</th> <th>-RT</th> </tr> </thead> <tbody> <tr> <td>2× RT Buffer</td> <td>10.0</td> <td>10.0</td> </tr> <tr> <td>20× RT Enzyme Mix</td> <td>1.0</td> <td>—</td> </tr> <tr> <td>Nuclease-free H₂O</td> <td>Q.S.† to 20 µL</td> <td>Q.S. to 20 µL</td> </tr> <tr> <td>Sample</td> <td>up to 9 µL</td> <td>up to 9 µL</td> </tr> <tr> <td>Total per Reaction</td> <td>20.0</td> <td>20.0</td> </tr> </tbody> </table> <p>† Quantity Sufficient</p> <p>Note: Prepare the RT reaction on ice. Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.</p> <p> WARNING CHEMICAL HAZARD. 2× RT Buffer may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate eyewear, clothing, and gloves.</p>	Component	Volume/Reaction (µL)		+RT	-RT	2× RT Buffer	10.0	10.0	20× RT Enzyme Mix	1.0	—	Nuclease-free H ₂ O	Q.S.† to 20 µL	Q.S. to 20 µL	Sample	up to 9 µL	up to 9 µL	Total per Reaction	20.0	20.0
Component	Volume/Reaction (µL)																					
	+RT	-RT																				
2× RT Buffer	10.0	10.0																				
20× RT Enzyme Mix	1.0	—																				
Nuclease-free H ₂ O	Q.S.† to 20 µL	Q.S. to 20 µL																				
Sample	up to 9 µL	up to 9 µL																				
Total per Reaction	20.0	20.0																				
3	Preparing the cDNA Reverse Transcription Reactions	<p>To prepare the cDNA RT reactions:</p> <ol style="list-style-type: none"> Aliquot 20 µL of RT reaction mix into each well, or tube. seal the plates or tubes. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles. <p>Place the plate or tubes on ice until you are ready to load the thermal cycler or Applied Biosystems Real-Time PCR system.</p>																				

<p>4</p>	<p>Performing Reverse Transcription</p>	<p>To perform reverse transcription:</p> <p>a. Using one of the required thermal cyclers listed in "Materials and Equipment" in <i>High Capacity RNA-to-cDNA Kit Protocol</i> (part number 4387951), program the thermal cycler conditions:</p> <p>IMPORTANT! These conditions are optimized for use with the High Capacity RNA-to-cDNA Kit.</p> <table border="1" data-bbox="711 373 1263 478"> <thead> <tr> <th></th> <th>Step 1</th> <th>Step 2</th> <th>Step 3</th> </tr> </thead> <tbody> <tr> <td>Temperature (°C)</td> <td>37</td> <td>95</td> <td>4</td> </tr> <tr> <td>Time</td> <td>60 min</td> <td>5 min</td> <td>∞</td> </tr> </tbody> </table> <p>b. Set the reaction volume to 20 µL.</p> <p>c. Load the reactions into the thermal cycler or Applied Biosystems Real-Time PCR system.</p> <p>d. Start the reverse transcription run.</p>		Step 1	Step 2	Step 3	Temperature (°C)	37	95	4	Time	60 min	5 min	∞
	Step 1	Step 2	Step 3											
Temperature (°C)	37	95	4											
Time	60 min	5 min	∞											
<p>5</p>	<p>Storing cDNA Reverse Transcription Reactions</p>	<p>You can store cDNA RT plates or tubes prepared using the High Capacity RNA-to-cDNA Kit for short-term or long-term storage.</p> <table border="1" data-bbox="711 672 1274 777"> <thead> <tr> <th>Storage Duration</th> <th>Storage Temperature (°C)</th> </tr> </thead> <tbody> <tr> <td>Short-term (up to 24 hours before use)</td> <td>2 to 8</td> </tr> <tr> <td>Long-term</td> <td>-15 to -25</td> </tr> </tbody> </table> <p>If required, briefly centrifuge the archive plates or tubes before storing to spin down the contents and to eliminate any air bubbles.</p>	Storage Duration	Storage Temperature (°C)	Short-term (up to 24 hours before use)	2 to 8	Long-term	-15 to -25						
Storage Duration	Storage Temperature (°C)													
Short-term (up to 24 hours before use)	2 to 8													
Long-term	-15 to -25													

7.8 qRT-PCR Protocol: Modified from ThermoFisher Scientific

qRT-PCR was carried out for analysing different primers and housekeeping genes on keratinocyte samples. A reaction mix for the Master Mix plus the assay primer and probe mix was prepared. Furthermore, a sample mix for each cDNA was prepared as well.

Table 5. Quantities of Master Mix, primer, cDNA and distilled water for every plate.

Reaction	Component	1x reaction	25x reaction (for 24 wells on one plate)
Reaction Mix	Master Mix	5 µL	125 µL
	Assay primer and probe mix	0.5 µL	12.5 µL
Sample Mix	cDNA diluted	1 µL	10 µL
	Distilled water	3.5 µL	35 µL

A fresh PCR fast plate was put on ice and 5.5 µL of the reaction mix were pipetted into the correct well until the plate was full. When it was ready 4.5 µL of sample mix were added to each row. Then the plate was covered with an adhesive film and centrifuged. The plate was put into the StepOnePlus™ Real-Time PCR System and the template was adjusted to the correct samples to be run and to analyse the results.

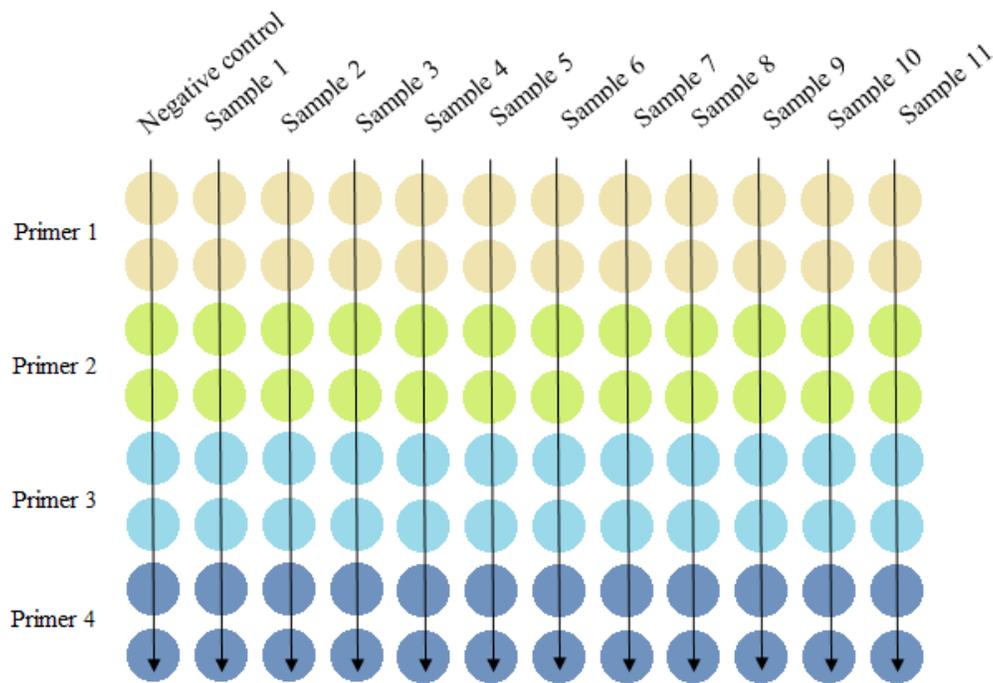


Figure 7. Drawing of the plate with the samples and primers.

7.9 siRNA Transfection Protocol: ThermoFisher Scientific

Protocol - Stealth RNAi or siRNA Transfection

Use this brief procedure to transfect **Stealth RNAi** or **siRNA** into mammalian cells in a **24-well format**. All amounts and volumes are given on a per well basis. Use this procedure as a starting point; optimize transfections as described in **Optimizing Stealth RNAi or siRNA Transfection**, especially if you are transfecting a mammalian cell line for the first time.

1. One day before transfection, plate cells in 500 μ l of growth medium without antibiotics such that they will be **30-50% confluent** at the time of transfection. **Note:** Transfecting cells at a lower density allows a longer interval between transfection and assay time, and minimizes the loss of cell viability due to cell overgrowth.
2. For each transfection sample, prepare oligomer-Lipofectamine 2000 complexes as follows:
 - A. Dilute 20 pmol Stealth RNAi or siRNA oligomer in 50 μ l Gibco™ Opti-MEM™ I Reduced Serum Medium without serum (resulting concentration of RNA in Opti-MEM is 400 nM). Mix gently
 - B. Mix Lipofectamine 2000 gently before use, then dilute 1 μ l in 50 μ l OptiMEM I Reduced Serum Medium. Mix gently and incubate for 5 minutes at room temperature. **Note:** Proceed to the next step within 25 minutes.
 - C. After the 5-minute incubation, combine the diluted oligomer with the diluted Lipofectamine 2000. Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy).
3. Add the oligomer-Lipofectamine 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.

Incubate the cells at 37°C in a CO2 incubator for 24-96 hours until you are ready to assay for gene knockdown. Medium may be changed after 4-6 hours.

Optimizing Stealth RNAi or siRNA Transfection

To obtain the highest transfection efficiency and low non-specific effects, optimize transfection conditions by varying RNA and Lipofectamine 2000 concentrations. Test 10-10pmol/ μ l RNA and 0.5-1.5 μ l Lipofectamine 2000 for 24-well format. Depending on the nature of the target gene, transfecting cells at higher densities may also be considered when optimizing conditions.

Scaling Up or Down Transfections

To transfect cells in different tissue culture formats, vary the amounts of Lipofectamine 2000, nucleic acid, cells, and medium used in proportion to the relative surface area, as shown in the table. With automated, high-throughput systems, a complexing volume of 50 μ l is recommended for transfections in 96-well plates.

Note: You may perform rapid 96-well plate transfections by plating cells directly into the transfection mix. Prepare complexes in the plate and directly add cells at twice the cell density as in the basic protocol in a 100 μ l volume. Cells will adhere as usual in the presence of complexes.

Culture vessel	Surf. area per well ¹	Shared reagents		DNA transfection		RNAi transfection	
		Vol. of plating medium	Vol. of dilution medium ²	DNA	Lipofectamine 2000	RNA	Lipofectamine 2000
96-well	0.3 cm ²	100 µL	2 x 25 µL	0.2 µg	0.5 µL	5 pmol	0.25 µL
24-well	2 cm ²	500 µL	2 x 50 µL	0.8 µg	2.0 µL	20 pmol	1.0 µL
12-well	4 cm ²	1 mL	2 x 100 µL	1.6 µg	4.0 µL	40 pmol	2.0 µl
6-well	10 cm ²	2 mL	2 x 250 µL	4.0 µg	10 µL	20pmol/µl	5 µL
60-mm	20 cm ²	5 mL	2 x 0.5 mL	8.0 µg	20 µl	200 pmol	10 µL
10-cm	60 cm ²	15 mL	2 x 1.5 mL	24 µg	60 µL	600 pmol	30 µL

7.10 Abstract for previously published work

The role of p63 isoforms in the epidermal development as replicated in cellular models for normal human skin and genetic skin diseases

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⁶ CECAD, University of Cologne, Cologne, Germany

Transcription factor p63 is a key regulator of epidermal development. TP63 codes for two major isoforms, TAp63 and Δ Np63. Mutations in TP63 are associated with severe diseases such as ectrodactyly/ectodermal dysplasia and cleft lip/palate (EEC) and ankyloblepharon/ectodermal defects and cleft lip/palate (AEC) syndromes. These diseases and knockout and transgenic mice pointed to a major role of p63 in ectodermal development. Details about the importance of the isoforms are not clear so far; TAp63 is present early in ectodermal development, Δ Np63 is involved in epidermal maturation. In order to study the importance of p63 for epidermal differentiation, we have analysed the expression of TP63 and its major isoforms in keratinocytes in vitro. Quantitative real time PCR revealed a minor increase early in Ca²⁺ induced differentiation of primary human normal epidermal keratinocytes and a decrease during terminal differentiation. Isoform-specific analysis showed that Δ Np63, the predominant isoform in epidermis, was present during differentiation with decreasing levels. TAp63, in contrast, showed an increase late in differentiation after 10d in culture. These results are in accordance with an association of Δ Np63 with basal epidermal keratins 5 and 14 and a declining expression in suprabasal epidermal layers as shown previously by immunohistochemistry. KLF, a transcription factor gene important for gene expression in terminally differentiating keratinocytes, showed an increase in parallel with the increase of differentiation markers such as transglutaminase 1 and filaggrin, which is in line with the supposed induction of KLF by p63. Keratinocytes of patients with autosomal recessive congenital ichthyosis (ARCI), a disorder of epidermal keratinisation, showed a similar pattern for the expression of TP63, indicating that the disturbed keratinisation seen in ARCI is restricted to proteins of terminally differentiating keratinocytes but does not influence their transcription factors. Our findings underpin the importance of switching p63 isoforms during epidermal development and confirm an additional role for TAp63 in terminal differentiation. Moreover, these results corroborate the importance of cellular models based on primary keratinocytes for the study of epidermal keratinisation. Studies with 3D human skin models will give further insight into the regulation of gene expression profiles during epidermal differentiation.