The Role of FOXK1 in Keratinocyte Differentiation

By

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Abstract

Cell differentiation is the process by which cells become more specialised to perform a specific function. Many intrinsic and extrinsic factors, such as signal transduction events and the surrounding environment, interplay to regulate this process. Previous work in the lab identified that loss of Rnd3 (a member of the Rho family of small GTPases) expression blocked epidermal keratinocyte differentiation suggesting that Rnd3 plays an important role in skin biology. Proteomic data identified the FOXK1 transcription factor, a member of the forkhead box transcription factors family, as one of several proteins with significantly altered expression in Rnd3 knockdown cells. This project aimed to identify whether FOXK1 is important for keratinocyte differentiation. Culturing keratinocytes in low Ca^{2+} media suppresses differentiation and switching them to high Ca^{2+} medium induces differentiation. Under these conditions, FOXK1 expression was increased during differentiation, however, knocking out FOXK1 did not prevent differentiation suggesting that whilst FOXK1 may be required for the expression of some differentiation-specific genes, it is not necessary for the initiation of differentiation. Furthermore, EdU-stained FOXK1 knockout cells were found to be 50% less proliferative than wild type cells. Next-generation RNA-sequencing (RNA-Seq) revealed that FOXK1 knockout cells had upregulated differentiation genes and downregulated cell proliferation and division genes, suggesting that FOXK1 is required for the proper maintenance of cellular proliferation and differentiation.

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This thesis was copy edited for conventions of language, spelling and grammar by PSUK Communications Ltd.

DECLARATIONS

The work carried out in this thesis is my own with exception of some of the data in chapters 4 and 5. CRISPR-Cas9 knockout genotyping screening was performed by the Sources Biosciences. RNA-Seq was performed by the University of Birmingham sequencing facilities. The initial analysis of the RNA-Seq data was done by Dr Aditi Kanhere. The ChIP quality check mentioned in Chapter 6 was performed by the University of Liverpool Centre for Genomic Research.

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ABBREVIATIONS

ATP- Adenosine Triphosphate

BrdU- Bromo-deoxyuridine

CDK- Cyclin-dependent kinase

CKI- Cyclins and Cyclins kinases inhibitor

CDC45- Cell division cycle 45

CRISPR- Clustered regularly interspaced short palindromic repeats

Cas9- associated protein 9

C2C12- Myoblast cells

ChIP- Chromatin immunoprecipitation

DVL- Dishevelled

DAPI- 4',6-Diamidino-2-Phenylindole

DBD- DNA binding domain

DAVID- The Annotation, Visualization and Integrated Discovery database

ECM- Extracellular matrix

EGF- Epidermal growth factor

EdU- 5-ethynyl-2′-deoxyuridine

FGF- Fibroblast growth factor

FOX- The forkhead box

FHA- Forkhead-associated domain

GAP- GTPase activating protein

GDI- Guanine nucleotide disassociation inhibitors

GDP-Guanosine Diphosphate

GEF- Guanine exchange factor

GPCRS- G protein coupled receptors

GTP- Gunosine 5’ triphosphate

G6pc- Glucose-6-phosphatase

HaCaT- Cultured Human Keratinocyte

H1ESC- H1 human embryonic stem cell line

Hela cells- Human cervical cancer cells

HDAC- Histone deacetylase

HDR- Homology-directed repair

ILF- Interleukin binding factor

IVL- Involucrin

IP- Immunoprecipitation

IRS- Insulin response sequences

JNK- Jun N-terminal Kinase

KHGs- Keratohyalin granules

KSC- Keratinocyte stem cells

K1- Keratin 1

K4- Keratin 4

K5- Keratin 5

K10- Keratin 10

KRT19- Cytokeratin 19

MAPK- Mitogen-activated protein kinase

MNF- Myogenic nuclear factor

mTOR- Mammalian target of rapamycin

mRNA- Messenger RNA

NSC- Non-silencing control

NGS- Next-generation sequencing

NHEJ- Non-homologous end joining

PI3K- Phosphatidylinositol 3-kinases

PCNA- Proliferating cell nuclear antigen

PHH3- Phospho-Histone 3

ROCK- Rho-associated coiled-coil containing kinase

RNA-Seq- RNA sequencing

REP- Repetitive extragenic palindromic

PDGF- Platelet-derived growth factor

PPGB- β-galactosidase

PP2A- Protein phosphatase 2A

SILAC- Stable isotope labelling using amino acids in cell culture

siRNA- Small interfering RNA

Shh- Sonic-Hedgehog

SY5- Involucrin

TF- Transmembrane tissue factor

TGF- Transforming growth factor

TP53- Tumor protein 53

TP63- Tumor protein 63

TGM1- Transglutaminase1

Chapter 1

1 Introduction

The skin is the largest organ in the body that serves many important functions, one of which is the protection of the internal organs and tissues against any external harm such as pathogens, toxins, and trauma (Liu et al., 2013; Kirby et al., 2015). The epidermis forms the outer layer of the skin and is comprised mainly of epithelial cells known as keratinocytes. Keratinocyte differentiation is crucial for epidermal stratification and forming the outermost barrier called the cornified envelope that provides the skin's protective function (Gutowska-Owsiak et al., 2020). Keratinocyte differentiation is strictly controlled by intrinsic and extrinsic factors. The extrinsic factors are the microenvironments that surround the cells, such as growth factors and cell-cell contact, whereas the intrinsic factors regulate intracellular signalling pathways that ultimately control transcription factors and, hence, gene transcription (Watt and Huck, 2013).

1.1 The dermis and the epidermis

The mammalian skin architecture consists of two major layers: the dermis, which is the lower layer and the epidermis, which is the upper layer, separated from each other by a specialised extracellular matrix known as the basal membrane (Figure 1.1). The interaction between these two layers is essential to its function and to regulate skin development and differentiation (Liu et al., 2013; Watt, 2015). The dermis is mainly composed of three major layers. The first layer is called the papillary layer, located directly under the basal membrane, and is rich in fibroblasts and a few thin collagen fibres. The second layer is the reticular layer, the thickest layer in the dermis and containing highly organised collagen fibres. The third layer is the hypodermis, containing white adipose cells (Rognoni et al., 2018). Other essential structures in the dermis

include the dermal papilla, the hair follicle, and the dermal sheath. The dermal papilla is an integral part of the hair cycle regulation that stimulates the epidermal cells to grow downward from the hair follicle. The dermal sheath is composed of connective tissue that wraps around the hair follicle and connects it with the arrector pili muscle, which causes the hair to erect during goosebumps when thermal regulation of the skin is needed (Figure 1.2) (Kretzschmar et al., 2014; Watt, 2015; Rognoni et al., 2018). The dermis also contains other types of cells, such as nerve cells, B cells, and macrophages and provides support and protection for the epidermis and the underlying tissue (Kretzschmar et al., 2014; Watt, 2015).

The epidermis is a heterogeneous tissue that consists of epithelial cells called keratinocytes and non-epithelial cells such as melanocytes that produce melanin which prevents UV-induced DNA damage to the skin. The epidermis also contains immune cells and Merkel cells that function as immune response cells and mechanosensors, respectively (Kashgari et al., 2018). Keratinocytes in the epidermis are found in multiple layers at differing stages of terminal differentiation. These layers are the basal layer, the spinous layer, the granular layer, and the stratum corneum, which is the outermost layer of the skin (Figure 1.1) (Fuchs, 2008). The keratinocyte stem cells reside in the basal layer and are attached to the underlying basal lamina via hemidesmosomes. Basal stem cells divide to give daughter cells and some of these cells remain in this basal layer as stem cells while others cease proliferating and migrate upwards through the epidermis. The migrating keratinocytes undergo terminal differentiation, passing through the spinous layer to the granular layers and finally to the cornified layer, where they form the outermost protective layer of the skin. Cells at each layer exhibit certain morphological and biochemical characteristics based on their stemness and stage of differentiation (Figure 1.3) (Eckert and Rorke, 1989; Eckhart et al., 2013).

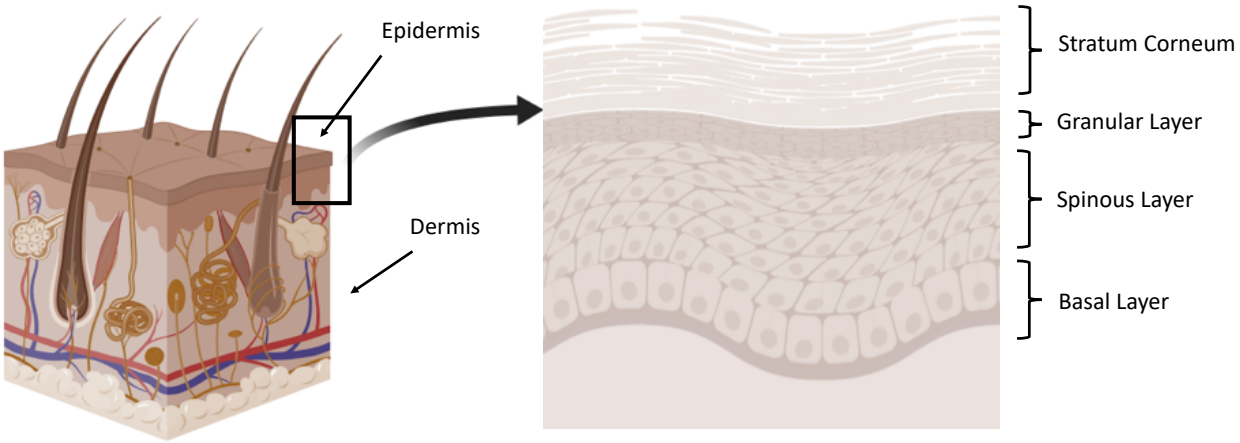


Figure 1.1 The mammalian skin

The skin protects the underneath tissues and organs form any external harm. It consists of two major layers, the dermis and the epidermis which is divided into four major layers according to the degree of cellular differentiation. (Adapted from Fuchs, 2008).

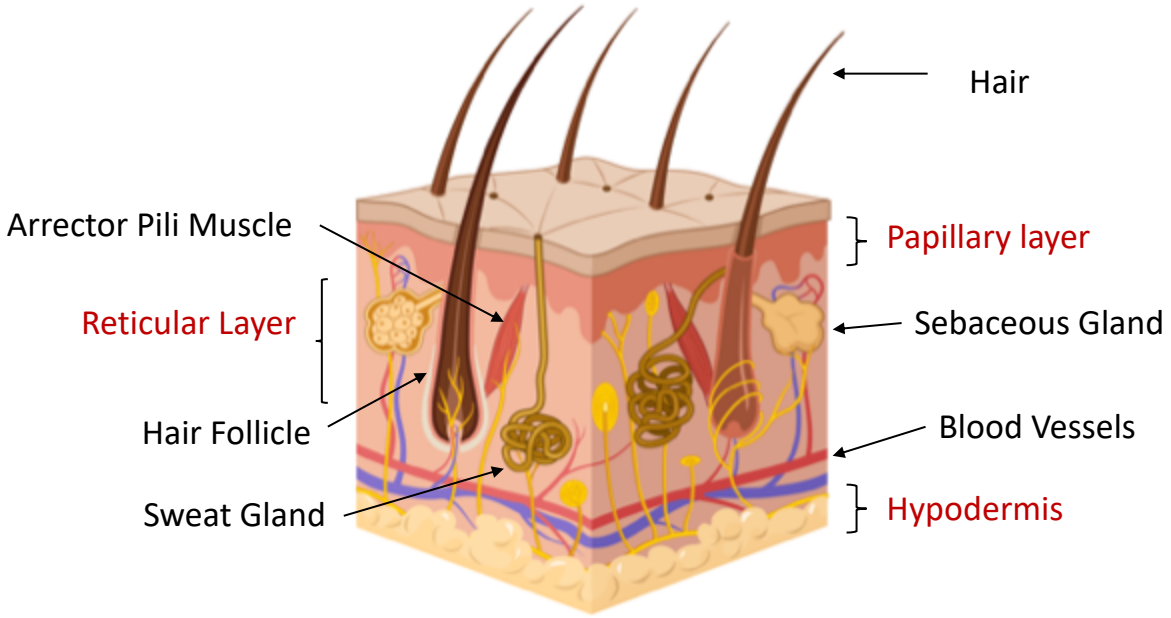


Figure 1.2 The dermis

The dermis is the lower part of the skin and consists of three major layers which are the papillary layer, the reticular layer and the hypodermis. (Adapted from Watt, 2015).

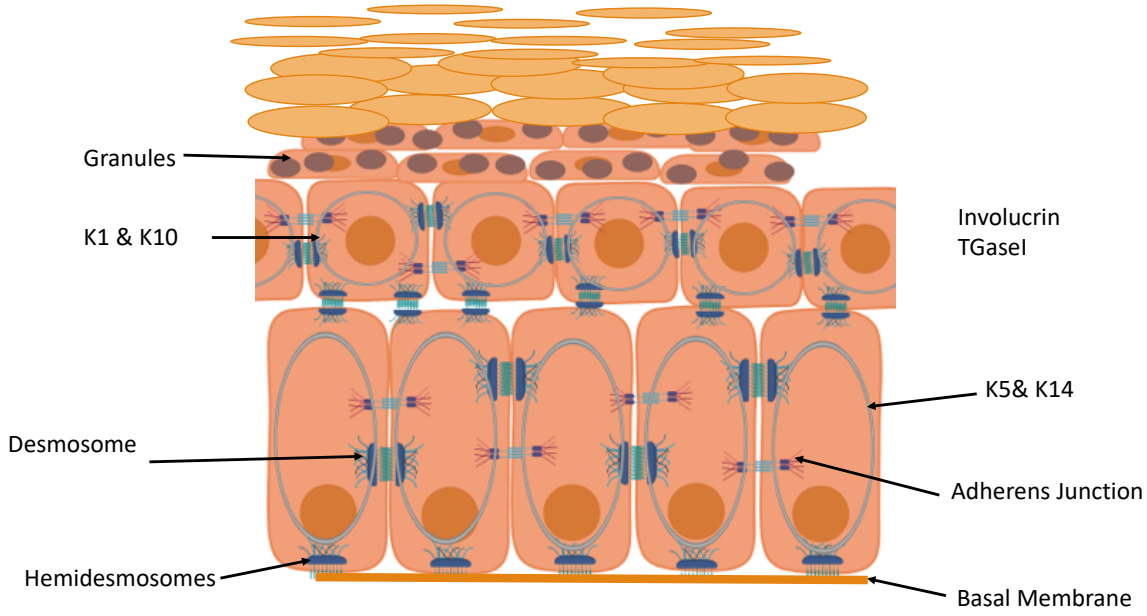


Figure 1.3 Markers of keratinocyte differentiation

The epidermis consists of multiple layers of keratinocytes with cells at varying degrees of terminal differentiation. In each layer, keratinocytes express specific sets of proteins which can be markers for the stem state or markers for differentiation. Non-keratinocyte cells have been omitted for clarity. (Adapted from Eckhart et al., 2013).

1.2 Markers of Basal Stem Cells and Keratinocyte Differentiation

Several biochemical and morphological changes occur as keratinocytes undergo terminal differentiation.

1.2.1 Morphological markers of keratinocytes

The degree of keratinocyte differentiation can be assessed by observing biochemical and phenotypical changes, such as changes in cell shape, cell size, the appearance of keratohyalin granules (KHGs) and the loss of nucleus and cell organelles (Gutowska-Owsiak et al., 2020).

1.2.1.1 Cell size and shape

There is an association between keratinocyte size and shape and the stage of terminal differentiation. In the basal layer, non-dividing keratinocytes are small and cuboidal in shape becoming spherical during cell division, which is critical for spindle orientation, thereby an important determinant for the type of cellular division, that is, symmetrical or asymmetrical (Kunda and Baum, 2009). Cells in the granular layer have a tetrahexadecahedron (diamond) shape that is optimal for space-filling, mechanical strength, and barrier formation and function. Cells in the cornified layer are larger and flattened in shape and are organised into columns in a honeycomb fashion, which is ideal for forming the outermost protective barrier of the skin (Luxenburg and Zaidel-Bar 2019).

1.2.1.2 Keratohyalin granules and cornification

The shrunken nucleus and the appearance of keratohyalin granules are distinct characteristics of the granular layer in the epidermis. Keratohyalin granules consist of profiligrin, keratin, and loricrin, which contribute to the keratinization and cornification of the cornified envelop during

keratinocyte differentiation (Scott and Miller, 2010). As cells proceed further upward during differentiation, corneocytes, the cells that constitute the cornified envelope, are formed by a unique form of programmed cell death and held together tightly via corneodesmosomes. During cornification, filaggrin and keratin are condensed throughout the cytoplasm, involucrin and loricrin are cross-linked, and DNA, mitochondria, and other organelles are degraded (Eckhart, et al., 2013; Ipponjima et al., 2020).

1.2.2 Biomolecular markers of keratinocyte differentiation

In addition to the previously mentioned physiological phenotypic markers of epidermal differentiation, keratinocytes undergo changes in their biochemical signatures during terminal differentiation. Basal stem markers, early and late terminal differentiation markers of the epidermis can be assessed easily using simple laboratory methods such as western blotting and immunofluorescent staining for specific proteins (Gutowska-Owsiak et al., 2020).

1.2.2.1 Integrins

Basal keratinocytes in the basal layer express several proteins and transcription factors that maintain their proliferative state. Cells in the basal layer are in contact with the extracellular matrix (ECM) that is part of the basal lamina and they express cell adhesion proteins such as integrins and the cytoskeletal keratins K5 and K14 (Watt and Huck, 2013; Deyrieux and Wilson, 2007). Integrins have an important role in maintaining epidermal homeostasis and integrity (Watt, 2002). Integrins are heterodimeric transmembrane receptors that are composed of an alpha (α) and a beta (β) subunit. They are indirectly linked to the cytoskeleton – keratin intermediate filaments in the case of hemidesmosomes and actin in the case of focal adhesions (Danen, 2013; Alberts et al., 2017). The most abundant integrins in the epidermis is $\alpha1\beta2$,

α3β1, α6β4 (found exclusively in hemidesmosomes) and αvβ5. One of the most important classes of integrins in keratinocytes are the β1 integrins, which are important in embryogenic development (Stephens et al., 1995). β1 integrin knockout mice failed to develop normally, and embryos died in E5-6. They have a role in cell-ECM adhesion (Larjava et al., 1990), serving not only to attach cells to ECM but are also involved in the regulation of intracellular signalling pathways including those that regulate differentiation. This regulation is evident in keratinocytes grown in suspension which lose their ability to attach to ECM, leading them to exit the cell cycle and enter terminal differentiation (Adams et al., 1990; Watt, 2013). In addition, β1 integrins regulate other protein kinase pathways such as MAPK and PI3K and other proteins that are key players in keratinocyte function, including small GTPase proteins such as Rac and Rho (Lü et al., 2016). Integrins are also involved in keratinocyte motility and migration essential for wound healing, with α3β1 and α6β4 expressed in the leading edge of migrating cells and for distal stabilisation of migrating cells, respectively (Watt, 2002). Also, α6β4 promotes cell migration through PI3K, MAPK and Rho GTPase pathways (Mainiero et al., 1997; O'Connor et al., 2000; Mercurio et al., 2001; Watt, 2002).

To summarise, integrins have a crucial role in maintaining epidermal structure and function, specially β1 integrins which have important roles in regulating cell adhesion, motility and migration. Additionally, integrins play a vital role in many important cellular pathways for regulating keratinocyte development, proliferation, differentiation and wound healing and repair.

1.2.2.2 P63 transcription factor

One of the main transcriptional factors associated with epidermal development and regulation is p63, a member of the p53 family of transcription factors. The p63 gene is expressed in the ectoderm surface at the beginning of the stratification and continues to be expressed during embryonic development until it becomes restricted to the basal layer in the adult epidermis (Koster et al., 2004). P63 regulates the expression of K5/K14 from K8/K18 during embryogenesis and p63 knockout mice have dysfunctional epidermal development leading to a lack of skin stratification and death soon after birth due to dehydration (Koster et al., 2004; Romano et al., 2009; Sotiropoulou et al., 2012). P63 exists in two isoforms, TAp63 and ΔNp63, the latter of which lacks the N-terminal activation domain (Romano et al., 2009). Each isoform is further subdivided into α, β, and γ due to alternative splicing in their C-terminal region (Hau et al., 2011). P63 function in keratinocytes is still relatively unclear but different epidermal functions have been identified for p63 and its isoforms. Truong et al. (2006) reported that p63 is critical in regulating keratinocyte differentiation and p63 knockout in normal human keratinocytes resulted in altered expression of various differentiation genes such as keratin 1, keratin 10, loricrin, and transglutaminase1. In addition, integrins β1 and β4 have been identified as targets of p63 (Carroll et al., 2006). Despite this, the exact role of p63 in epidermal differentiation and stratification is unclear (Sotiropoulou et al., 2012). For example, it was found that TAp63 is the first isoform expressed during embryogenesis in E7.5 and induces the expression of K5/K14. At the same time, ΔNp63 is the main isoform expressed in E9.5 and predominantly expressed in the mature epidermis suggesting that ΔNp63 regulates and maintains the proliferative quality of the mature epidermal stem cells (Koster et al., 2004). ΔNp63α is involved in the regulation of epidermal senescence by inhibiting p53 and regulating

other genes involved in cellular senescence (Hau et al., 2011). Furthermore, p63 isoforms counteract each other. TAp63 controls the initiation of epithelial stratification during embryonic development and is induced during differentiation as it has been detected in the suprabasal layers (Nylander et al., 2002; Truong and Khavari, 2008). In contrast, ΔNp63 maintains the proliferative potential of the keratinocyte stem cells and its expression decreases during differentiation (Nylander et al., 2002; Truong and Khavari, 2008; Soares & Huiqing, 2018; Yi et al., 2020).

In conclusion, the exact roles of p63 in epidermal proliferation and differentiation have not been fully elucidated. Nonetheless, distinct roles have been identified for each p63 isoform, with ΔNp63 mainly involved in regulating basal stem cell proliferation, while TAp63 regulates epidermal embryonic development and differentiation. All in all, p63 is an important transcription factor for regulating epidermal function.

1.2.2.3 Keratins

Keratins are a subfamily of the intermediate filaments in the epithelial cells which are found in abundance in the epidermis. K5 and K14 are type II (basic) and type I (acidic) keratins, respectively, expressed in the active proliferative epidermal basal cells including stem cells (Alam et al., 2011). During embryonic development, basal stem cells switch from expressing K8/K18 to K5/K14 as the epidermis becomes more stratified (Romano et al., 2009; Liu et al., 2013). The p63 isoforms may be responsible for K5/K14 induction, where ΔNp63 is directly responsible for inducing K5/K14 expression in keratinocyte stem cells while TAp63 induces K14 expression indirectly through AP-2g (Romano et al., 2009; Liu et al., 2013). K5/K14 are involved in regulating intracellular pathways such as PI3K/Akt and Notch1. PI3K/Akt and

Notch1 pathways are activated in K14 knockdown cells and associated with induced expression of differentiation markers such as involucrin and K1. It has been suggested that K14 might regulate differentiation through these two pathways (Alam et al., 2011). β1 integrins, K5 and K14, are downregulated when keratinocytes begin to differentiate, with differentiated cells expressing a different set of keratins, K1 and K10 (Deyrieux and Wilson, 2007). K1(type II) and K10 (type I) are also members of the intermediate filaments in the epidermal cells (Syder et al., 1994) and are the most abundant keratins in terminally differentiated keratinocytes, thus considered early differentiation markers (Gutowska-Owsiak et al., 2020). K1/K10 knockout mice suffer from fragile skin and die after birth, indicating the important role of K1/K10 in maintaining skin integrity (Wallace et al., 2012). The importance of K1/K10 for maintaining healthy skin is illustrated by the identification of mutations in K1/K10 genes in individuals with epidermolysis hyperkeratosis, a disorder characterized by abnormal thickening of the skin (Syder et al., 1994). K1/K10 are important in cornified envelope formation because keratin intermediate filaments (IF) provide a platform for other differentiation protein assembly such as for involucrin and loricrin (Wallace et al., 2012; Moravcová et al., 2013). Involucrin is a protein expressed in late terminally differentiated keratinocytes and is cross-linked with loricrin by transglutaminase to form the cornified envelope (Banks-Schlegel and Green, 1981; Chou et al., 2004; Eckhart et al., 2013).

In conclusion, there are many types of keratins in the epidermis. However, the main keratins considered as epidermal basal and differentiation markers are K5/K14 and K1/K10. The main keratins expressed in basal keratinocytes are K5/K14, and their role is to regulate and maintain basal stem cell function. However, the primary types of keratins expressed in terminally differentiated keratinocytes are K1/K10. They are critical for cornified envelope formation by

providing the structures necessary for other differentiation proteins to assemble. Overall, keratins play a crucial role in skin barrier development, maintenance, and differentiation.

1.3 Overview of The Cell Cycle

The cell cycle is the process by which cells reproduce and involves a sequence of tightly regulated events whereby cells double their contents, including organelles and then divide into two daughter cells (Alberts, 2017). Cell proliferation is strictly regulated by intrinsic and extrinsic factors to ensure tissue homeostasis. The cell cycle comprises four main steps: gap G_1 phase, S phase where DNA replication takes place, gap G_2 and, finally, M phase, which is mitosis, the stage at which cells divide. Depending on the environment and tissue turnover, the cell cycle continues to G_1 or enters a quiescent state G_0 (Figure 1.4) (Liu et al., 2019; Malumbres, 2020). The progression of these stages is mainly regulated by enzyme-substrate phosphorylation/dephosphorylation of specific protein kinases called cyclin-dependent kinases (CDKs) with their cyclins and cyclins kinase inhibitors (CKIs) (Lim and Kaldis, 2013; Liu et al., 2019; Malumbres, 2020).

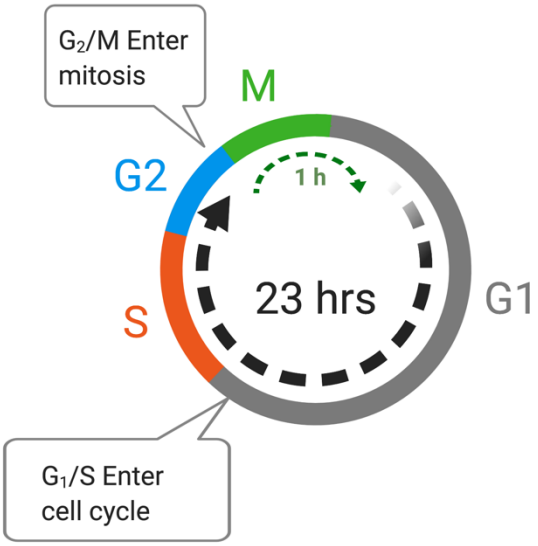


Figure 1.4 An Overview of the eukaryotic cell cycle

The cell cycle involves four main steps, two of which are major changes in the cell: the S phase where DNA is replicated and doubled, and the M phase where cells divide into two daughter cells. Gap phases G_1 and G_2 separate these phases to allow cell maintenance and growth. Cell cycle checkpoints, G_1/S and G_2/M are located in gap phases where their role is to ensure the correctness of process before proceeding to the next phase. G_1, S and G_2 together may take 23 hours to be completed, while the M phase takes 1 hour to be completed. (Adapted from Alberts, 2017).

1.3.1 CDKs, Cyclins and CKIs

Cyclin-dependent kinases are serine/threonine kinases that require activation through the binding with their cyclin substrates (Alberts, 2017; Malumbres, 2020). There are more than 20 members of the CDK family in mammalian cells, four of which have been identified as critical for cell cycle progression, CDK1, CDK2, CDK4 and CDK6. Other CDKs bind to other proteins and regulate multiple events in the cell cycle (Lim and Kaldis, 2013; Alberts, 2017; Malumbres, 2020). Four main cyclins bind to specific sets of CDKs depending on the cell cycle stage: cyclin E, A, B and D from G_1/S-cyclins, S-cyclins, M-cyclins and G_1-cyclin classes, respectively (Table 1.1) (Alberts, 2017). Cyclin-dependent kinase inhibitors (CKIs) function is to balance the cyclin activation effect on CDKs by inhibiting their activity. CKIs are divided into two families based on their properties: the INK4 family comprising $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ which inhibit the binding of CDK4 and CDK6 with their cyclins, and the Cip/Kip family of $p21^{Cip1}$ (another name p21Waf1), $p27^{Kip1}$, and $p57^{Kip2}$ which inhibit the cyclin-CDK complex rather than binding to the individual unbound CDKs (Alberts, 2017; Malumbres, 2020).

1.3.2 The cell cycle

The cell cycle is driven by micro-and macro-stimuli such as growth factors and begins in the G_1 phase, where cyclin D expression is upregulated, which then binds to and activates CDK4/CDK6. In the late G_1 phase, the expression of G_1/S cyclins (cyclin E) is also upregulated and leads to the binding and activation of their partner CDK1/CDK2, thereby inactivating specific transcription factors such as E2F and gene activation associated with promoting S phase entry and progression. During the S phase, DNA replication occurs to double the genetic material for cell segregation at the end of the cell cycle. In the G_2 phase, cells are prepared for

entry to mitosis (M phase) by cyclin E-CDK2 complex activation. Cyclin B translocates from the cytoplasm into the nucleus, binds to its partner CDK1 and promotes mitosis (chromatids division) and cytokinesis (a division of cell continent) into two identical daughter cells. The CKI families (INK and Cip/Kip) play their regulatory roles throughout the cell cycle (Figure 1.5) (Alberts, 2017; Liu et al., 2019).

Table 1.1 The cyclins with their CDK partners

(adapted from Alberts, 2017)

Cyclin-CDK complex	Cyclin	CDK
G_1-CDK	Cyclin D	CDK4, CDK6
G_1/S-CDK	Cyclin E	CDK2
S-CDK	Cyclin A	CDK1, CDK2
M-CDK	Cyclin B	CDK1

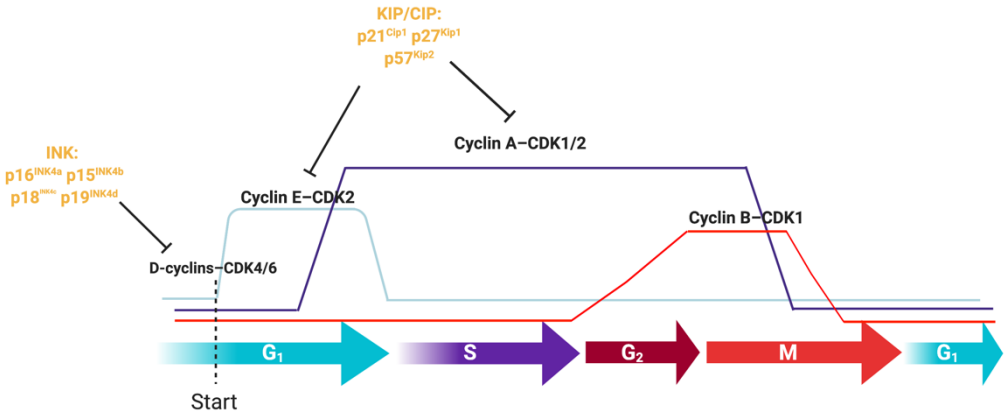


Figure 1.5 Summary of cell cycle regulation

(Alberts, 2017; Liu et al., 2019)

1.4 Symmetrical and Asymmetrical Division

Skin integrity is maintained through the tight regulation of the self-renewal ability of the epidermal basal cells and the process of terminal differentiation. Keratinocyte stem cells can divide symmetrically or asymmetrically, creating a heterogeneous pool of cells in the basal layer (Eckhart et al., 2013). Symmetrical division occurs when dividing cells give rise to two daughter cells, one of which remains attached to the basal membranes maintaining the proliferative cell pool, while the other migrates upward entering terminal differentiation, or divides to give two differentiated progenies. Asymmetrical division is when an epidermal progenitor cell divides to make one progenitor cell and one differentiated cell called committed or transit-amplifying cells (TACs), which can only divide a certain number of times before leaving the cell cycle and entering terminal differentiation (Figure 1.6) (Poulson and Lechler, 2012; Kashgari et al., 2018).

It is theorised that the cell cycle length might dictate the type of division in cells. Calegari and Huttner proposed "The Cell Cycle Length Hypothesis" in 2003, which suggests three possible outcomes of cell cycle length on cell division. First, the cell favours symmetric cell division when the cell cycle is very short. Second, when the cell cycle becomes longer, the fate determinants have enough time for one of the cells to favour differentiation which leads to asymmetric division. Third, both cells favour differentiation and divide symmetrically with an even longer cell cycle, as the fate determinates have sufficient time to induce differentiation in both daughter cells (Calegari and Huttner, 2003; Calegari et al., 2005; Barui and Datta, 2019).

The spindle orientation during cell division is one of the critical regulators for either type of division. The mitotic spindles are oriented to drive the division to be perpendicular or parallel with the basal membrane which determines tissue development and morphogenesis such as

tissue elongation, branching, and stratification (Figure 1.7) (Poulson and Lechler, 2012). What pushes cells to either type of division, however, is dependent on a majority of intrinsic and extrinsic stimuli and niches within the tissue, such as tissue development during embryogenesis or the need for replenishing cell pools following an injury. Although the spindle orientation during division has been extensively investigated, the exact mechanism of orientation determination and division regulation is still unclear due to the interconnection complexity of these fate determinants. Many factors of division preparation and organisation govern the outcome of cell division, such as correct centromere positioning, centromere migration, midbody retainment (the midbody is a ring-shaped structure responsible for abscission during cytokinesis) and chromosomal segregation (Inaba and Yamashita, 2012; Poulson and Lechler, 2012; Majumdar & Liu, 2020; Rai et al., 2021).

To summarise, keratinocyte stem cells can divide symmetrically or asymmetrically creating a heterogeneous pool of cells in the basal layer. There are many factors involved in regulating cells' decision to undergo each type of cellular division. For that, the connection between cell division type and proliferation and differentiation has yet to be fully understood. Therefore, research into these biological processes is crucial for understanding skin health and diseases.

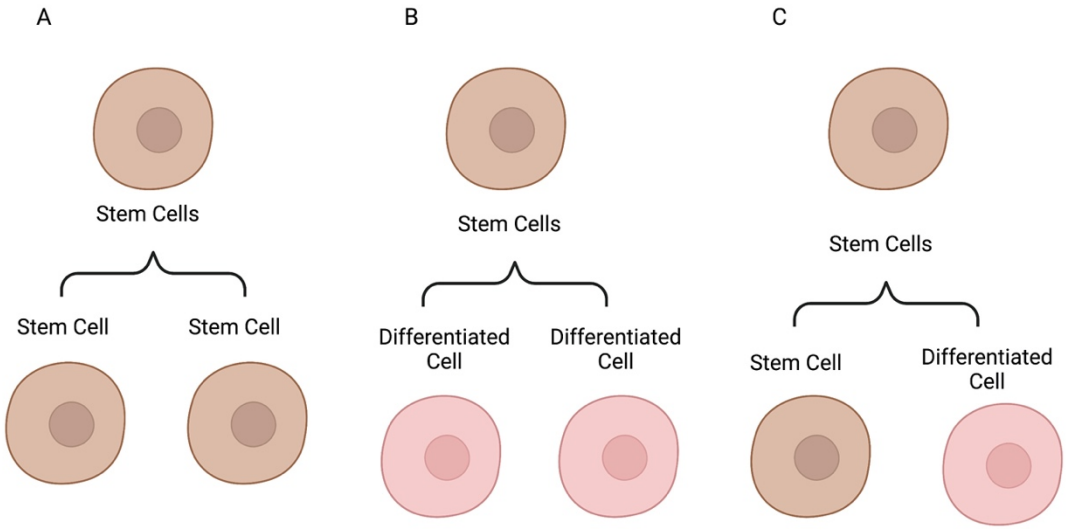


Figure 1.6 Symmetrical and asymmetrical division

(A) Symmetrical division of a stem cell giving two stem cells**. (B)** Symmetrical division of a stem cell to give two differentiated cells. **(C)** Asymmetrical division of a stem cell to form one stem cell and one differentiated cell. (Created with BioRender.com).

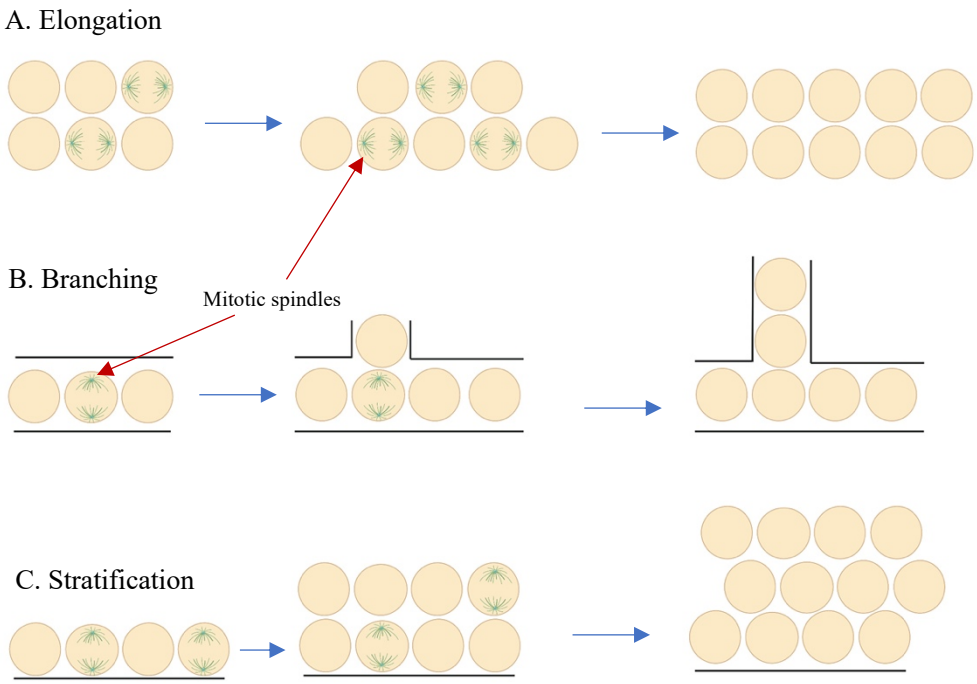


Figure 1.7 Spindle orientation and morphological tissue development

The spindle orientation determines the tissue morphology and type of division. **(A)** Cell division along the axis results in elongated tissue. **(B)** Tissue branching is caused when the division occurs perpendicular to the tissue underneath. **(C)** Tissue stratification resulting from the perpendicular orientation of cell division along a basal membrane. (Adapted from Poulson and Lechler, 2012).

1.5 Cell Cycle and Terminal Differentiation Coupling

The epidermis is structured into multiple cell layers with varying degrees of terminal differentiation (Eckhart et al., 2013). Terminal differentiation is a complex process that involves the interaction of several distinct processes that are regulated at multiple levels, the time-dependent coordination of gene expression changes and intracellular and intercellular changes in response to a wide range of cell stimuli. This tight regulation maintains the epidermis constantly intact in the correct structure, and that terminal differentiation only occurs when epidermal regeneration is necessary. The balance of cell proliferation and cell differentiation is crucial for tissue homeostasis and integrity, with any disturbance resulting in tissue dysfunction and abnormality including cancer and tissue degeneration. Numerous studies have been conducted to elucidate the cell cycle and cell differentiation coupling but the processes of regulation and cell fate decision-making factors of proliferation and differentiation remain unknown. The signals that control cell division and terminal differentiation may be affected by the type of division, as well as the type of cells tested, such as testing embryonic stem cells (ESCs) or somatic stem cells (Musacchio and Helin, 2013). Advances in the field of stem cells and regenerative medicine have been greatly accelerated by utilising pluripotent stem cells. These cells exhibit distinct characteristics that enable them to be self-renewing and differentiate into many cell types. However, the regulation of all these processes is more complex because these decision-making signals are dependent on the tissue (Soufi and Dalton, 2016).

Nonetheless, advancements in the area have identified several factors that influence the chromosomal organisation, epigenetic changes, and gene expression, all of which influence cell fate. To transition cells from one state to another, the epigenetic architecture of the cells

must be altered by transcriptional reprogramming. Several molecular regulators of proliferation and differentiation have been identified such as MYC, NOTCH, and WNT (Bretones et al., 2015; Muhr and Hagey, 2021), as well as the transcription factors SOX2, NANOG, OCT4, P63, E2F, and RB (Boward et al., 2016; Muhr and Hagey, 2021) and cell cycle regulators, the cyclins, CDKs and CKIs such as CDK4/CDK6-cyclin D and the CDK2-cyclinA/E complexes and CKI P21 and P27 (Roccio et al., 2013; Soufi and Dalton, 2016; Ter Huurne and Stunnenberg, 2021).

An intriguing observation that has a significant effect on cell fate is that the interaction of the previously stated effectors influences the duration of the cell cycle. ESCs are known for their high replicative capacity and short cell cycle duration (they divide once every 3–4 hours) because these cells spend most of their cell cycle in the S and M phases with little or no time for gap phases; later in development, the cell cycle lengthens due to the increased time spent in gap phases. Hence, the longer G_1 phase is thought to affect cell division symmetrically or asymmetrically, hence affecting cell fate (Calegari and Huttner, 2003; Calegari et al., 2005). Cellular G_1, S and G_0 phases are all thought to have implications for cell proliferation and cell differentiation (Roccio et al., 2013; Liu et al., 2019; Hwang et al., 2021). One important finding is that the transition of G_1 to S phases is crucial for cell decisions to exit the cell cycle and inter-terminal differentiation (Soufi and Dalton, 2016). It is thought that the differentiation signals are activated at the G_1 phase and the length of this phase has a crucial effect on cell division in murine and human ESCs as well as in induced pluripotent stem cells (iPSCs). In the early stages of the G_1 phase when cells are most sensitive to differentiation signals, but because the ESCs are known for their short G_1 phases, cells pass these signals and do not enter terminal

differentiation. However, prolonged G1 phases induce terminal differentiation (Gao and Liu, 2019; Ter Huurne and Stunnenberg, 2021).

To summarise, proliferation and differentiation regulation is a very complex process due to the interplay of multiple factors and effectors in the tissue. These factors are crucial for tissue health and integrity. Several molecular regulators have been identified that control the cell's decision towards proliferation or differentiation, such as gene expression and cell division type. However, research in this field has yet to fully elucidate the complex regulation of proliferation and differentiation and how that affects cell fate.

1.6 Healing and Repair

Any skin damage must be repaired rapidly to prevent harmful materials such as pathogens from reaching the inner organs, consequently, the study of skin structure, development and differentiation is critical for understanding wound healing and tissue repair. The wound healing process consists of four phases, haemostasis, inflammation, proliferation and remodelling. Immediately after wounding, haemostasis and inflammatory phases are triggered to form blood clots and stop the bleeding by recruiting immune cells such as neutrophils, macrophages and lymphocytes and releasing multiple proinflammatory cytokines and growth factors such as transforming growth factor (TGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) (Guo et al., 2010; Dekoninck and Blanpain, 2019). The clotted tissue serves as a matrix by which cells in and around the wound proliferate and migrate to close the wound and restore tissue integrity. Keratinocytes at the edge of the wound do not proliferate but only migrate to close the wound, whereas cells located around 0.5-1.5 mm farther from the wound edge are proliferative cells (Dekoninck and Blanpain,

2019). Depending upon the wound size and depth, many cell types in addition to keratinocytes basal stem cells contribute to restoring the normal skin structure. For example, bone marrow-derived cells give rise to many types of stem cells, which differentiate to restore the hair bulge, adipose tissue and blood vessels (Guo et al., 2010). Finally, the remodelling phase involves the regular differentiation of keratinocytes whereby the downregulation/upregulation of basal and differentiation molecules and signalling pathways occurs (Martin, 1997).

1.7 Proliferation and differentiation in skin disorders

The integrity of the epidermis is dependent on tight regulation between proliferation and differentiation which involve more complex networks of cellular processes such as cell cycle, cell division, senescence, and apoptosis. These networks are interwoven through signalling pathways, chromosomal organisation, transcription control, and DNA damage response, and disorders occur if any of these parameters are disrupted. Genetic studies have identified different causes of various skin diseases. For example, cancers arise from mutations that lead to uncontrolled proliferation, disrupted differentiation, and resistance to apoptosis (Lopez-Pajares et al., 2013). Atopic dermatitis is caused by mutations in keratin and filaggrin protein expression and dysregulation of their expression causes incorrect structuralising of the epidermal layers, hence barrier malfunctions (Lefèvre-Utile et al., 2021). The epidermis not only provides physical protection but also chemical protection by producing antimicrobial peptides, including cathelicidin, defensin, and psoriasin. These antimicrobial peptides inhibit the colonisation of harmful microorganisms on the skin surface, hence protecting against microbial infection. Disruption in the inflammatory response causes inflammatory diseases such as psoriasis, thus an understanding of the biology of these processes is necessary to

improve our understanding and treat disorders and injuries (Table 1.2) (Lopez-Pajares et al., 2013; Lefèvre-Utile et al., 2021; Komine et al., 2022).

Table 1. 2 Epidermal disorders with associated mutations and processes affected

(adapted from Lopez-Pajares et al., 2013 and Lefèvre-Utile et al., 2021).

Disease	Mutated gene	Loss or gain of function	Process affected	Phenotype
AR congenital ichthyosis	TGM1	Loss	Cornified envelope	Abnormal skin scaling over the whole body
Epidermolysis bullosa simplex	KRT5 and KRT14	Loss	Intermediate filament formation	Trauma-induced blistering with minimal scarring
Hailey-Hailey disease	ATP2C1	Loss	Ca^{2+} signaling	Hyperkeratosis affecting the flexural areas
Lethal congenital epidermolysis bullosa	JUP	Loss	Desmosomes	Generalised blistering and erosions, alopecia, and onycholysis
Multiple self-healing squamous epithelioma	TGFBR1	Loss	Signal transduction	SCC-like invasive tumours
Noonan syndrome	PTPN11 and KRAS	Overexpression	MAPK signaling	Heart, skeletal, haematologic and cognitive defects, distinctive facial dysmorphisms, Hyperkeratosis, and lymphoedema
Palmoplantar keratoderma	JUP, DSP, DSG1, KRT9, KRT16, and KRT1	Loss	Cell adhesion, Desmosomes, Intermediate filaments	Hyperkeratosis affecting palms and soles, curly hair, and arrhythmia

1.8 Rho Family GTPase

One family of small GTPase proteins implicated in the control of keratinocyte function is the Rho family. Rho proteins are a Ras homology (Ras-related) group of small GTPases discovered in 1985 in Aplysia by Madaule and Axel, who named the first family member Rho (Hall, 2012). Further Rho genes have been identified in rats and humans (Hall, 2012, Narumiya and Thumkeo, 2018). The Rho family consists of six subfamilies: Rho, Rac, Rnd, Cdc42, RhoBTB and RhoT/Mario and their members are closely related by their amino acid sequences (Figure 1.8) (Bustelo et al., 2007). They share a CAAX motif at their C-terminal (C for cysteine, A for aliphatic amino acids and X for any other amino acids), which is either farnesylated or geranylgeranylated, affecting the protein localization and stability (Foster et al., 1996).

1.8.1 Rho GTPase and the GTP-GDP cycling

Rho GTPase proteins act as molecular switches, cycling between the active and inactive states by binding to GTP and GDP. This process is regulated and controlled by guanine nucleotide exchange factors (GEF), GTPase activating proteins (GAP) and guanine nucleotide dissociation inhibitors (GDI). GEFs facilitate the exchange of GDP with GTP, which activates GTPases, whereas GAPs and GDIs inhibit the activity of GTPases. GAPs hydrolyse GTP into GDP, while GDIs prevent the interaction between GTPases and GEFs. The switch between the active GTP and inactive GDP-bound forms is called GTP-GDP cycling (Figure 1.9) (Jaffe and Hall, 2005; Ryan et al., 2012; Lawson and Ridley, 2018).

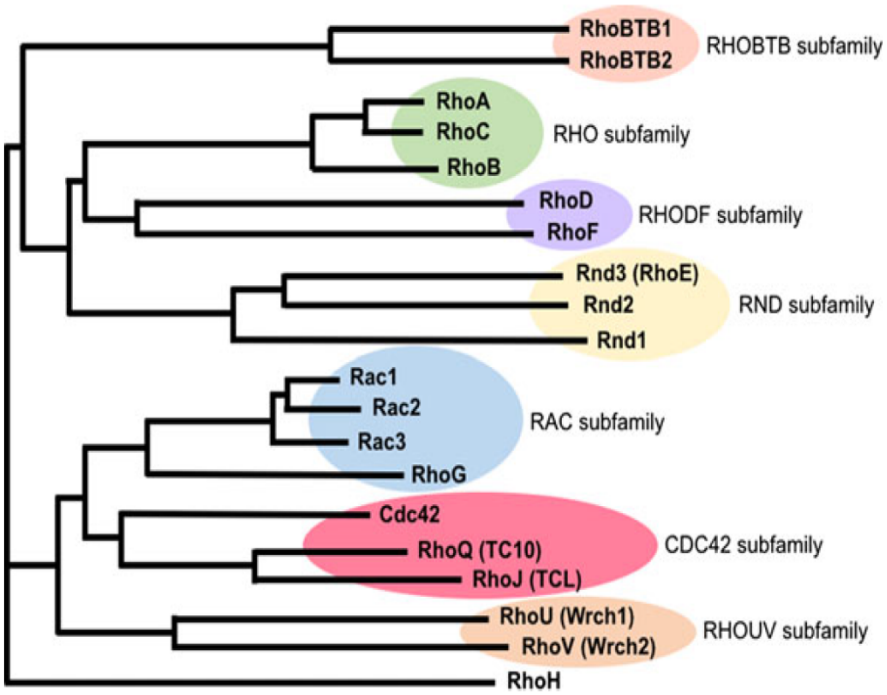


Figure 1.8 The evolutionary tree of the Rho family and its sub-families

Rho GTPases are encoded by twenty genes into six sub-families Rac, Rho, Rnd, CDC42, RhoT/Mario and RhoBTB. (Adapted from Narumiya and Dean, 2018).

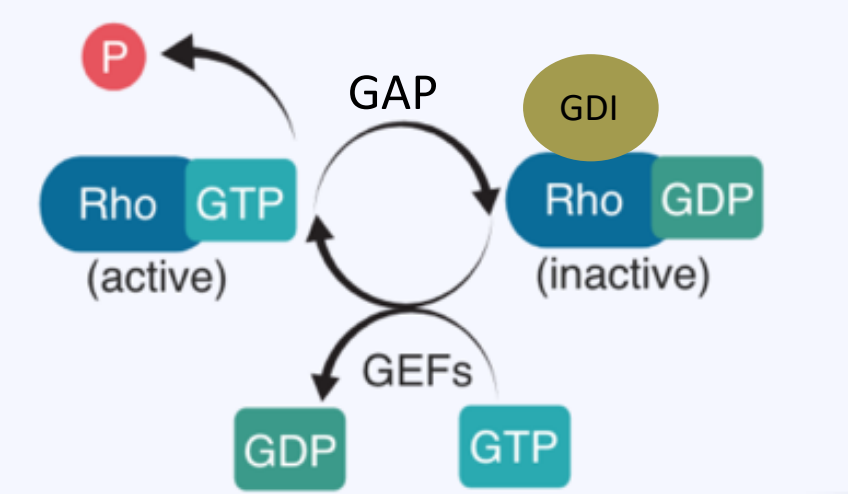


Figure 1.9 The molecular switching of a typical Rho GTPase

Rho proteins are activated by binding to GTP and Rho becomes inactive by the hydrolysis of GTP to GDP. Rho protein activity is regulated by GAPs, GDIs and GEFs. (Adapted from Lawson and Ridley, 2018).

1.8.2 Rho GTPases in biology

The majority of Rho GTPase research has focused on RhoA, Rac1, and Cdc42, and has shown that Rho family GTPases are involved in regulating many cellular processes including the cytoskeleton, cell cycle, cell shape and cell migration (Wennerberg and Channing, 2004; Chardin, 2006). RhoA and Cdc42 proteins are involved in the cell cycle and proliferation. P21/PAK, a protein kinase activated through the ERK-MAP kinase pathway, is a downstream target of RhoA and Cdc42, but the exact pathway has not been fully elucidated. It was suggested that RhoA and/or CDC42 inhibit p21, thus promoting cell cycle progression (Sahai et al., 2001). Furthermore, Rho, Rac1, and CDC42 activate P38, JNK, and MAPK pathways, although the interaction might depend on the cell type (Symons, 1996; Jaffe and Hall, 2005; Mosaddeghzadeh and Ahmadian, 2021).

RhoA, Rac1, and Cdc42 are considered the master regulators of cell cytoskeleton organisation and focal adhesion formation, which are critical in keratinocyte proliferation and differentiation. RhoA induced focal adhesion formation facilitates cell adhesion to the ECM via integrins (Narumiya and Thumkeo, 2018). Rac1 and CDC42 induce filament structure formation via the activation of the downstream pathway of WASP and WAVE, which is important in lamellipodia (Narumiya and Thumkeo, 2018; Mosaddeghzadeh and Ahmadian 2021). Rac1 is responsible for forming the leading-edge extension of the migrating cell, while CDC42 recognises the extracellular environment of the migrating cell. RhoA regulates the cellular shape by promoting focal adhesion and F-actin fibre formation (Wennerberg and Channing, 2004). RhoA promotes the formation of actomyosin filaments through the ROCKII/mDia signalling pathway and mDia is a mammalian diaphanous-related FH-containing protein which promotes actin polymerisation and myosin II formation (Lock et al.,

2011; Hall, 2012, Narumiya and Thumkeo, 2018). The RhoA/ROCKII pathway is critical in keratinocyte differentiation, as blocking Rho inhibited keratinocyte differentiation and induced proliferation. Inhibiting ROCKII in keratinocytes resulted in increased proliferation and blocked differentiation, whereas activation and overexpression of ROCKII in cell suspension resulted in cell cycle arrest and increased keratinocyte differentiation gene expression (McMullan et al., 2003; Lock and Hotchin, 2009).

Overall, Rho GTPase, specially RhoA, Rac1 and CDC42 have critical roles in cell cytoskeleton organisation and focal adhesion formation, which are critical in keratinocyte proliferation and differentiation. They are also involved in regulating cell cycle, cell shape and migration.

1.9 Rnd Proteins

1.9.1 Atypical Rho GTPases

Rnd proteins are not activated or inactivated in the same way as the rest of Rho GTPases. Since Rnd1, Rnd2, and Rnd3 contain 30 amino acid extensions in both their N- and C-terminals and cannot hydrolyse GTP to GDP, they are always bound to GTP. Second, they are farnesylated, a posttranslational modification process by adding a 15-carbon lipid motif, not geranylgeranylated. The GTP-bound state of Rnd proteins makes them almost always active in cells and their activity is regulated by gene expression (transcription/translation) and posttranslational modification (Wennerberg and Channing, 2004; Chardin, 2006; Hall, 2012; Ryan et al. 2012; Paysan et al., 2016). Finally, Rnd proteins are not found in the cytosol but are bound to the membrane. The phosphorylation of the C-termini of Rnd proteins followed by the binding of 14-3-3 promotes their localisation to the cytosol (Paysan et al., 2016; McColl et al., 2016).

1.9.2 Rnd subfamily

Rnd1, Rnd2 and Rnd3 are expressed in different tissues such as in the liver and the brain where they are believed to be important for development (Chardin, 2006; Riou et al., 2010). Although Rnd proteins are part of the Rho family proteins, they might have different functional roles from RhoA, Rac and Cdc42. Rnd1/3 is found in a nonphosphorylated state bound to membranes, while the phosphorylated forms are found in the cytosol (Chardin, 2006). Overexpression of Rnd1/3 in epithelial cells inhibits RhoA and leads to loss of focal adhesions and cell-extracellular matrix binding, hence cell rounding and migration (Nobes, 1998; Haga and Ridley, 2016).

1.9.3 Rnd3 roles in multiple cellular processes

Rnd3 is involved in the cell cycle, differentiation, cytoskeleton regulation and apoptosis, and affects cell cycle progression through the downregulation of Cyclin D1 (Villalonga and Ridley, 2009). In a study in NIH-3T3 cells, induced Rnd3 expression caused accumulation in cells in the G_1 phase and overexpression of Rnd3 prevented G_1/S transition (Villalonga and Ridley, 2009; Paysan et al., 2016). It was suggested that Rnd3 promotes cell cycle arrest by inhibiting cyclin D1 or p21 in the ROCKI/RhoA dependent-inhibition pathway. However, Rnd3 does not bind to or affect the gene expression of cyclin D1 but it might affect cyclin D1 at the posttranscriptional level (Jie et al., 2015). Rnd3 is believed to be a downstream target for the Notch pathway, which is active in proliferative stem cells, promoting cell cycle progression and blocking differentiation (Zhu et al., 2014; Dong et al., 2017). Rnd3 is a downstream target of the zymogen serine protease (VIIa) and when VIIa binds to its transmembrane tissue factor (TF), it triggers the coagulation cascade activation, that is, the cleavage of fibrinogen to fibrin to form blood clots. In addition, VIIa signals for gene expression of cellular proliferation and

migration during proliferation and the remodelling phases of wound healing, and upregulates Rnd3, suggesting the role of Rnd3 in wound healing (Camerer et al., 2000; Chardin, 2006). Rnd3 interacts with ROCKI and regulates desmosome function and keratinocyte cell death. Rnd3 is a p53 transcriptional target (DNA damage-induced apoptosis pathway), implying its involvement in apoptosis, however, the precise role has yet to be determined (Ryan et al., 2012). Other findings which connect Rnd3 to apoptosis are that Rnd3 depletion promotes drug resistance in several types of cancers while increased Rnd3 expression induces apoptosis in response to tumour drugs or UV (Riou et al., 2010).

1.9.4 Rnd3 and cancers

Dysregulation of Rnd3 expression in many cancers has led to the suggestion that Rnd3 functions both as a tumour suppressor or as an oncogene depending on cellular context (Table 1.3) (Paysan et al., 2016). Interestingly, this apparent conflict is most likely related to the cancer cell lineage and/or mutational state. Examples of cancers which have been linked to Rnd3 dysregulation include skin cancer, squamous cell carcinoma, prostate cancer and colorectal carcinoma (Paysan et al., 2016). Rnd3 expression and its crucial role in cytoskeletal organization contribute to tumour invasion and metastasis. For example, DNA microarray screening showed that the Rnd3 expression is involved in the high metastatic characteristic of prostate cancer (Bektic et al., 2005). In gastric cancer cells, Rnd3 expression caused the downregulation of E-cadherin thus promoting tumour cell migration (Jie et al., 2015). These findings suggest that Rnd3 could be a target for cancer treatment, however, more investigations are needed to have a clearer understanding of Rnd3 expression behaviour and function.

To conclude, Rnd3 is involved in regulating many cellular processes in the cell, such as cell cycle and wound healing, and dysregulation of Rnd3 expression has a role in many types of cancers.

Table 1.3 The effect of Rnd3 dysregulation in different types of cancers

(adapted from Paysan et al., 2016).

Cancer type	Cell line	Loss or gain of function experiment	Effect of cell proliferation	Effect on cell invasion
Prostate cancer	DU-145	Overexpression	Decrease	Not determined
Gastric cancer	SGC7901-M	Loss	No impact	Decrease
Gastric cancer	SGC7901-NM	Overexpression	No impact	Increase
Glioma	U87	Loss	Decrease	Decrease
Glioma	U251	Overexpression	Decrease	Not determined
Melanoma	A375	Overexpression	Decrease	Not determined
Melanoma	WM793, WM115	Loss	No impact	Decrease
Sarcoma	Cen3tel	Loss	Not determined	Increase
Skin squamous cell carcinoma	A431	Loss	Not determined	Decrease

1.9.5 Rnd3 and regulation of the cytoskeleton

Rnd3, like other Rho GTPase proteins, has a critical role in cytoskeleton organisation and focal adhesion formation. Interestingly, Rnd3 depletion changed the morphology of HaCaT keratinocytes. Rnd3 depleted HaCaTs were more compact and showed increased expression of several desmosomal proteins such as desmocollin 2/3 and desmoglein 3. These cells had chromosome compaction and were resistant to cisplatin, a chemotherapeutic drug that induces apoptosis through DNA damage (Ryan et al., 2012). Rnd3 also interacts with plexin-B2 and promotes cell rounding in HeLa cells, and plexin-B2 depletion reduces cell rounding, indicating that plexin-B2 is a downstream effector of Rnd3 (McColl et al., 2016). A cell rounding phenotype was observed in the Rhabdomyosarcoma alveolar subtype (ARMS), which is an aggressive subtype of this form of cancer and exhibits rampant metastases and resistance to chemotherapy and radiotherapy (Thuault et al., 2016). Using microarray data analysis, it was found that ROCK activation is associated with the rounding and evasive nature of ARMS, and these cells exhibit downregulation of Rnd3 (Williamson et al., 2010; Thuault et al., 2016).

ROCKI and ROCKII are well-established effectors of some Rho GTPases regulating the proliferation, differentiation, and migration of keratinocytes. This is linked to their role in regulating focal adhesion and stress fibre formation (Guasch, 1998). RhoA activated ROCKII and induces actin assembly and keratinocyte terminal differentiation, whereas blocking ROCKII inhibits keratinocyte differentiation and induces proliferation (McMullan et al., 2003; Liebig et al., 2009; Lock and Hotchin, 2009). Rnd3 antagonises the function of RhoA indirectly through the binding and activation of p190RhoGAP inducing inactive GDP-RhoA formation or through direct binding and inhibition of ROCKI (Piquet et al., 2018). Rnd3 inhibition of RhoA results in the loss of stress fibre formation and affects actomyosin contractility, which

are important in muscle cell function as well as in myoblast fusion (Riou el al., 2010; Piquet et al., 2018). Interestingly, Rnd3 is phosphorylated by ROCKI promoting its localisation and stabilisation in the cytosol, and inhibition of ROCKI using Y-27632, a well-known ROCKI and ROCKII inhibitor, reduces Rnd3 expression and localisation (Riento et al., 2005; Ryan et al., 2012; Thuault et al., 2016).

Rnd3 is involved in regulating keratinocyte proliferation and differentiation. Rnd3 expression is upregulated during keratinocyte terminal differentiation induced through Ca^{2+} and in suspension, while Rnd3 depletion induces keratinocyte proliferation and delays differentiation (Liebig et al., 2009; Jie et al., 2015; Begum, 2017). Overexpression of Rnd3 also promotes keratinocyte stratification (Liebig et al., 2009). As previously stated, cell size is considered a differentiation marker (Luxenburg and Zaidel-Bar 2019), and Rnd3 upregulation increases cell size in stratifying keratinocytes (Liebig et al., 2009). Interestingly, Rnd3 depletion did not reduce the cell size and did not prevent keratinocyte stratification with Ca^{2+}-induced differentiation, suggesting that Rnd3 does not affect stratification in committed keratinocytes, which are already affected by fate determinants, but it is critical for newly committed cells (Liebig et al., 2009).

Previous work in the lab established an interesting connection between Rnd3 and keratinocyte regulation (Ryan et al., 2010; Begum, 2017). Rnd3 depleted HaCaT cells displayed stemness characteristics such as smaller cell size, increased adhesion to ECM and clustered in tight colonies (Ryan et al., 2010; Begum, 2017), as well as increased expression of the basal stem marker p63, increased desmoplakin expression and reduced differentiation (Begum, 2017). As part of this work, SILAC-mass spectrometry was used to identify proteins that were altered in expression when Rnd3 was depleted using RNAi. A list of proteins that were significantly

upregulated and downregulated is given in Table 1.4. Further analysis of these proteins identified that some are involved in regulating the transcription of differentiation genes, including the forkhead box (FOX) transcription factor FOXK1 (Begum, 2017). All in all, the evidence suggests that the Rnd3 function in keratinocytes is to block the cell cycle and induce terminal differentiation.

Table 1.4 A list of the proteins which are found to be upregulated and downregulated in Rnd3 depleted keratinocytes.

(Begum, 2017).

Upregulated	
Protein	**log2 Ratio**
SARG	1.946168966
NUP153	1.982278431
LAMC2	2.05338809
SLC1A3	2.066030329
LCP1	2.077404076
SERPINB2	2.158288909
MINA	2.224397188
QTRT1	2.456640299
ZFP36L1	2.627016235
PTP4A2	2.884005307
FOXK1	3.248335228
HOOK1	3.433004909
DMD	3.777433612
Downregulated	
Protein	**log2 ratio**
S100A8	-4.251794823
OR3A2	-3.67468662
S100A9	-3.265166816
PPP3CA	-2.97652861
LTF	-2.267835392
KIF14	-2.063192281
JUNB	-1.667574266
C9orf64	-1.443659691
SERPINB4	-1.423363028
SCD	-1.420940456
PKDREJ	-1.372729271
SUN2	-1.366196301
HK2	-1.185486004
H2AFV	-0.947179878

1.10 Forkhead Transcription Factors

The forkhead box (FOX) is a large family of transcription factor proteins first identified in 1990 in Drosophila when mutations resulted in flies with a forkhead-shaped (Jackson et al., 2010). Another group identified hepatocyte nuclear factor (HNF-α), later named FOXA1, in rat hepatocytes, which shared similar structural homology with forkhead proteins (Kaestner, 2000). In 2000, the nomenclature "FOX" for forkhead box was adopted (Carlsson and Mahlapuu, 2002). Fifty-five genes encode forkhead box proteins in the human genome and the family members share a well-conserved DNA binding domain, also known as a winged-helix domain, of approximately 100 amino acids. Based on the conserved sequences, FOX proteins were sub-grouped into nineteen subfamilies based on structural similarity (Figure 1.10) (Lalmansingh, 2012).

1.10.1 Forkhead Box Domain

In 1993, Clark et al. solved the FOXA1 DNA binding domain structure by X-ray crystallography, indicating that this domain is composed of 110 amino acids organized in three α-helices (H1, H2 and H3), three antiparallel β-sheets (S1, S2 and S3) and two non-ordered wing-like structures (W1 and W2). The order of these secondary structures gave this family a butterfly-like wing shape and is where the name "winged-helix" arises from (Figure 1.11) (Clark et al., 1993; Kaufmann and Knöchel, 1996; Marsden et al., 1998). FOX proteins bind to DNA as monomers where H3 is the structure responsible for DNA binding at the major groove, the loop between H2/3 consists of seven to twelve amino acids which are responsible for the DNA sequence recognition, while the minor groove phosphate backbone binds with the C-terminal wing of FOX proteins (Marsden et al., 1998).

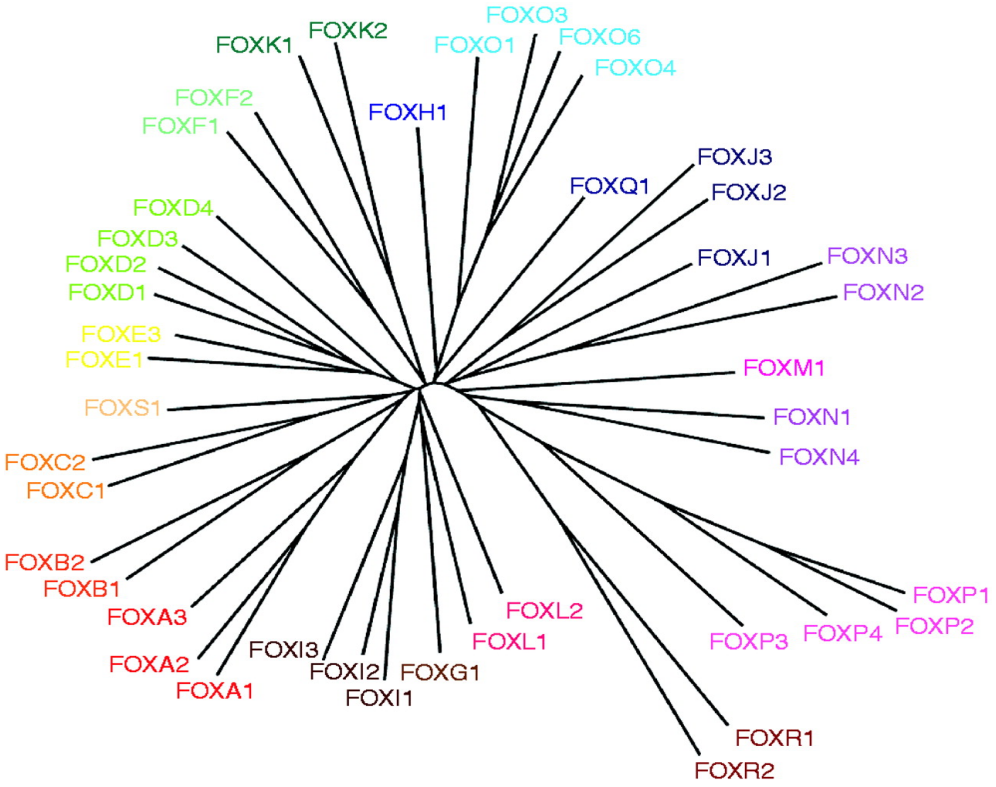


Figure 1.10 Forkhead box transcription factor protein family tree

They are grouped into nineteen sub-families which all share a conserved forkhead box domain (adapted from Uhlenhaut and Mathias Treier, 2011).

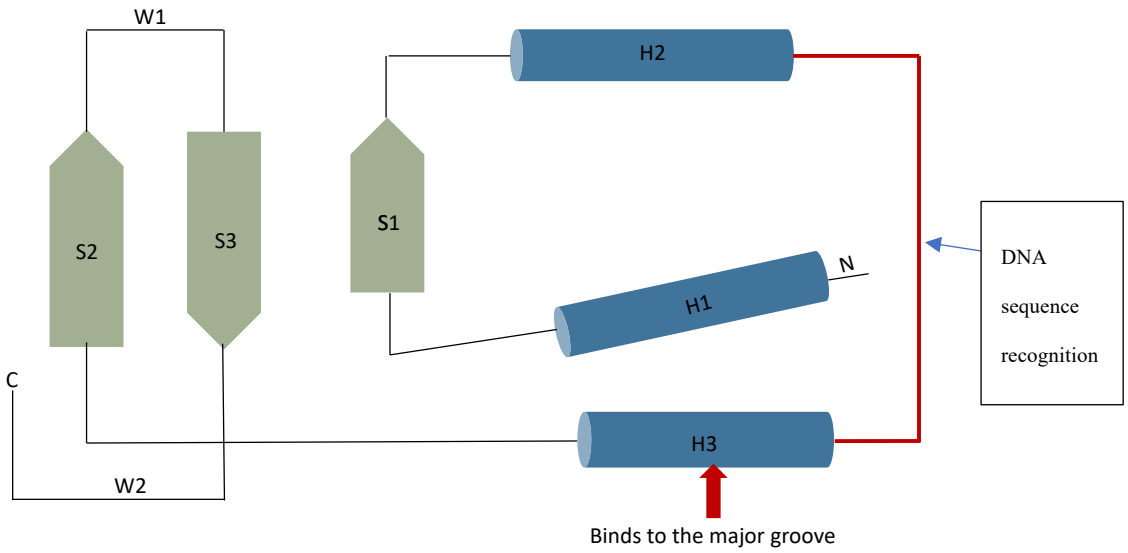


Figure 1.11 Illustration of the Forkhead box domain

(Clark et al., 1993).

1.10.2 FOX transcription factors

Studies on FOX transcription factors have established their involvement in many cellular processes such as development, proliferation, differentiation, cell cycle regulation, metabolism and apoptosis (Kaufmann and Knöchel, 1996; Fetterman et al., 2008). FOXM1 showed an interesting cell cycle regulation function being part of the dimerisation partner, RB-like, E2F and multi-vulval class B (DREAM) complex. Members of the DREAM complex (p130, p107, E2F, BMYB and FOXM1) bind to their target genes, repress them during the G_0 phase, and allow the expression of the same genes only during cell cycle phases (Sadasivam and De Caprio, 2013; Golson and Kaestner, 2016). FOX transcription factors are part of multiple major signalling pathways such as TGF-β, MAPK, Sonic-Hedgehog (Shh) pathway, Wnt/β-catenin and insulin growth factor pathways (Benayoun et al., 2011). For example, the FOXO subgroup is well-studied because of its importance in glucose homeostasis. FOXO proteins bind to insulin response sequences (IRS) and activate gene expression of gluconeogenic enzymes, however, when insulin binds to IRS, Akt is activated by PI3K, which phosphorylates FOXO proteins and facilitates their transport out of the nucleus, therefore, deactivating gluconeogenesis (Hannenhalli and Kaestner, 2009; Lalmansingh et al., 2012). FOX proteins are also involved in chromatin remodelling, where they are believed to recruit chromatin remodelling factors such as p100, NuRD and HDACs (Lalmansingh et al., 2012). FOXA proteins contain two flanking transactivation (TA) domains that bind histones H3 and H4 in the compacted chromatin, allowing the binding of other transcription factors (Golson and Kaestner, 2016). Mutations in FOX genes are associated with multiple diseases such as cancer, developmental defects and metabolic disorders (Fetterman et al., 2008; Lalmansingh et al.,

2012). In summary, FOX transcription factors are critical for regulating many cellular processes in different tissues.

1.11 FOXK1 and FOXK2

FOXK1, also known as myogenic nuclear factor (MNF) is a member of the FOX family transcription factors. FOXK1 and FOXK2 differ from other FOX members in that both share a forkhead-associated domain (FHA) domain. The FHA domain is about 55 to 75 amino acids and is located at the N-terminal of the DNA binding domain (DBD) and recognises and binds to phosphoproteins (Hofmann and Bucher, 1995). In mouse muscle stem cells, MNF, the ortholog of FOXK1, is expressed in two isoforms by alternative splicing from the *Mnf* gene to produce MNF-a and MNF-b; however, only one isoform of FOXK1 is expressed in humans (Garry et al., 2000). FOXK2 is a 660 amino acid protein and was identified in the 1990s as an interleukin binding factor (ILF) which binds specifically to purine-rich motifs in the HIV long terminal repeat (LTR) (Li et al., 1991; Nestal de Moraes et al., 2019).

1.11.1 FOXK2

The functions of FOXK1 and FOXK2 are yet to be fully understood, although there is more known about FOXK1 than FOXK2. FOXK2 genome-wide ChIP-Seq analysis showed that FOXK2 has unique DNA binding sites compared to the other FOX members. FOXK2 plays a role in AP-1-mediated gene expression, regulating cell-cell contact, adhesion, motility, metabolism, apoptosis, and cancer (Ji et al., 2012; Nestal de Moraes et al., 2019). ChIP-Seq analysis for FOXK2 in the H1 human embryonic stem cell line (H1ESC) identified a chromatin remodelling function. FOXK1 and FOXK2 bind to Sin3A and HDAC1 suggesting the role of FOXK1/2 in gene repression. However, further analysis of FOXK2 indicated that FOXK2

association with Sin3A resulted in more active chromatin (Bowman et al., 2014; Ji et al., 2021). Immunoprecipitation experiments using Flag-tagged FOXK2 indicated that BAP1 is a binding partner for FOXK2. The binding of FOXK2 and Sin3A recruits BAP1 transcription factors, leading to local histone deubiquitylation (Ji et al., 2021). Furthermore, FOXK2 might have a role in DNA repair as it can bind to G/T mismatches (Fuiji and Nakamura, 2010; Ji et al., 2014). To conclude, although research on FOXK2 are scarce, evidence suggests that it has a role in regulating cell cycle, chromatin remodelling and DNA repair.

1.12 FOXK1

1.12.1.1 FOXK1 function in proliferation and differentiation

FOXK1 is important in muscle proliferation, differentiation, and wound healing (Garry et al., 2000; Hawke et al., 2003). FOXK1 expression is upregulated in proliferating myoblasts and downregulated during differentiation (Shi et al, 2010) and knockout of FOXK1 in mice resulted in impaired muscle stem cell function and severe muscle atrophy (Garry et al., 2000). In addition, wound repair and gene expression were compromised and dysregulated (Garry et al., 2000; Hawke et al., 2003). FOXK1 represses differentiation by interaction and repression of several transcription factors and regulates muscle progenitor differentiation through its interaction with another transcription factor, SRF. FOXK1 represses muscle cell differentiation by binding to the MADS-domain of SRF and repressing smooth muscle α-actin and protective protein or β-galactosidase (PPGB) genes (Freddie et al., 2007). Recently, a long noncoding RNA (lncRNA) named FOXK1-AS was shown to regulate FOXK1 expression in C2C12 muscle cells. FOXK1-AS overexpression reduced the expression of FOXK1 mRNA and protein which in turn, induced muscle differentiation and muscle cell regeneration following

an injury, supporting the previous finding of the role of FOXK1 in myogenic proliferation and differentiation (Li et al., 2022). This paper provided an interesting viewpoint on FOXK1 regulation as well as an approach to investigating the effectiveness of lncRNAs in treating muscle injuries (Li et al., 2022).

1.12.1.2 FOXK1 function in cell cycle

FOXK1 and G_0 Phase

FOXK1 regulates the cell cycle and muscle cell proliferation. A study on muscle cell progenitors and C2C12 myoblasts showed that FOXK1 interacts with FOXO4 and Mef2 and represses their function, which leads to increased proliferation of muscle cell progenitors and repression of cellular differentiation (Shi et al., 2012). Additionally, binding between FOXK1 and FOXO4 resulted in FOXO4 repression and a subsequent decrease in p21 expression; $p21^{CIP}$ inhibits cell cycle progression by promoting cell cycle arrest in the G_0 phase. Overexpression of FOXK1 showed a low number of cells in the G_0/G_1 phases, and a high number of cells in the S phase, and the expression of p21 was decreased. In contrast, FOXK1 knockout cells showed increased numbers of cells in the G_0/G_1 phase, decreased numbers of cells in the S phase, and increased p21 expression (Hawke et al., 2003; Shi et al., 2010; Shi et al., 2012; Li et al., 2017). Furthermore, FOXK1 knockout caused cell cycle arrest at the G_1/S checkpoint and showed decreased expression of many cell cycle regulation proteins such as CDK1, CDK4, CDK6, cyclin D1 and cyclin E1 (Shi et al., 2012; Wencong et al., 2020).

Similarly, it was discovered that FOXK1 promotes the re-entry of quiescent satellite cells into the cell cycle via the Sox15-Fhl3 interaction. Sox transcription factors are similar to Fox proteins in that they are expressed in many tissues and regulate multiple cellular processes.

One of the members is Sox15, which is mainly expressed in satellite cells and is involved in FOXK1 regulation. Sox15-binding between Sox15 and the adaptor protein Fhl3 recruits FOXK1 to its target genes. Knockout studies showed that $Sox15^{-/-}$satellite cells have decreased FOXK1 activity and disturbed proliferation potential (Meeson et al., 2007).

FOXK1 and G_1/M checkpoint

FOXK1 also regulates the cell cycle by promoting progression through the G_2/M checkpoint. FOXK1 knockout cells showed slower cell cycle progression compared to the wild type with more accumulation of cells in the G_2/M checkpoint (Tang et al., 2020). Another study identified FOXK1 as a protein target for JLP (JNK-associated leucine zipper protein), which is known as a scaffolding protein that interacts with other proteins that regulate cellular processes such as the cell cycle (Ramkumar et al., 2015). In this study, SILAC and quantitative LC-MS/MS analysis revealed that JNK interacts with PLK1 (Polo-like kinase 1), and binds to the FHA domain of FOXK1 and recruits it predominantly in the G_2/M phase compared to the G_1 or S phases. Interestingly, JNK knockdown increased FOXK1 protein levels with no change in mRNA levels suggesting that JNK regulates FOXK1 translation or degradation. Additionally, FOXK1 increased expression caused decreased p21 expression in JNK knockdown cells. These data supported other studies in C2C12 muscle cells which reported that FOXK1 regulated the cell cycle by inhibiting p21 (Ramkumar et al., 2015).

To summarise, evidence from the literature indicates that FOXK1 has a crucial role in promoting cell cycle and cell proliferation and blocking differentiation in muscle cells.

1.12.1.3 FOXK1 and retinal development

Furthermore, FOXK1, like other FOX members, is involved in embryonic development. FOXK1 expression is upregulated in retinal stem cells during mouse retinogenesis from E15 onwards, suggesting its involvement in stem cell maintenance and function. However, it is not clear yet whether FOXK1 is involved in retinal differentiation in adult cells or whether loss of FOXK1 would disturb retinal development. Hence, further research on FOXK1 in different tissues is required to understand its function (Sel et al., 2013).

1.12.1.4 FOXK1 function in chromatin remodelling

FOXK1 is involved in chromatin remodelling and histone modification. One recent study showed that FOXK1/2 facilitates PR-DUB-Polycomb repressive deubiquitinase-complex recursion into its target genes, notably, genes involved in metabolism, such as in glycolysis and mitochondrial metabolism. Interestingly, promoting and repressing gene expression via FOXK1/2 recruitment were observed. However, the overlapping function of FOXK1/2-PR-DUB complex members is yet to be elucidated (Kolovos et al., 2020). Histone modification of FOXK1 also occurs through FOXK1 interaction with the Snail transcription factor (Sin3A and Sin3b). Sin3A and Sin3b have different activities depending on different protein binding (Ji et al., 2021). In one study, FOXK1 interacted with sds3 through the protein-protein binding between the FHA domain of FOXK1 and phospho-Thr49 of Sds3, serving as an adaptor to recruit Sin3b and form a repression complex causing myogenic progenitor activity (Shi and Garry, 2012; Shi et al, 2012). In other studies, co-immunoprecipitation resulted in pulling down FOXK1/2 and Sin3A, which were coupled with their core components (HDAC1, HDAC2, Sap30, Sds3, Rbbp4 and Rbbp7), while binding between Sin3A and FOXK2 alone increased chromatin activity (Ji et al., 2021).

FOXK1/2 negatively regulates autophagy, a process crucial for cellular homeostasis and repair. Autophagy is regulated posttranslationally through phosphorylation, ubiquitination, and acetylation. FOXK1 represses the autophagy gene Fbox32 by recruiting Sin3A and its components, causing histone deacetylation for H4 (Bowman et al., 2014). A more recent study showed that FOXK1/2 regulated DNA damage and autophagy through the CHK2-FOXK pathway (Chen et al., 2020). Under normal conditions, FOXK1/2 is located in the nucleus as a repressor for autophagy-related genes (ATGs). When a DNA damage response is induced, ATM phosphorylates checkpoint kinase 2 (CHK2), a serine-threonine kinase involved in DNA repair. CHK2 binds to FOXK1/2 through its FHA domain, thereby phosphorylating FOXK1/2, which in turn, facilitates the binding of 14-3-3g, causing FOXK1/2 translocation to the cytoplasm from the nucleus, hence, activation of ATGs and autophagy. Interestingly, FOXK1/2 phosphorylation is linked to autophagy chemoresistance, making the CHK2-FOXK1/2 pathway a potential target for tumour drug design (Chen et al., 2020).

FOXK1 and HDAC3 have recently been linked to the type I IFN response pathway. After detecting viral infection, type I IFN activates the JAK/STAT pathway to induce an innate immune response. HDACs regulate type I IFN but the exact mechanism is not yet understood. Nonetheless, HDAC3 interacts with FOXK1 via the FHA domain, forming a transcriptional complex which activates STAT1 and STAT2 expression, promoting the activation of immune response pathways. Interestingly, HDAC3 inhibited the lysosomal degradation of FOXK1 protein, suggesting another pathway for FOXK1 posttranslational regulation. It is speculated that HDAC3/FOXK1 might be a potential target for treating disorders that involve HDAC inhibitors, such as metabolic disorders and antiviral infections (Yang et al., 2022).

1.12.1.5 FOXK1 function in signal transduction

FOXK1 is a downstream target of Wnt/β-catenin, mTORC1, and TGF-β signalling pathways, which are involved in cell proliferation, differentiation, and tissue homeostasis. FOXK1 binds to Dishevelled through the FHA domain and promotes DVL translocation into the nucleolus. Dishevelled then inhibits the formation of the destruction complex, stabilising β-catenin, which, in turn, activates the Wnt/β-catenin pathway (Wang et al., 2015). FOXK1 and mTOR are associated with cell differentiation, metabolism, and cancer (Bowman et al., 2014; Nakatsumi et al., 2017). Interestingly, both phosphorylation and dephosphorylation of FOXK1 by mTORC1 were reported, giving an insight into FOXK1 upstream regulation (Bowman et al., 2014; Nakatsumi et al., 2017). The mammalian target of rapamycin (mTOR) is a central signalling pathway important for cell proliferation, growth, and metabolism regulation. It is still unclear whether mTORC1 activates or inactivates FOXK1 phosphorylation. A study showed that mTORC1 phosphorylates FOXK1, which results in an inhibition of autophagy genes (Bowman et al., 2014). However, another study showed that mTORC1 activates FOXK1 through dephosphorylation by protein phosphatase 2A (PP2A) (Nakatsumi et al., 2017). mTORC1 recruits PP2A and dephosphorylates FOXK1 in multiple cell lines, which causes the activation of C-C chemokine ligand 2 (CCL2), a proinflammatory chemokine that promotes the accumulation of macrophages on inflammation sites. mTORC1-FOXK1-CCL2 is thought to be an effector in many diseases, such as type 2 diabetes, atherosclerosis, and cancer, and suggests a role of FOXK1 in the inflammatory response (Nakatsumi et al., 2017). Indeed, in a recent paper investigating atopic dermatitis (AD), FOXK1 was enriched in binding sites in differentially expressed genes in chronic AD lesions. However, it is not known how FOXK1

is involved in the immune response, but it was speculated that it might regulate IL-13 (Tsoi et al., 2020).

FOXK1 is an upstream target in the Smad/TGF-β signalling pathway. FOXK1 promoter analysis resulted in identifying several binding sequences for c-jun, smad3 and smad4, promoting their functional activity. TGF-β induction promotes E-cadherin loss and promotes epithelial-to-mesenchymal transition (EMT) in epithelial tissues (Peng et al., 2016). Increased EMT is linked with invasion and metastasis, as well as a poor prognosis in many types of cancers, highlighting the role of FOXK1 in cancer as a biomarker and as a drug target (Wu et al., 2016; Cao et al., 2019; Xu et al., 2021).

1.12.1.6 FOXK1 function in metabolism

FOXK1 is a regulator of glucose metabolism in muscle cells. Gene set enrichment analysis of embryogenic muscle fibroblast (EMFs) showed that many sugar metabolic pathways were significantly altered when FOXK1 was knocked down, such as glycolysis, gluconeogenesis, and pyruvate metabolism (Sakaguchi et al., in 2019; Wang et al., 2019). FOXK1 knockdown causes a reduction in glucose uptake in these cells, supporting the hypothesis that FOXK1 regulates glucose metabolism (He et al., 2018). A connection between FOXK1 and metabolism was suggested when it was found that FOXK1 is one of the mTORC1 targets. In the presence of nutrients, oxygen, and growth factors, mTORC1 drives cells to anabolism and represses catabolism, maintaining cellular homeostasis (Bowman et al., 2014; He et al., 2018). Also, mTORC1 promotes FOXK1 dephosphorylation by inhibiting the GSK3 signalling pathway. Activated FOXK1 is accumulated in the nucleus and, therefore, induces Hif1-α, which regulates glucose and nucleotide metabolism, while inhibition of mTORC1 results in activation

of the Gsk3 pathway. Phosphorylated Gsk3 accumulates in the cytoplasm and phosphorylates FOXK1, promoting the binding of 14-3-3 to FOXK1 and preventing its translocation into the nucleus (Figure 1.12) (He et al., 2018).

However, other studies showed independent Gsk3 phosphorylation and inhibition of FOXK1 without suppression of mTORC1, suggesting further complicated FOXK1 network pathways. Interacting proteins frequently rearranged in advanced T lymphomas 1 and 2 (FRAT1 and 2) preventing nuclear Gsk3 phosphorylation and exclusion of the nucleus, thus promoting FOXK1 phosphorylation and inhibition. However, the detailed network of these pathways has yet to be fully understood (He et al., 2019).

FOXK1 is involved in metabolism regulation as a downstream effector in insulin signalling. FOXK1 functions in an opposite manner to other family members, the FOXOs (FOXO1, FOXO3 and FOXO4), which promote gluconeogenesis, cell cycle arrest and apoptosis. FOXK1/2 were both translocated into the nucleus upon Akt-mTOR pathway activation in the presence of insulin in liver cells. Insulin stimulates the PI3K/Akt pathway, which causes FOXO phosphorylation and exclusion of the nucleus (Sakaguchi et al., 2019). At the same time, Akt phosphorylates mTOR, which stimulates the Gsk3/FOXK1 pathway, regulated by mitochondrial fatty acid oxidation, cell proliferation, and survival (Figure 1.13) (Sakaguchi et al., 2019). Additional information on the reciprocal relationship between FOXO1/FOXK1 in metabolism was identified in hepatocytes during fasting/feeding states through the regulation of glucose-6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase (Pck1) genes. During starvation, FOXO1 is located in the nucleus promoting the expression of G6pc and Pck1 genes, whereas, in feeding states, insulin is produced causing the nuclear exclusion of

FOXO1, translocation of FOXK1 into the nucleus and repression of G6pc and Pck1genes (Wang et al., 2019).

A recent study has found that during starvation, FOXK1/2 regulate aerobic glycolysis by promoting enzyme production required for this process (hexokinase-2, phosphofructokinase, pyruvate kinase and lactate dehydrogenase) and preventing oxidation of pyruvate in the mitochondria by increasing the activity of pyruvate dehydrogenase kinase 1/4 (Sukonina et al., 2019). When wild type and C57BLMice were starved for 16 hours, they had elevated levels of FOXK1/2 in muscle and in adipose tissue, as well as in obese mice and skeletal muscles after exercise. Overexpression of FOXK1/2 in 3T3-L1 adipocytes, C2C12 and L6 myoblasts showed increased glucose uptake due to increased levels of GLUT1/4 as well as increased triglyceride synthesis. However, most glucose was used in aerobic glycolysis rather than the TCA/Kreps cycle. Furthermore, FOXK1/2 knockdown caused the opposite results where cells had reduced insulin stimulation, glucose uptake, and triglyceride synthesis (Sukonina et al., 2019).

To conclude, metabolism is a crucial biological process for growth and survival, therefore, tight regulation is required. Understanding the role of FOXK1/2 in the regulation of metabolism might give new insights into the treatment of diseases where metabolism dysregulation is involved such as cancer, diabetes and obesity.

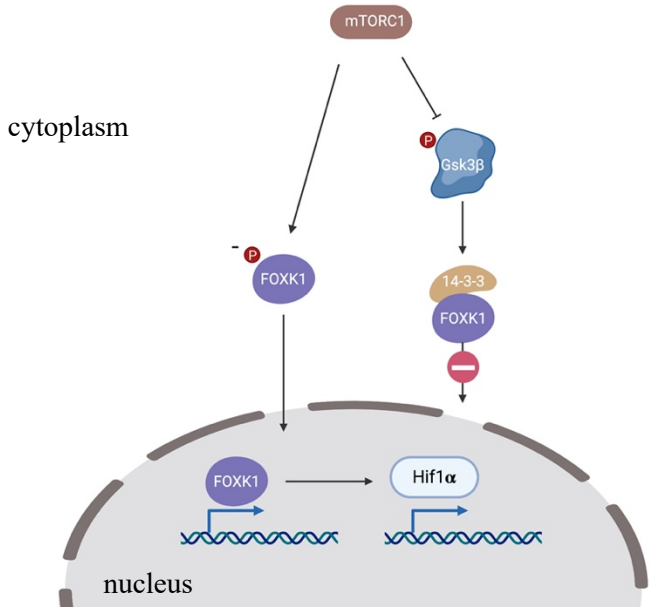


Figure 1.12 An illustration of the mTORC1 regulation of FOXK1 activity

When mTORC1 is active, Gsk3 is phosphorylated and inhibited. mTORC1 dephosphorylates FOXK1, promoting gene expression of Hifα dependent on active FOXK1 which accumulates in the nucleus. However, active Gsk3 phosphorylates FOXK1 at Ser402 which promotes 14-3-3 binding preventing FOXK1 from translocating into the nucleus and suppressing its function (adapted from He et al., 2018).

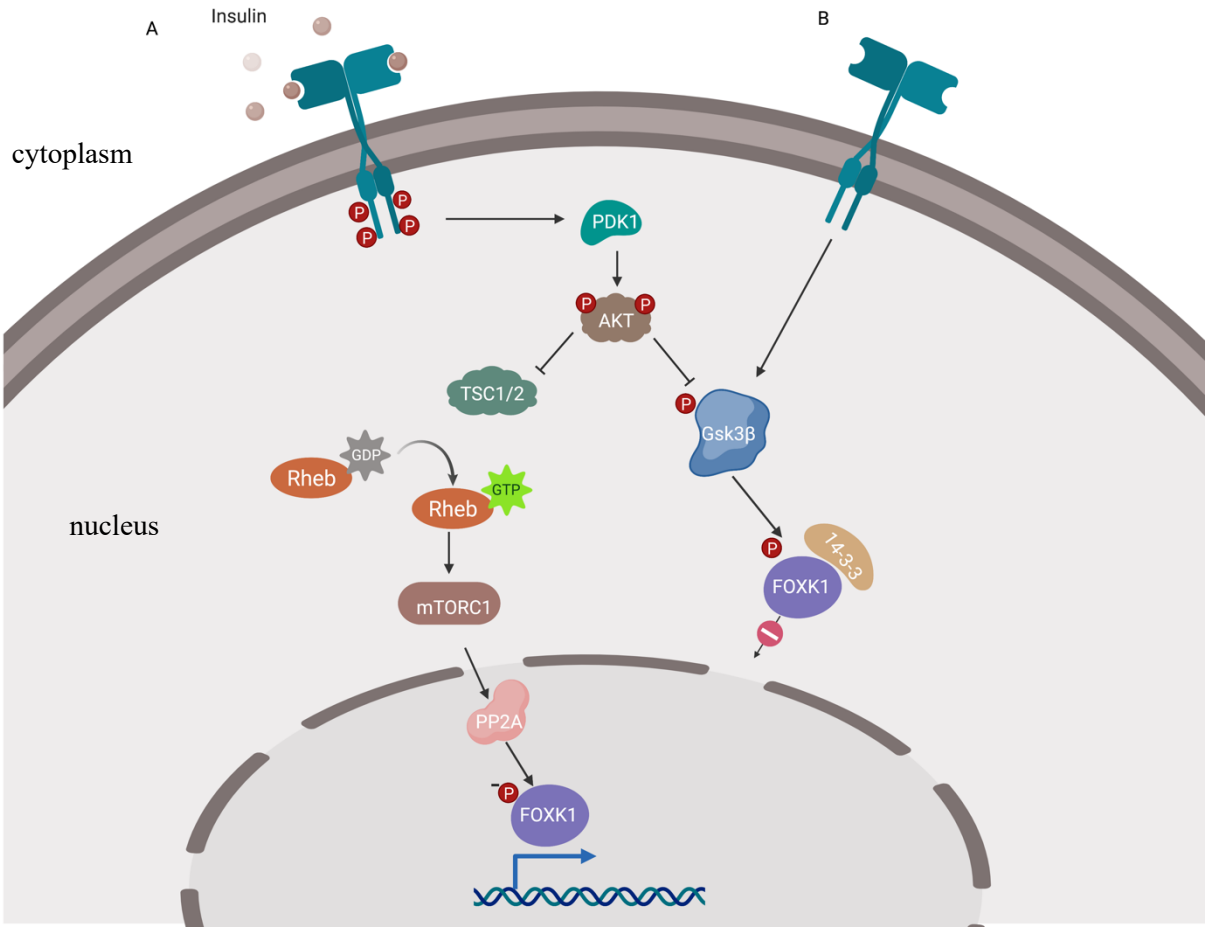


Figure 1.13 Schematic model for the insulin regulation of FOXK1

A) In the fed state, insulin stimulates the PI3K/Akt to phosphorylate and inactivate Gsk3β, promoting the mTORC1 pathway which promotes the dephosphorylated active FOXK1 in the nucleolus. **B)** In the case of starvation, Gsk3β independently phosphorylates FOXK1 which facilitates the binding between 14-3-3 and FOXK1, trapping it in the cytoplasm and inhibiting its function (adapted from Sakaguchi et al., 2019).

1.12.1.7 FOXK1 in cancer

FOX transcription factor members have been detected in several tumours and are involved in proliferation, migration, metastasis, and drug resistance. However, due to their nature as repressors and activators of transcription, studies have shown conflicting roles in cancer (Wang et al., 2018).

FOXK1 can act as an activator or a repressor of tumours depending on its function in a specific tissue. FOXK1 upregulation was detected in several cancers such as gastric cancer, colorectal carcinoma (Peng et al., 2016; Wu et al., 2016; Wang et al., 2018; Wang et al., 2020), papillary thyroid carcinoma (Xu et al., 2021), prostate cancer (Gu et al., 2021), ovarian cancer (Li et al., 2017; Sun et al., 2020), hepatocellular carcinoma (Cao et al., 2019), and melanoma (Yang et al., 2020). Its overexpression is associated with increased proliferation, EMT, invasion and metastasis and poor prognosis (Xu et al., 2021). Moreover, FOXK1 upregulation was associated with chemoresistance in ovarian cancer through the inhibition of cellular senescence and the promotion of glycolysis via the Aurora-A/SOX8/FOXK1 pathway (Sun et al., 2020). Conversely, FOXK1 expression is downregulated in breast cancer, however, FOXK1 overexpression was associated with a better prognosis. RNA-Seq data analysis showed that FOXK1 regulates genes involved in cell survival, migration, invasion, and angiopoiesis, therefore, FOXK1 is considered a tumour suppressor in breast cancer (Sun et al., 2016).

In vivo and in vitro studies revealed a promising approach for cancer treatment and drug resistance by inhibiting autophagy (O'Donovan et al., 2011; Ma et al., 2014; Li et al., 2017; Wang et al., 2020). Autophagy suppresses tumour growth by eliminating defective organelles and proteins in the early stages of tumorigenesis. FOXK1 expression was associated with tumour proliferation and invasion in gastric cancer by inhibiting autophagy. Moreover, gastric

cancer cell lines expressing FOXK1 have lower levels of autophagy-associated proteins LC3-II and Beclin1, and higher levels of p62, whereas knocking down FOXK1 in these cell lines showed higher levels of LC3-II and Beclin1, and lower levels of p62, confirming its role in promoting gastric cancer by inhibiting autophagy; however, the exact mechanism or regulation remains unknown (Wang et al., 2020).

Additionally, Chen et al. (2020), examined the link between FOXK1/2 phosphorylation and chemoresistance caused by the induction of autophagy. Under normal conditions, FOXK1/2 are repressors for autophagy-related genes, with DNA damage inducing CHK2 to phosphorylate FOXK1/2 and promote their translation and trapping in the nucleus, thus, inducing autophagy. However, tumour cells which were FOXK1/2 mutants are continually phosphorylated and are most resistant to cisplatin treatment than WT cells expressing FOXK1/2, while cells which harbour FOXK1/2 mutations prevent their phosphorylation and constantly repress autophagy-related genes in the nucleus were the most sensitive to cisplatin. Interestingly, a combination of cisplatin and autophagy inhibition drugs killed cancer cells completely (Chen et al., 2020).

Recently, it was found that FOXK1 interacts with 53BP1, suggesting its involvement in the DNA damage response. Intriguingly, FOXK1 knockout cells were more sensitive to ionising radiation DNA damage, suggesting that FOXK1 promotes cell survival. Indeed, FOXK1 overexpression impaired 53BP1 accumulation in DNA damage foci promoting cell proliferation and growth. The status of 53BP1 and its related proteins play a crucial role in determining the efficacy of PARPi-based cancer treatment, which is utilised to treat a variety of malignancies with HR pathway disorders. In another study on myeloid leukaemia, FOXK1 was shown to affect BAP1 function. The BAP1-ASXL1-FOXK1 complex represses polycomb

repressive complex 1 (PRC1)-mediated histone H2A ubiquitylation, thus, antagonising its effect on gene expression. For that, FOXK1 could be a potential target for cancer therapy, particularly when combined with PARPi cancer therapy and other BRCA1-deficient or HR-deficient tumour treatments (Tang et al., 2020; Xia et al., 2021).

To conclude, it is speculated that FOXK1 could be a potential target for cancer therapy, however, FOXK1 studies in animal models are inadequate to fully understand its role in normal and cancer tissues.

1.13 Thesis Aim

Rnd3-depleted keratinocytes do not differentiate and Rnd3 depletion leads to increased FOXK1 protein expression in keratinocytes. FOXK1 has a role in muscle progenitore differentiation, therefore, it is speculated that it has a similar role in regulating keratinocyte differentiation. The current research on FOXK1 showed its important involvement in muscle, liver and adipose tissue proliferation, differentiation and survival. In addition, there is evidence for a role in many types of cancers. However, the role of FOXK1 in keratinocyte proliferation and differentiation has yet to be investigated. The overarching aim of this project is to analyse the role of FOXK1 in epidermal keratinocyte proliferation and differentiation. Understanding its role in these cells would improve our knowledge of how FOXK1 functions and provide insight into treating diseases in which FOXK1 is involved.

Project aims:

- Establish an understanding of FOXK1 expression in keratinocytes during Ca^{2+}-induced differentiation.

- Investigate the effect of knocking out FOXK1 on HaCaT keratinocytes using the CRISPR-Cas9 gene editing technique.
- Identify genes directly or indirectly regulated by FOXK1 during keratinocyte differentiation using Next Generation RNA-Sequencing (RNA-Seq).

Chapter 2

2 Materials and Methods

2.1 Materials

Table 2.1 Buffers and Solutions

Buffer	Experiment	Reagents	Manufacturer
Cell lysis buffer	Cell lysing	20 mM Tris-HCl pH 7.5, 0.5% Triton Y-100, 0.5% deoxycholate, 10 mM EDTA, 150 mM NaCl + 1 tablet of protease inhibitor cocktail per 10 ml	
RIPA lysis buffer	Cell lysing	20mM Tris pH7.5, 150mM NaCl, 0.5% deoxycholic acid, 0.5% Triton X-100	
Bradford assay	Protein concentration quantification	Detergent-compatible Bradford assay reagent	Thermo scientific
12% or 10% Acrylamide gel	SDS PAGE	30%	
10X Running buffer	SDS PAGE	288 g glycine, 60.4 g Tris-base, 20 g SDS, 1.8 L ddH_2O	
Transfer buffer	Western blotting	Premixed transfer buffer	BioLab
Blocking buffer	Western blotting	Odyssey blocking buffer or 5% BSA in PBS	

Antibodies dilution solution	Western blotting	Odyssey blocking buffer or 5% BSA in PBS	
PBST	Western blotting washing solution	Phosphate buffered saline, 0.05% Tween 20	
Coverslip fixing solution	Immunofluorescence	Methanol	
Coverslip washing solution	Immunofluorescence	Phosphate-buffered saline (PBS)	Thermo Scientific
Blocking buffer	Immunofluorescence	1% BSA in PBS	
Antibodies dilution solution	Immunofluorescence	3% BSA in PBS	
CutSmart Buffer	Cloning		BioLab
T4 Ligation buffer	Cloning		BioLab
LB broth	Bacterial culture	25 g LB, 1 L of dH_2O	Sigma
Agar	Bacterial Culture	12.5 g LB, 17.5 g agar, 1L dH_2O	
1% agarose	Agarose gel electrophoresis	0.9 g agarose, 90 ml 1X TAE	
2% agarose	Agarose gel electrophoresis	1.6 g agarose, 80 ml 1X TAE	

50X TAE	Agarose gel electrophoresis Buffer		BioLab
Trizol	RNA extraction		Thermo Fisher
Chloroform	RNA extraction		Invitrogen
Phenol	RNA extraction		
100% ethanol	RNA extraction		Invitrogen
75% ethanol	RNA extraction	7.5 ml 100% ethanol, 2.5ml dH_2O	
DNase1	RNA extraction		Thermo Fisher
Sodium acetate	RNA extraction	24.6 g sodium acetate (anhydrous), 100 ml dH_2O	

Table 2.2 Primary Antibodies

Antibody	Antibody	Molecular weight (kDa)	Supplier	Cat. No	Species	Dilution for WB	Dilution for IF
FOXK1	FOXK1	97	Cell signalling	12025S	Rabbit	1:500	1:500
FOXK2	FOXK2	79	Cell signalling	12008S	Rabbit	1:500	
RohE	Rnd3	37	Proteintech	1234-1-AP	Rabbit	1:500	
TGM1	E-6	68	Santa Cruz Biotechnology		Rabbit	1:1000	
TGM1	TGM1	68	Proteintech	12912-3-AP	Rabbit	1:1000	
Alph-Tubulin		55			Mouse	1:10000	
Keratin1	K1	70	Proteintech		Mouse	1:500	
Kertatin5	D4U8Q	62	Cell Signalling		Rabbit	1:1000	
Kertatin10			Abcam		Rabbit	1:1000	
Involucrin	SY5	90			Mouse	1:1000	1:500
Cyclin D1	E3P5S	36	Cell Signalling		Rabbit	1:1000	

Cytokeratin 19	A53-B/A2.26	40	Diagnostic Biosystems		Mouse	1:1000	
Proliferating cell nuclear antigen (PCNA)	D3H8P	36	Cell Signaling		Rabbit	1:1000	
DeltaN p63	E6Q3O	60	Cell Signaling		Rabbit	1:500	

Table 2.3 Secondary Antibodies

Antibody	Supplier	Raised in	Dilution for WB	Dilution for IF
IRDye® 680RD Goat anti-Mouse IgG	Licor	Goat	1:10,000	
IRDye® 800CW Goat anti-Rabbit IgG	Licor	Goat	1:10,000	
Alexa Fluro 488 (Anti-mouse)	Abcam	Goat		1:100
Alexa Fluor 635 (Anti-rabbit)	Abcam	Goat		1:100

Hoechst 33342	Invitrogen			1:5000

Table 2.4 sgRNA used for FOXK1 CRISPR

Position	sgRNA sequence	PAM	Strand	off -target
Exon 2	CCAGTTCACGTCGCTCTATCACA	CCA	+	28
Exon 2	ACATCCCGGAGCCGGACCTCCGG	CGG	+	65
Exon 2	CCCTCTGAAGATCCACATCCCGG	CCG	+	75

Off-target is the site in the genome that is similar to the target site at which CRISPR might cut and it contains a PAM (NGG) followed by a sequence similar to the original sgRNA sequence. The off-targets are calculated based on the number of mismatches in the sequences and on their proximity to the PAM (Hsu et al., 2013). One way commonly used by researchers to predict the off-targets of their designed sgRNA is by using bioinformatic tools. These tools predict the potential off-target sites based on the similarity of the template sequences with other sequences throughout the genome (Bao et al., 2021).

Table 2.5 Plasmids used in this study

Plasmid name	Description	Source
PX459 V2.0	9.7 kb vector containing a puromycin selection marker, ampicillin resistance and multiple restriction enzymes sites	
pJET1.2/blunt	cloning vector with multiple cloning sites containing two BglII restriction sites and a T7 promoter	Thermo Scientific

Table 2.6 Primers

Oligos	Supplier	Forward Sequence
CDC45	Thermo Fisher	F: 5'TGGAGCTGTCCAAGGACA3' R: 5'CAGGACACCAACATCAGTCACG3'
CDK1	Thermo Fisher	F: 5'GGCTCTTGGAAATTGAGCGG3' R: 5'CATGGCTACCACTTGTGT3'

FOXK1	Thermo Fisher	F: 5'CTGTACCCCCAGATGTCCCC3' R: 5'GCTGCAAACTCTGCTGCCA3'
GAPDH	Thermo Fisher	F: 5'CTCTGCTCCTCCTGTTCGAC3' R: 5'ACCAAATCCGTTGACTCCGA3'
IVL	Thermo Fisher	F: 5'TGTGAGTCTGGTTGACAGTAGC3' R: 5'GGAGGAACAGTCTTGAGGAGC3'
KRT19	Thermo Fisher	F: 5'TAAGGCTGGGACAAATCGAC3' R: 5'TAAGGCTGGGACAAATCGAC3'
ΔNP63	Thermo Fisher	F: 5'GAAGAAAGGACAGCAGCATTGAT3' R: 5'GGGACTGGTGGACGAGGAG3'

2.2 Cell Culture

2.2.1 HaCaT cell culture

HaCaT cells were cultured and maintained in low Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% chelated foetal bovine serum (FBS, Labtech), 2% 200 mM L-Glutamine (Sigma), 1% penicillin/streptomycin (P/S, Gibco) and 5 ml of 0.03 mM

Ca^{2+}. Cells were incubated with 5% CO_2 at 37°C and harvested at 100% confluence by adding 3 ml of 0.05% 1X trypsin-EDTA for 20 minutes. The cell suspension was centrifuged, the supernatant discarded, and the cells resuspended in 0.03 mM Ca^{2+} DMEM P/S or low Ca^{2+} FBS + 10ml DMSO freezing media and stored at -80°C.

2.2.2 Calcium induced differentiation assay

HaCaT cells were seeded in 10 cm culture plates in 0.03 mM low Ca^{2+} DMEM until they reached 100% confluence. Then, low Ca^{2+} DMEM was aspirated and 10 ml of high Ca^{2+} DMEM media (2.8 mM) was added to the plates. The cells were lysed on days 0, 1, 3 and 6. The media was refreshed every 48 hrs.

2.2.3 RNAi Transfection of FOXK1 into HaCaT cells

RNAi transfection for FOXK1 was performed in 6-well plates. First, 15 nM of FOXK1 oligos were mixed in 500 µl Low Ca^{2+} Opti-mem and incubated at room temperature for 5 min. Then, 5 µl of Lipofectamine RNAi MAX was added to 500 µl of 60% confluent cells in 0.03 mM Ca^{2+} DMEM without penicillin/streptomycin. The transfected cells were incubated at 37°C with 5% CO_2 for 24 hours, and then fresh low Ca^{2+} (0.03mM) DMEM media was added to each well.

2.2.4 RNAi in High Ca^{2+} (2.8mM) DMEM

To induce differentiation in FOXK1 knockdown cells, transfection media was replaced with high Ca^{2+} (2.8 mM) DMEM 24 hours after transfection. The cells were lysed on days 1, 3 and 6 after differentiation induction.

2.3 Biochemistry

2.3.1 Bradford assay

The protein concentration was quantified using Bradford assays (the Coomassie blue detergent compatible protein assay) (ThermoFisher Scientific), using Bovine serum albumin (BSA) to generate a standard curve as per the manufacturers protocol. The absorbance was measured at 570 nm using the Tecan Infinite F200 pro plate reader.

2.3.2 Western blotting

10% and 12% polyacrylamide gels were prepared for SDS-PAGE, then 15-25 μg of samples were loaded in each well with 2 μl of PageRuler Pre-stained Protein Ladder used as a molecular weight marker. Samples were run in Tris-glycine running buffer for 2 hours before transfer to nitrocellulose or PVDF membrane using the BioRad Trans-Blot Turbo transfer system. Membranes were blocked with Odyssey blocking buffer or 5% BSA in PBS for 1 hour at RT. Membranes were incubated with primary antibodies overnight at 4°C with shaking, then washed with 10 ml PBST three times for 2 minutes and incubated with secondary antibodies for 1 hour at RT. Membranes were washed with 10 ml PBST three times for 2 minutes and with 10 ml of PBS for 5 minutes. The protein bands were detected by the LI-COR Biosciences detection system.

2.3.3 Quantification and normalisation of the western blot

Protein expression was quantified by measuring the bands signal using Image Studio Lite and band signals were normalised to the control (α-tube). Then the protein expression was calculated as either percent change or log transformed Y=log(Y) to perform statistical analysis.

2.3.4 Immunofluorescence

Cells were seeded on 13 mm glass coverslips and the differentiation assay was performed as described. Coverslips were collected and washed 3 times with 2 ml cold PBS. Cells were fixed with 2ml cold methanol. Coverslips were stored in methanol at -20°C until they were needed. Cells were washed 3 times with PBS, then blocked with 1% BSA in PBS for 10 minutes at RT. After that, cells were washed with PBS and incubated with the primary antibody in 3% BSA in PBS dilution of 1:500 and incubated for 1 hour at RT. Cells were washed with PBS 3 times and then incubated with fluorescent secondary antibody in addition to Hoechst 33342 nuclear staining for 1 hour at RT. Finally, cells were washed with PBS and mounted using Mowiol and stored at -20°C. Images were taken at 40X by a Nikon A1R confocal microscope (Nikon, UK Ltd). FOXK1 expression was measured using Fiji software. To minimise bias, images were divided into four sections and then nine cells from each section were chosen at random to get thirty-six cells in total for each repeat. The signal was obtained by drawing a field around the cells. Signals were adjusted to the background and the ratio normalised to quantify the expression change.

2.3.5 EdU labelling

Cells were plated in autoclaved glass slips overnight in 0.03 mM Low Ca^{2+} DMEM. EdU labelling was performed using the Invitrogen Click-iT® EdU Imaging Kit according to the manufacturer's protocol. Briefly, cells were incubated in a final concentration of 10 μM EdU for 24 hours, then fixed with 3.7% formaldehyde in PBS for 15 minutes at room temperature. Coverslips were washed twice with 3% BSA followed by a permeabilization step where they were incubated with 0.5% Triton® X-100 for 20 minutes at room temperature. Cells then were

incubated with 500 µL of Click-iT® reaction cocktail per coverslip for 30 minutes away from light. Then, the coverslips were washed once with 1 mL of 3% BSA in PBS and stained with Hoechst 33342 diluted 1:2000 in PBS for 30 minutes at room temperature away from light. Coverslips were washed twice with 1 mL of PBS and mounted using Mowiol before storage at -20ºC. Images were obtained using a Nikon A1R confocal microscope (Nikon, UK ltd) at 10X. The number of EdU (positive) cells over Hoechst from three independent experiments was quantified using Fiji software.

2.3.6 Cell proliferation and cytotoxicity assay

In 96-well µclear tissue culture plates (655098, Greiner), 5 x 10^3 cells per well were plated and incubated overnight. A mixture of 2X RealTime-Glo™ MT Cell Viability Assay and CellTox™ Green Cytotoxicity Assay reagents were prepared according to the manufacturer's instructions. At specific time points (0 h, 24 hrs, 42 hrs and 72 hrs), luminescence and fluorescence at 485/535 nm (2X RealTime-Glo™) and (CellTox™ Green) were recorded using a Tecan Infinite F200 Pro plate reader at room temperature. Normalised viability and cytotoxicity per cent were calculated as suggested by Kamiloglu et al. (2020):

$$\% \text{ Viability} = \frac{\text{luminescence reading at xh}}{\text{luminescence reading at 0h}} \text{ X } 100$$

$$\% \text{ Cytotoxicity} = \frac{\text{fluorescence reading at xh}}{\text{fluorescence reading at 0h}} \text{ X } 100$$

2.4 CRISPR-Cas9 for FOXK1

2.4.1 sgRNAs design

Three sequences of guide RNA for each FOXK1 were designed using the Wellcome Trust Sanger Institute (WTSI) Genome Editing tool and sgRNAs were chosen based on the proximity to the translation start codon and the lowest off-target effect. The sgRNAs were ordered from Eurofins Genomics.

2.4.2 sgRNA annealing

sgRNAs top and bottom strands were annealed by mixing 100 μM of forward and reverse sgRNAs with 1 μl of 10X T4 ligation buffer, 1 μl of T4PNK and 6 μl dH_2O. Ligation was performed as follows:

37°C	30 minutes
95°C	5 minutes
Cooled to 25°C	5°C per minutes

Oligos then were diluted 1:200 and stored at -20°C.

2.4.3 Plasmid digestion and oligo ligation

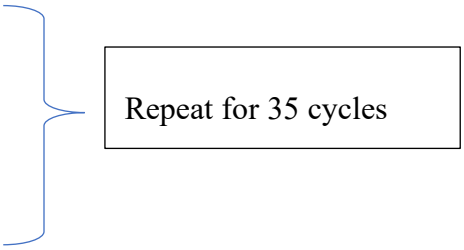
pSpCas9(BB)-2A-Puro (PX459) V2.0 was digested and gRNA oligos were annealed to form the DNA vector. Then, 2 μg of pSpCas9 vector was digested by adding 2 μl of 10X Cutsmart

buffer (BioLab), 1 µl of BbsI restriction enzyme and 16 µl of dH_2O. The mixture was incubated for 2 hours at 37°C. The digested vector digest was purified using 1% agarose gel (0.9 g agarose dissolved on 90 ml 1X TAE). SYBR Safe DNA gel stain (Invitrogen) was added in the dilution of 1:10,000. The correct band size was cut and gel extraction was performed as indicated in the Qiagen kit. After that, sgRNA oligos were ligated to the digested vector and vectors containing oligos and control vectors were prepared. The mixtures consisted of 2 µl of 10X T4 DNA ligation buffer (BioLab), 1 µl of T4 DNA ligase (BioLab), 100 ng of the cut vector, 2 µl of each oligo insert made up to 20 µl with dH_2O. The ligation was performed overnight at 16°C.

2.4.4 Bacterial transformation

Briefly, 5 µl of each of the ligated DNA vectors and vector control were transformed in 50 µl of competent *E. coli* DH5α. Bacteria were incubated on ice for 30 minutes, then heat shocked at 42°C for 45 seconds and returned to the ice for 2 minutes before 500 µl of SOC media was added and incubated at 37°C for 1 hour. Then, 50 µl and 200 µl of bacterial suspension were spread onto LB/Amp agar plates. The ampicillin dilution was 100 µg/ml. Agar plates were incubated at 37°C overnight. PCR was then used to screen for bacterial colonies which contain the correct construct. Three colonies were selected at random and diluted in 50 µl of dH_2O. The PCR master mix was prepared for each colony as follows: 12.5 µl of 2X ReadyTaq, 2 nm of MT5470-U6 promoter, 2 nM of each reverse oligo, 5 µl of the bacterial colony and 2.5 µl of dH_2O. The PCR cycling conditions were as follows:

95°C	5 minutes
95°C	30 seconds
55°C	30 seconds
72°C	30 seconds
72°C	5 minutes



PCR products were separated in 2% agarose gels (1.6 g of agarose dissolved in 80 ml 1XTAE with 1:10,000 of SYPR Safe DNA gel stain). The remaining 45 µl of the bacterial colonies was incubated in 4ml LB/Amp (100 µg/ml) at 37°C under shaking for 18 hours.

2.4.5 DNA extraction

The bacterial colonies (100 µl) were incubated with 100 ml LB/Amp (100 µg/ml) at 37°C in a shaking incubator for 6 hours. The bacteria were collected by centrifugation and DNA was extracted using the Sigma Midiprep kit protocol according to the manufacturer's instructions. The DNA was quantified using the NanoDrop and stored at -20°C until further use.

2.4.6 HaCaT cell nucleofection of CRISPR-Cas9 FOXK1

CRISPR-Cas9 for FOXK1 was performed using the Nucleofection Kit V (Lonza) as indicated by the manufacturer's protocol. GFP and JARD2 DNA were used as negative and positive control respectively. After 24 hours of nucleofection, complete 0.03 mM Ca^{2+} DMEM with

P/S was refreshed. Cell selection was performed using 0.5 μg/ml of puromycin. Puromycin media was refreshed every day for three days. On the 4th day, the cells were kept in 0.03 mM Ca^{2+} DMEM with P/S and cell growth was monitored for a week. Confluent mixed cell populations were trypsinised and seeded into 25 cm^2 flasks. When cells reached 100% confluence, they were harvested and frozen at -80°C.

2.4.7 FOXK1 CRISPR single-cell dilution

In a 96-well plate, 1 X 10^4 cells/ml were serially diluted and seeded into each well to get one cell per well. Wells were visually screened to select only wells containing a single colony. Single cells were grown in low Ca^{2+} medium and then transferred to a 48-well plate, 24-well plate, 12-well plate and finally to a 6-well plate. Knockout colonies were screened by western blotting.

2.4.8 PCR amplification and genotyping screening

DNA was isolated for FOXK1 CRISPR knockout clones and HaCaT WT using the PureLink Genomic DNA Mini Kit (Invitrogen). Amplification of targeted loci was performed using the Pfu DNA Polymerase kit (Promega) and 100 ng of DNA was amplified using specific primers (Table 2.6) targeting exon 2 of the FOXK1 gene. PCR products were extracted from the gel and cloned into a pJET1.2 blunt vector (ThermoFisher Scientific). The purified RT-PCR product (50ng) was ligated into the pJET1.2 blunt vector (3:1 molar ratio of insert to vector) using T4 DNA ligase. The mixture was incubated overnight at 16°C. Then, 5 μl of the ligation reaction was used to transform competent DH5-α E. coli cells and plated on agar plates containing ampicillin. At least 12 bacterial colonies were picked and grown in LB broth with 100 μg of ampicillin for 18 hours. Plasmids were extracted using the Plasmid Plus Midi Kit

(QIAGEN) and ~100 ng of DNA was sent to Sources Biosciences for sequencing https://www.sourcebioscience.com/home.

2.4.9 Wound Migration Scratch Assay

HaCaT WT and FOXK1 knockout cells were seeded in a 6-well plate in low Ca^{2+} (0.03 mM) DMEM media. Scratches using 1000 µl pipette tips were used to create the wounds when cells reached 100% confluence. A Confocal Nikon A1R microscope was used to monitor the wound closure for 24 hours and the wound areas of HaCaT WT and FOXK1 knockout cells were measured using Fiji.

2.5 RNA-Seq

2.5.1 RNA extraction

Cells were grown in 10 cm^2 culture plates in low Ca^{2+} (0.03 mM) DMEM media and high Ca2+ (2.8 mM) DMEM media for Days 0 and 3 of differentiation respectively. Media was removed and cells were washed with cold PBS twice before 1 ml of Trizol was added to each plate. The cells were scraped and collected in 2 ml Eppendorf tubes before 500 µl chloroform was added to each tube and vortexed for 15 s. The cells were incubated at RT for 3 min, then centrifuged at the highest speed for 15 min at 4°C and 600 µl of the upper layer was transferred into a 1.5 ml Eppendorf tube containing 500 µl isopropanol. The tubes were inverted several times to mix gently and incubated for 10 min at RT before centrifugation for 10 min at 4°C at the highest speed. The supernatant was discarded and the RNA pellets were washed with 75% ethanol twice and air dried at RT before resuspension in 40 µl nuclease-free water. The RNA samples were stored at -80°C.

2.5.2 DNase1 purification of RNA

For each 8 µl of RNA, 1 µl of DNase1 was added into 1.5 µl Eppendorf tubes and incubated at RT for 15 min. DNase1 was denatured by adding 1 µl of stop buffer for each sample and the volume was made up to 200 µl by adding nuclease-free water. Trizol/chloroform was added to samples in a ratio of 1:1 and the tubes were vortexed for 15 s. The samples were centrifuged at the highest speed for 10 min at 4°C and the supernatants were collected into new 1.5 Eppendorf tubes. Then, 3 M sodium acetate was added to each sample at 1/10 and 2.5 vols of 100% ethanol was added and the samples were incubated for 1.5 hours at -20°C before centrifugation for 20 min at 4°C. The RNA pellets were washed with 70% ethanol twice spinning each time at the highest speed for 10 min, then air dried at RT and resuspended in 40 µl nuclease-free water and stored at -80°C. For mRNA sequencing, RNA samples were submitted to the RNA sequencing facility (Institute of Cancer and Genomic Medicine at the University of Birmingham) and sequenced using the Illumina HiSeq 2500 system. Libraries were prepared using TruSeq stranded mRNA library prep for the Neoprep kit (Illumina). RNA sequencing analysis and de novo motif using Homer software were performed by Dr Aditi Kanhere. Analysis of functional annotation was carried out using Database for Annotation, Visualization and Integrated Discovery (DAVID) https://david.ncifcrf.gov/tools.jsp.

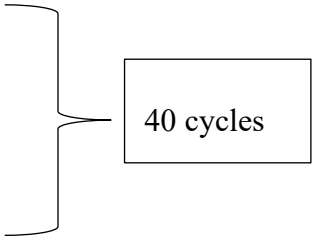
2.5.3 Primer design

Primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). They were designed with melting temperatures (Tm) between 55°C (min.) and 62°C (max.), and a 50% GC ratio. All primer sequences were synthesised by Thermo Fisher (Table 2.5).

2.5.4 One-step qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the qPCRBIO SyGreen 1-Step Go Lo-ROX kit (PCRBiosystems). RNA samples (50 ng/well) were added into the PCR tubes in triplicate with 10 µl 2x qPCRRBIO SyGreen 1-step mix, 0.8 µl Forward primer, 0.8 µl Reverse primer (10 µM) and 1 µl of 20x RTase Go. The reaction volumes were made up to 20 µl and the qRT-PCR reaction conditions were as follows:

45°C	10 min
95°C	2min
95°C	5 seconds
60°C	30 seconds



The qRT-PCR data were analysed using the $2^{-(\Delta Ct)}$ method. The Ct values of each triplicate were averaged and subtracted from the average Ct values of 18S rRNA or GAPDH (ΔCt = Ct value of the target gene – Ct value of the housekeeping gene). The relative expression of the target gene was determined by $\Delta\Delta Ct=2^{-(\Delta Ct)}$.

2.6 Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

The SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) from Cell signaling was used to prepare the samples.

2.6.1 Cross-linking and sample preparation

Cells were grown in 15 cm^2 culture plates in low Ca^{2+} (0.03 mM) DMEM media and then switched to high Ca^{2+} (2.8 mM) DMEM media for days 1 and 3 of differentiation respectively. Cells were cross-linked using 540 µl of 37% formaldehyde to each culture dish containing 20 ml of medium and incubated on a shaker for 10 minutes at room temperature. After that, 2 ml of 10X glycine was added to each dish to quench, the media was aspirated and cells were washed twice with 10 ml ice-cold PBS. Then, 20 ml of ice-cold PBS was added to each dish and cells were scraped and collected into 15 ml Falcon tubes and centrifuged at 2000 x g for 5 min at 4°C. The supernatants were removed and cell pellets were stored at -80°C.

2.6.2 Nuclei preparation and chromatin digestion

Cells were resuspended in 1 ml of ice-cold 1X Buffer A cocktail and incubated on ice for 10 min with mixing by inversion every 3 minutes. The nuclei were pelleted by centrifugation at 2000 x g for 5 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 1 ml ice-cold 1X Buffer B cocktail and centrifuged at 2000 x g for 5 min at 4°C. The supernatant was removed and pellets were resuspended in 100 µl 1X Buffer B cocktail and transferred to a 1.5 ml Eppendorf tube before 0.5 µl of Micrococcal Nuclease was added and incubated for 20 min at 37°C with mixing by inversion several times to digest DNA to a length of approximately 150-900 bp. After that, 10 µl of 0.5 M EDTA was added to stop digestion and the samples were centrifugated at 16000 x g for 1 min at 4°C. The supernatant was

discarded and the pellet was resuspended in 100 µl of 1X ChIP buffer and incubated on ice for 10 min. The samples (500 µl) were sonicated in 8 cycles of 30-second on-off pulses to shear the chromatin. Lysates were cleared by centrifugation at 9400 x g for 10 min at 4°C and transferred to new Eppendorf tubes and stored at -80°C

2.6.3 Chromatin immunoprecipitation

In 1.5 ml Eppendorf tubes, 400 µl of 1X ChIP Buffer was added to each 100 µl sample and 10 µl was removed into new Eppendorf tubes. This is the 2% input sample which was stored at -20°C until further use. To each immunoprecipitation sample, 10 µl of antibody was added and the samples were incubated overnight at 4°C with rotation. The next day, 30 µl of Protein G Magnetic Beads was added to each IP reaction and incubated for 2 hours at 4°C with rotation. Protein G magnetic beads were pelleted by placing the tubes in a magnetic separation rack and the supernatant was removed. Beads were washed three times with 1 ml of low salt buffer and incubated at 4°C for 5 min with rotation before a final wash with 1 ml of high salt wash at 4°C for 5 min with rotation. The supernatant was removed by placing the tubes in a magnetic separation rack.

2.6.4 Elution of chromatin from antibody/protein G magnetic beads and reversal of cross-links

In each of the IP and 2% input samples, 150 µl 1X ChIP elution buffer was added and the chromatin was eluted from the protein G magnetic beads by gentle vortexing using a thermomixer which was heated to 65°C for 30 min. After that, beads were pelleted in the magnetic rack and the cleared solution was carefully collected into new 1.5 ml Eppendorf

tubes. To reverse the cross-linking, 6 µl of 5 M NaCl and 2 µl of Proteinase K were added to all chromatin and 2% input samples and heated at 65°C for 2 hours.

2.6.5 DNA purification using spin columns

For each chromatin sample, 750 µl of DNA binding buffer was added and transferred into a DNA column collecting tube and centrifuged at 18500 x g for 30 seconds. The liquid was discarded and 750 µl of DNA wash buffer was added, followed by centrifugation at 18500 x g for 30 seconds. The DNA was eluted in 50 µl of DNA elution buffer and centrifuged at 18500 x g for 30 seconds. The purified DNA was stored at -20°Cuntil further use.

2.6.6 ChIP quality check

University of Liverpool Centre for Genomic Research https://cgrlms.liv.ac.uk/gsle/mainPage

2.7 Illustrations and Charts

Illustrations and charts were created using the Biorender website https://biorender.com/

2.8 Statistical Analysis

Student's t-test, one-sample t-test, one-way and two-way ANOVA were performed using GraphPad Prism software version 9 (ns = $P>0.05$, * = $P\leq0.05$, ** = $P\leq0.01$, *** = $P\leq0.001$, **** = $P\leq0.0001$).

2.9 Venn diagram

Jvenn tool http://jvenn.toulouse.inra.fr/app/index.html (Bardou et al., 2014).

2.10 Cellular component and KEGG pathway enrichment

ShinyGO http://bioinformatics.sdstate.edu/go (Ge et al., 2020).

KEGG https://www.genome.jp/kegg/pathway.html (Kanehisa et al., 2017).

Chapter 3

3 Analysis of FOXK1 Expression During Keratinocyte Differentiation

3.1 Introduction

FOXK1 is a member of the FOXK subfamily of forkhead box family transcription factors (Hofmann and Bucher, 1995). FOX transcription factors are involved in many biological processes such as development, cell proliferation, apoptosis, cell cycle, signal transduction and metabolism (Kaufmann and Knöchel, 1996; Fetterman et al., 2008; Benayoun et al., 2011; Lalmansingh et al., 2012). FOXK1 is a transcription factor, one of two members of the FOXK subfamily, that regulates cellular proliferation, differentiation, and wound healing in muscle stem cells and skeletal muscle cells (Garry et al., 2000; Freddie et al., 2007; Shi et al, 2010; Li et al., 2022). It interacts with other transcription factors to inhibit their function and increase cellular proliferation while repressing differentiation (Li et al., 2017; Li et al., 2022).

The previous finding from the Hotchin lab on keratinocyte differentiation showed an interesting connection between Rnd3 knockdown and FOXK1 (Begum, 2017). Rnd3 was involved in regulating keratinocyte proliferation and differentiation (Liebig et al., 2009; Begum, 2017). Rnd3 expression was shown to be upregulated during keratinocyte terminal differentiation induced through Ca^{2+} and in suspension, while Rnd3 depletion induced keratinocyte proliferation and delayed differentiation (Liebig et al., 2009; Jie et al., 2015; Begum, 2017). Rnd3 depleted HaCaT cells showed increased expression of the basal stem marker p63, increased desmoplakin expression and reduced differentiation (Begum, 2017). SILAC mass spectrometry in Rnd3 RNAi cells showed an upregulation of FOXK1 protein

relative to control cells. The current research on FOXK1 has shown its important involvement in muscle, liver and adipose tissue proliferation, differentiation and survival. In addition, there is evidence for a role in many types of cancers. However, the role of FOXK1 in keratinocyte proliferation and differentiation has yet to be investigated. In this chapter, we established an understanding of FOXK1 expression in keratinocytes during Ca^{2+}-induced differentiation. FOXK1 expression is increased during keratinocyte differentiation, however, knockdown cells were able to differentiate indicating that FOXK1 might not be critical for the induction of differentiation in keratinocytes.

3.2 Results

3.2.1 FOXK1 expression is increased during Ca^{2+}–induced differentiation

Ca^{2+} is a well-known agent that causes keratinocytes to undergo terminal differentiation (Popp et al., 2014). A differentiation assay for the HaCaT cell line was established to analyse FOXK1 expression during differentiation. HaCaT cells were cultured and maintained in low Ca^{2+} (0.03 mM) DMEM media and when they reached 100% confluence, the concentration of Ca^{2+} was increased to induce differentiation by switching the media to high Ca^{2+} (2.8 mM) DMEM. The cells were lysed at different time points after switching to high Ca^{2+} on days 0, 1, 3 and 6 and expression of two differentiation markers, involucrin (IVL) and transglutaminase1 (TGM1), was assessed by immunoblotting. Figure 3.1A and B show that FOXK1 expression was significantly increased with differentiation, with little or no FOXK1 detected on D0, and a gradual increase in expression on days 1, 3, and 6 of induced differentiation by switching to high Ca^{2+} medium. An increase in transglutaminase (TGM1) and involucrin (IVL) was also observed over the same time course, confirming that differentiation did occur after switching to high Ca^{2+}(Figure 3.1A, C, D). The increase in FOXK1 expression suggests that FOXK1 might have a role in keratinocyte differentiation.

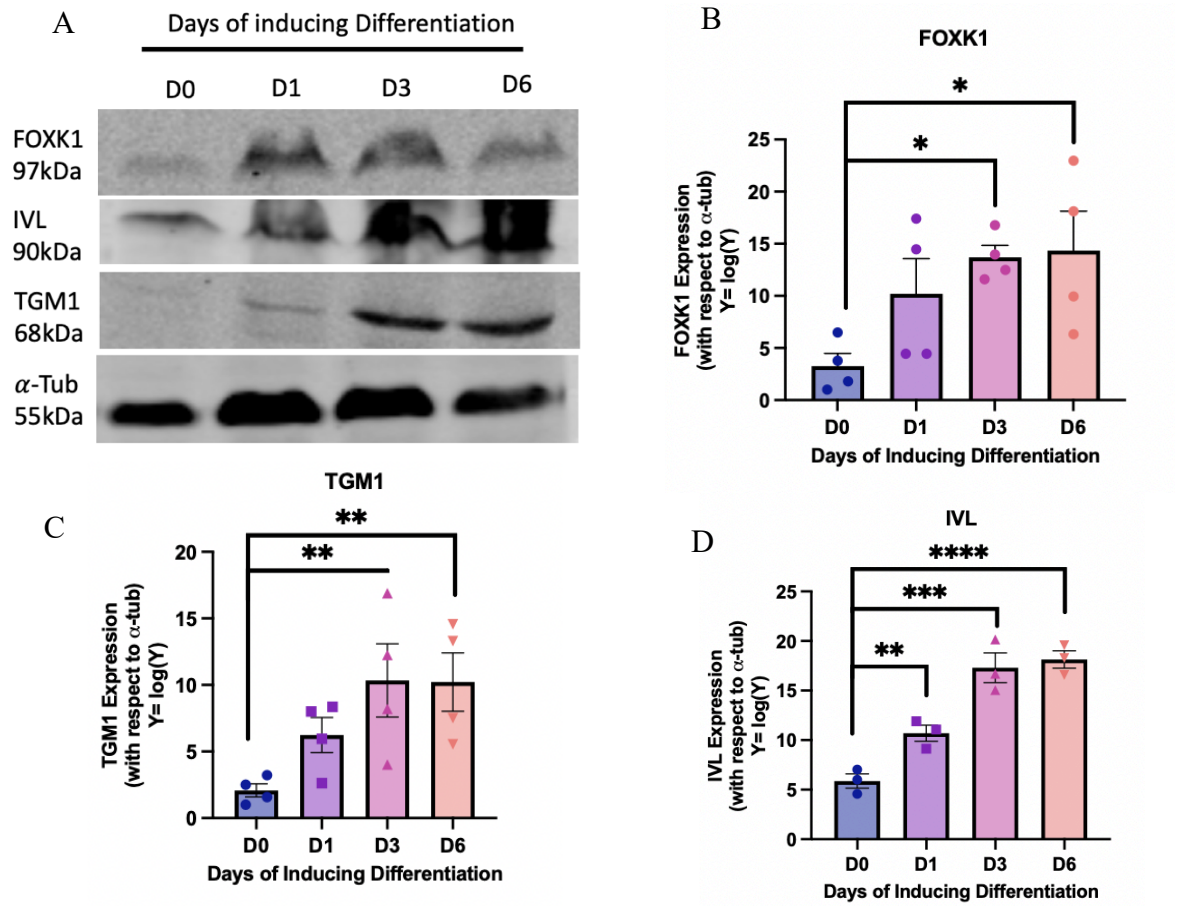
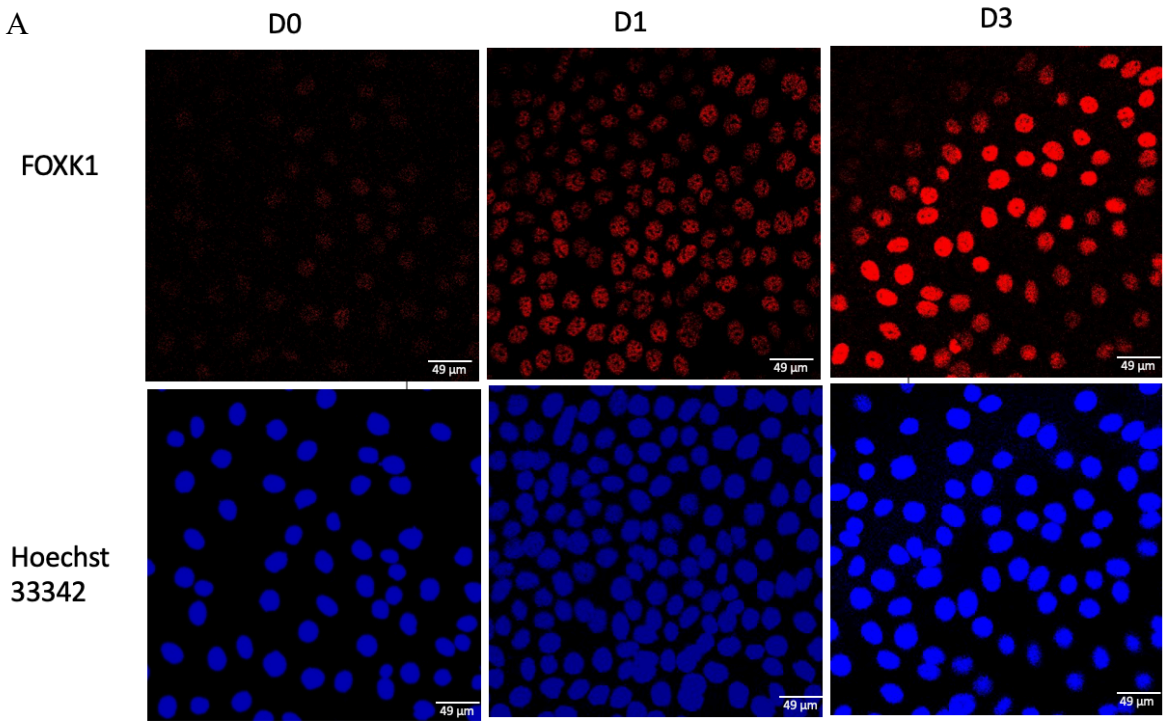


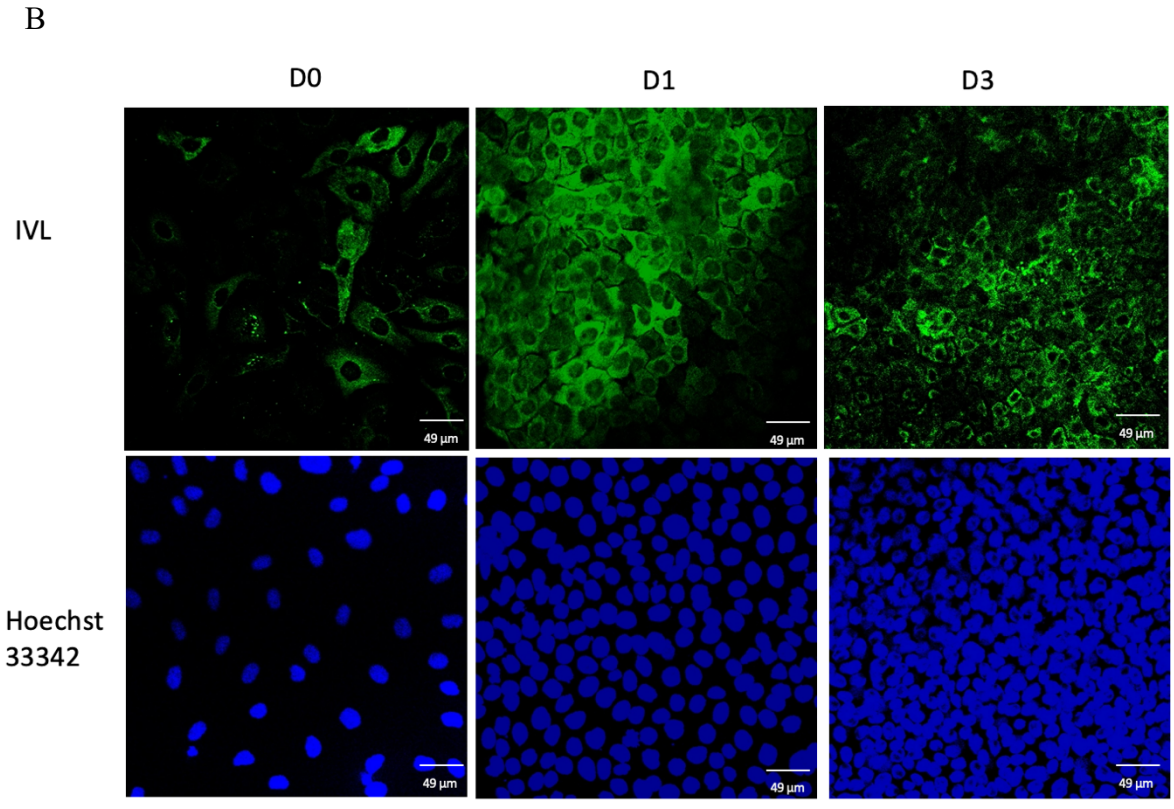
Figure 3.1 FOXK1 expression is increased during Ca^{2+}-induced differentiation of HaCaT keratinocytes

(A) HaCaT cells were cultured in low Ca^{2+} media and differentiation was induced by switching to high Ca^{2+} media. Whole cell lysates were collected 0, 1, 3 and 6 days after switching to a high Ca^{2+} medium and analysed by western blotting using specific antibodies against FOXK1, transglutaminase (TGM1) and involucrin (IVL). Expression of α-tubulin (**α**-Tub) was assessed as a loading control. **(B)** Quantification of FOXK1 protein during differentiation adjusted to α-tub using Image Studio Lite. (**C-D**) IVL and TGM1 protein levels were significantly increased on D3 and D6 compared to control D0. Data are presented as the mean ± SE and multiple comparisons were calculated using one-way ANOVA (*P≤0.05, **P<0.01, ***P<0.001 and ****P<0.0001). The blot picture in A is representative of an experiment which was repeated four times for FOXK1 and TGM1 (n=4) and three times for IVL (n=3).

3.2.2 Immunofluorescence staining for FOXK1 shows that its expression is increased during Ca^{2+}-induced differentiation

Immunofluorescence staining for FOXK1 during Ca^{2+}-induced differentiation was performed to investigate FOKX1 expression. HaCaT cells were seeded on glass coverslips and Ca^{2+}-induced differentiation was performed for 0, 1 and 3 days. Cells were fixed with methanol, stained for FOXK1 and involucrin (IVL) as a differentiation marker and were viewed by confocal microscopy at 40X. Figure 3.2A of the cells on days 0, 1 and 3 which were stained for FOXK1 in red, and cells stained in green are for involucrin (IVL) as a differentiation marker. FOXK1 expression was increased following Ca^{2+}-induced differentiation. Increased expression of IVL confirms differentiation induction. Figure 3.2C Fiji was used to quantify the intensity of FOXK1 expression during differentiation (****P<0.0001).


A
D0
D1
D3
FOXK1
Hoechst 33342
49 µm


B
D0
D1
D3
IVL
Hoechst 33342
49 µm

C

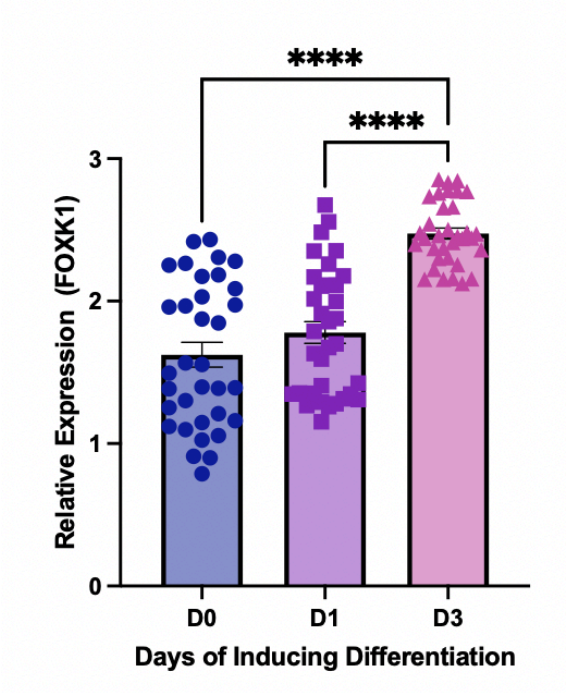


Figure 3.2 FOXK1 expression is increased during Ca^{2+}-induced differentiation

(A-B) HaCaT cells were induced to differentiate by switching to a high calcium medium for 3 days and fixed cells were stained for FOXK1 and involucrin (IVL). Hoechst 33342 was used to enable visualisation of the nucleus. **(C)** Quantification of FOXK1 expression during differentiation. The intensity of FOXK1 expression in cells was measured using Fiji software. The images in A and B are a representative of an experiment which was repeated three times (n=3). Statistical analysis was performed using one-way ANOVA (****P<0.0001).

3.2.3 FOXK2 protein expression is decreased when keratinocytes are switched to high calcium medium

To analyse FOXK2 expression after switching to high calcium media, HaCaT cells were cultured and maintained in low Ca^{2+} (0.03 mM) DMEM media. When cells reached 100% confluence, cells were switched to high Ca^{2+} (2.8 mM) DMEM media to induce differentiation. Cells were lysed at different time points on days 0, 1 and 3 and FOXK2 expression was assessed by immunoblotting. Figure 3.3 A and B show that FOXK2 expression was decreased after three days of switching to high Ca^{2+} compared to D0 where cells were maintained in low Ca^{2+} media. Figure 3.3 A and C also showed a gradual increase in involucrin (IVL) over time confirming that differentiation did occur after switching to high Ca^{2+}. Decreased expression of FOXK2 suggests that it might have a role in the basal stem state in keratinocytes.

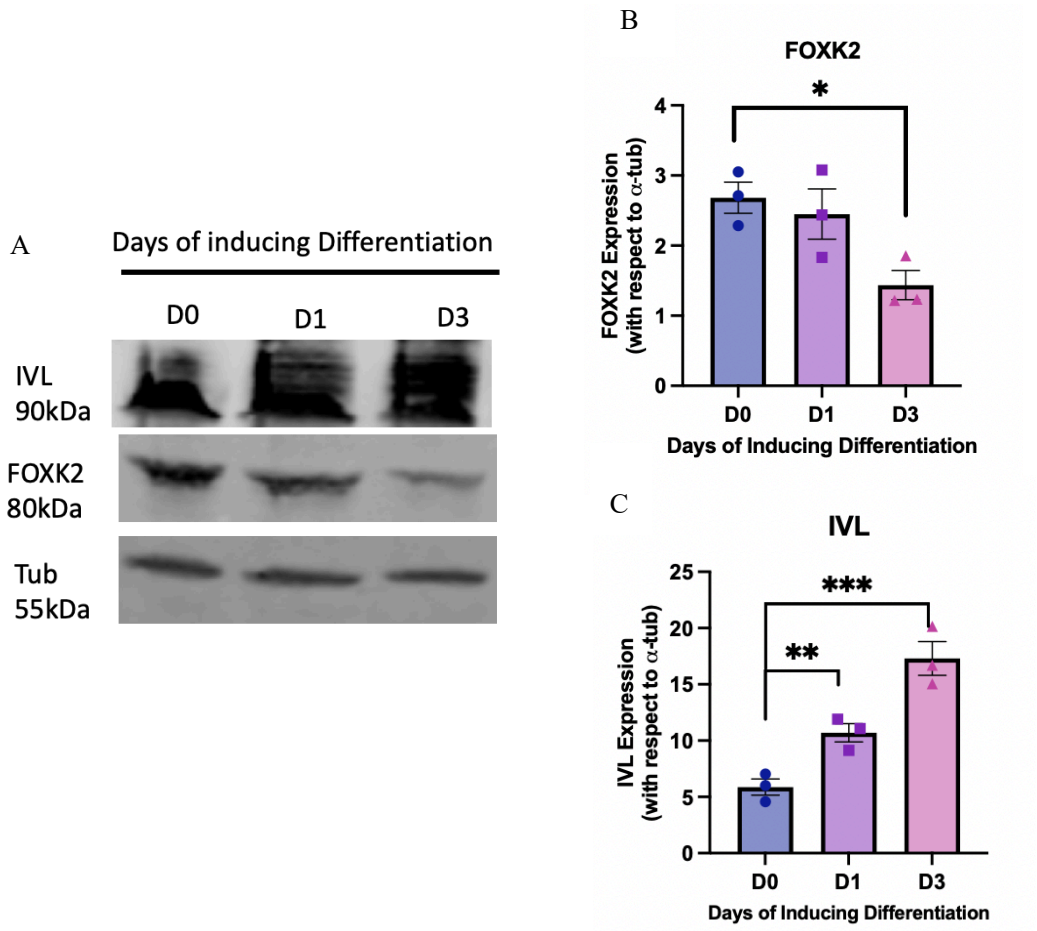
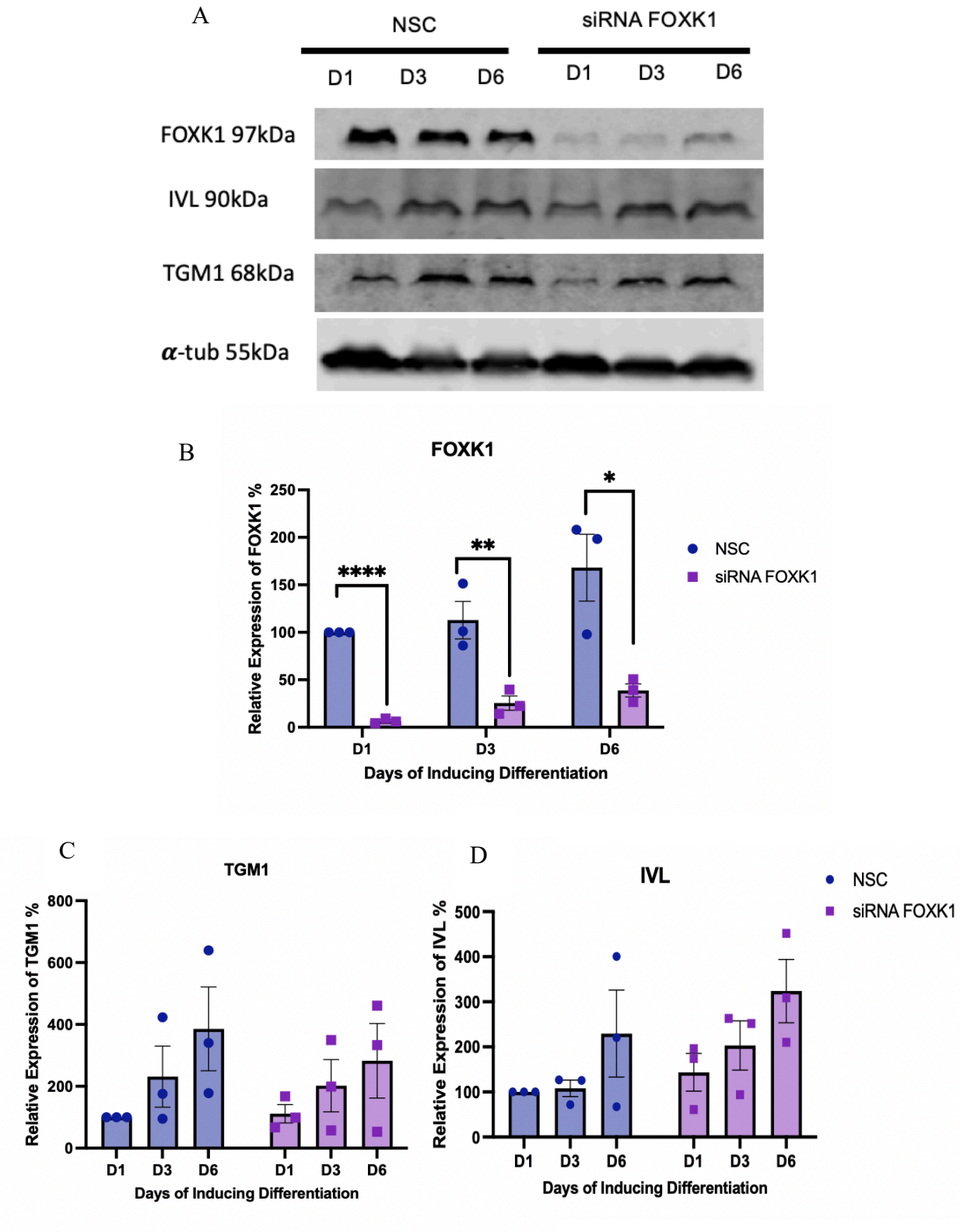


Figure 3.3 FOXK2 expression is decreased when keratinocytes are switched to high calcium medium

(A) HaCaT cells were cultured in low Ca^{2+} media, then switched to high Ca^{2+} to induce differentiation. Whole cell lysates were collected 0, 1 and 3 days after switching to high Ca^{2+} medium and analysed by western blotting using specific antibody against FOXK2. Expression of **α**-tubulin (**α**-Tub) was assessed as a loading control. **(B)** Quantification of FOXK2 protein levels during differentiation adjusted to α-tub using Image Studio Lite. **(C)** IVL protein levels were significantly increased on D1 and D3 compared to control D0. The blot picture in A is representative of an experiment which was repeated three times (n=3). Data are presented as the mean ± SE and multiple comparisons were calculated using one-way ANOVA (**P<0.01, ***P<0.001).

3.2.4 FOXK1 depletion did not affect the differentiation of HaCaT cells

To analyse the role of FOXK1 in keratinocyte differentiation, FOXK1-depleted cells were generated using RNAi and FOXK1 RNAi oligos and non-silencing control oligos (NSC). Differentiation of FOXK1-depleted and NSC control cells was induced by switching cells to high calcium medium for up to 6 days and whole cell lysates were prepared and analysed by western blotting, confirming reduced FOXK1 expression in cells transfected with FOXK1 oligos (Figure 3.3A). To analyse the differentiation, the expression of involucrin (IVL) and transglutaminase1 (TGM1) was analysed at 1, 3 and 6 days after switching to a high calcium medium. Repeated experiments showed FOXK1 knockdown was stable even at 4 to 7 days of RNAi transfection. Quantitative analysis of FOXK1 knockdown showed a 70-80% reduction in expression (Figure 3.3B). Expression of transglutaminase1 (TGM1) and involucrin (IVL) increased after switching to a high calcium medium indicating differentiation was induced. No significant difference in the expression of involucrin or transglutaminase was observed when control and FOXK1 depleted cells were compared, suggesting that depletion of FOXK1 expression did not affect differentiation. This data indicates that FOXK1 may have a role in differentiation but may not be required for inducing differentiation.



Caption is on the next page

Figure 3. 4 FOXK1 depletion did not affect HaCaT cells differentiation in high Ca2+ condition

HaCaT cells were transfected with FOXK1 oligos and non-silencing control (NSC) oligos using lipofectamine 3000 RNAi MAX. Cells were seeded in low Ca^{2+} media for 24 hours and then the Ca^{2+}-induced differentiation protocol was performed. **(A**) The analysis of whole cell lysates by western blotting using specific antibodies against FOXK1 and differentiation markers TGM1 and IVL and **α**-Tub as loading control. These blots are representative of three separate experiments **(B**) Densitometric quantification of FOXK1 protein levels in FOXK1 RNAi knockdown are relative to the non-silencing control (**C-D)** Densitometric quantification of IVL and TGM1 protein expression in FOXK1 RNAi are relative to NSC. Quantification of protein levels during differentiation are adjusted to α-tub. Densitometric quantification was performed using Image Studio Lite. These data are from three separate experiments (n=3). Data are presented as the mean ± SE and multiple comparisons were calculated using two-way ANOVA (*P≤0.05, **P<0.01 and ****P<0.0001).

3.3 Discussion

The FOXK1 transcription factor was discovered in mouse muscle cells and was shown to be critical for muscle stem cell proliferation and differentiation (Garry et al., 2000). Most research on FOXK1 has been conducted on muscle stem cells, so its role in keratinocytes is unknown. A potential connection between the Rho GTPase family member Rnd3 and FOXK1 was identified in mass spectrometry experiments where the expression of FOXK1 was identified as one of the significantly upregulated proteins in Rnd3 depleted keratinocytes (Figure 1.6) (Begum, 2017). This project aimed to identify whether FOXK1 is critical for keratinocyte differentiation. For this, HaCaT keratinocytes were chosen as a model cell line and a differentiation assay for these cells was established. Previously, it was reported that when HaCaT cells are cultured in low Ca^{2+} (0.03 mM) DMEM media, they become a homogenous population of undifferentiated cells and that differentiation can be induced by switching cells to a high Ca^{2+} (2.8mM) DMEM medium (Wilson, 2013).

The western blotting analysis showed increased expression of transglutaminase 1 and involucrin following the switch to a high calcium medium, confirming the results of Wilson (2013) and establishing this as a valid differentiation model. Also, FOXK1 expression was increased after switching to high Ca^{2+} and this increased expression of FOXK1 during differentiation suggests that it has an important role in controlling differentiation.

Immunofluorescent staining was performed on days 0 to 3 of differentiation showing that FOXK1 expression was increased in HaCaT cells during differentiation. These results confirmed the western blotting analysis showing increased FOXK1 expression during differentiation. Interestingly, it was reported that FOXK1/2 can translocate between the cytoplasm and the nucleus by binding to Dishevelled (DVL), causing its translocation out of

the nucleus and the subsequent activation of Wnt/b-catenin pathway (Wang et al., 2015). Furthermore, it was shown that mTORC1 and Gsk3β regulate FOXK1 function through dephosphorylation/phosphorylation which causes FOXK1 translocation in and out of the nucleus (He et al., 2018; Sakaguchi et al., 2019). Therefore, it was speculated that FOXK1 could be found in and out of the nucleus during Ca^{2+}-induced differentiation. However, immunofluorescent staining revealed that FOXK1 is always located in the nucleus, thus FOXK1 may be regulated through transcription in keratinocytes rather than a dephosphorylation/phosphorylation mechanism.

Increased FOXK1 expression during differentiation is interesting because mass spectrometry experiments revealed that FOXK1 expression was upregulated in Rnd3-depleted keratinocytes (Figure 1.6) (Begum, 2017), therefore, it was speculated that FOXK1 expression is higher in the basal undifferentiated state, and its expression is decreased during differentiation. However, the results in Figure 3.1 were the opposite, possibly because FOXK1 is a downstream target for Rnd3 where its depletion could have disturbed the signalling pathways that impact FOXK1 expression. FOXK1 is known to promote muscle cell proliferation and block differentiation (Garry et al., 2000; Freddie et al., 2007; Shi et al, 2010; Li et al., 2022) and Rnd3 may inhibit FOXK1 function in regulating the cell cycle causing cells to exit the cell cycle and enter terminal differentiation. Another explanation is that Rnd3 is involved in the regulation of actin cytoskeleton dynamics which are known to play a role in regulating gene expression and signal transduction, which could also impact FOXK1 expression (Williamson et al., 2010; Ryan et al., 2012; Thuault et al., 2016).

Overall, FOXK1 expression and function in keratinocytes are not fully understood, so further research is required to determine the connection between Rnd3 and FOXK1 and their roles in keratinocyte proliferation and differentiation.

FOXK1 is one of two members of the FOXK subfamily of FOX transcription factors. FOXK2 shares a specific similar structural forkhead associated domain (FHA) in addition to the forkhead domain that is present in all forkhead transcription factors. FOXK2 is a 660 amino acid protein and was identified in the 1990s as an ILF which binds specifically to purine-rich motifs in the HIV long terminal repeat (LTR) (Li, et al., 1991; Nestal de Moraes et al., 2019). While FOXK1 is mainly studied in muscle progenitor cells and is known to regulate proliferation, differentiation and cell cycle, FOXK2 function has not been fully elucidated. FOXK1 and FOXK2 share about 50% of amino acid identity and have similar functions. Both are involved in metabolism regulation, aerobic glycolysis, autophagy, signal transduction and chromatin remodelling (Bowman et al., 2014; Nestal de Moraes et al., 2019; Sukonina et al., 2019; Wang et al., 2019). However, because of the 50% sequence identity, studies have shown that FOXK2 also has distinct functions. Although FOX transcription factors share similar binding motifs, genome-wide ChIP-Seq analysis showed that FOXK2 has unique binding sites in the DNA from the other FOX members. FOXK2 plays a role in AP-1-mediated gene expression which regulates cell-cell contact, adhesion, motility, metabolism, apoptosis and cancer (Ji et al., 2012; Nestal de Moraes et al., 2019). There are no studies on FOXK2 and cell differentiation; therefore, I wanted to investigate FOXK2 expression during keratinocyte differentiation. Western blotting demonstrated that FOXK2 expression was gradually decreased after three days of switching to high Ca^{2+}; however, this decrease suggests that FOXK2 might be important for regulating the basal stem state and not required during

differentiation. For that, I focused on identifying the role of FOXK1 during keratinocyte differentiation.

To determine whether FOXK1 was required for differentiation, FOXK1 knockdown using RNAi was performed to significantly deplete FOXK1 expression but no effect on differentiation was observed after switching cells to a high Ca^{2+} medium to induce differentiation. These results suggest that FOXK1 might not be critical for inducing differentiation. TGM1 and IVL expression were gradually increased during Ca^{2+}-induced differentiation in FOXK1 RNAi and NSC. However, using the RNAi method does not result in 100% knockdown so maybe the existing FOXK1 was enough to induce differentiation. Furthermore, there are some limitations to using RNAi in this type of differentiation assay. Opti-MEM (Gibco) used to perform the knockdown contains 1.3 nM of calcium chloride per ml media and for HaCaT cells to be maintained in an undifferentiated state, they have to be kept in concentrations of calcium below 0.03 mM. Thus, it is possible that the calcium in the Opti-MEM was sufficient to induce differentiation before the knockdown of FOXK1 occurred. To address this, Ca^{2+} was deleted from Opti-MEM and knockdown was performed but this method resulted in high cell death and poor knockdown, suggesting that low calcium might affect the transfection efficiency. Therefore, a different approach to generate FOXK1 knockout keratinocytes is needed to produce more conclusive data.

In summary, FOXK1 expression is increased during keratinocyte differentiation, suggesting a potential involvement in this process. However, RNAi knockdown of FOXK1 did not prevent differentiation suggesting that FOXK1 might not be critical for inducing terminal differentiation in keratinocytes. However, it is still of interest to understand the cause of

increased expression during differentiation and what exactly its function during differentiation is.

Chapter 4

4 The Role of FOXK1 in Keratinocyte Differentiation

4.1 Introduction

In Chapter 3, RNAi was used to investigate the functional role of FOXK1 in keratinocyte differentiation. Using this technique, however, only 70% knockdown was achieved for FOXK1. Because gene knockdown only reduces protein levels, proteins could theoretically still be functional even with very low levels of expression. A gene knockout would eliminate this possibility, and a knockout of FOXK1 in keratinocytes might provide a better understanding of FOXK1 function in keratinocytes. Therefore, the aim of this chapter is to produce a FOXK1 KO cell line and investigate the effect of losing FOXK1 function upon keratinocyte differentiation.

4.2 CRISPR-Cas9.

Many gene-editing techniques have been established and performed over the years, each of which has advantages and limitations. Clustered Regularly Interspaced Short Palindromic Repeats Cas9 (CRISPR-Cas9) is currently one of the most commonly used techniques in biology. Cas9 is naturally found in bacteria and archaea, wherein it functions as a defensive tool against bacteriophages (Anzalone et al., 2020). The mechanism behind the CRISPR-Cas9 system, in general, is that Cas9 nuclease can be guided by a synthetic RNA sequence (sgRNA) to target a specific family of DNA sequences in the genome called protospacer adjacent motif (PAM). PAM is a short DNA sequence (NGG) which guides Cas9 to cut DNA 3–4 bp downstream. Thereafter, the action of Cas9 induces DNA repair mechanisms through two

pathways: the non-homologous end joining (NHEJ) pathway or the homology-directed repair (HDR) pathway. The NHEJ pathway is desirable for knockout gene experiments because it causes a single-nucleotide deletion or frameshift mutation, which leads to gene silencing or deletion; meanwhile, the HDR pathway is used to introduce a small DNA sequence into the genome (Ran et al., 2013; Pickar-Oliver et al., 2019; Anzalone et al., 2020).

In this chapter, CRISPR-Cas9 was used to produce a FOXK1 knockout cell line with which to investigate the role of FOXK1 in keratinocyte differentiation. FOXK1 KO cells were able to differentiate following a high Ca^{2+} switch, whereby indicating that FOXK1 is not critical for inducing differentiation. Further experiments on cell proliferation, viability, cytotoxicity and migration were carried out to determine the effect of knocking out FOXK1 upon keratinocytes.

4.3 Results

4.3.1 A CRISPR-Cas9 targeting FOXK1

The CRISPR-Cas9 system was used to target the FOXK1 gene to produce FOXK1 knockout cell lines. Three guide RNA sequences were designed targeting exon 2 in the FOXK1 gene (Table 2.4) (Chapter 2, section 2.4.1). CRISPR-Cas9 for FOXK1 was performed using Nucleofection Kit V (Lonza), as described in the manufacturer's protocol and in Materials and Methods (Chapter 2, section 2.4). Single cells were grown in low Ca^{2+} medium and expanded before screening for a knockout by means of western blotting. Around thirty expanded cell clones from the three sgRNAs were screened to confirm FOXK1 knockout, however, only one clone was a successful knockout. Interestingly, clone 2.1 showed a decrease in FXOK1 expression after differentiation induction. This could be contamination from wild-type cells grown during the expansion step or it could be that there was a mix-up during gel loading of samples between WT and KO. However, whether Cas9 had caused a modification of FOXK1 expression in this cell line or there was a mix-up or contamination was not confirmed because this screening was performed only to produce a FOXK1 knockout; therefore, any cell line generated which was not a knockout was discarded.

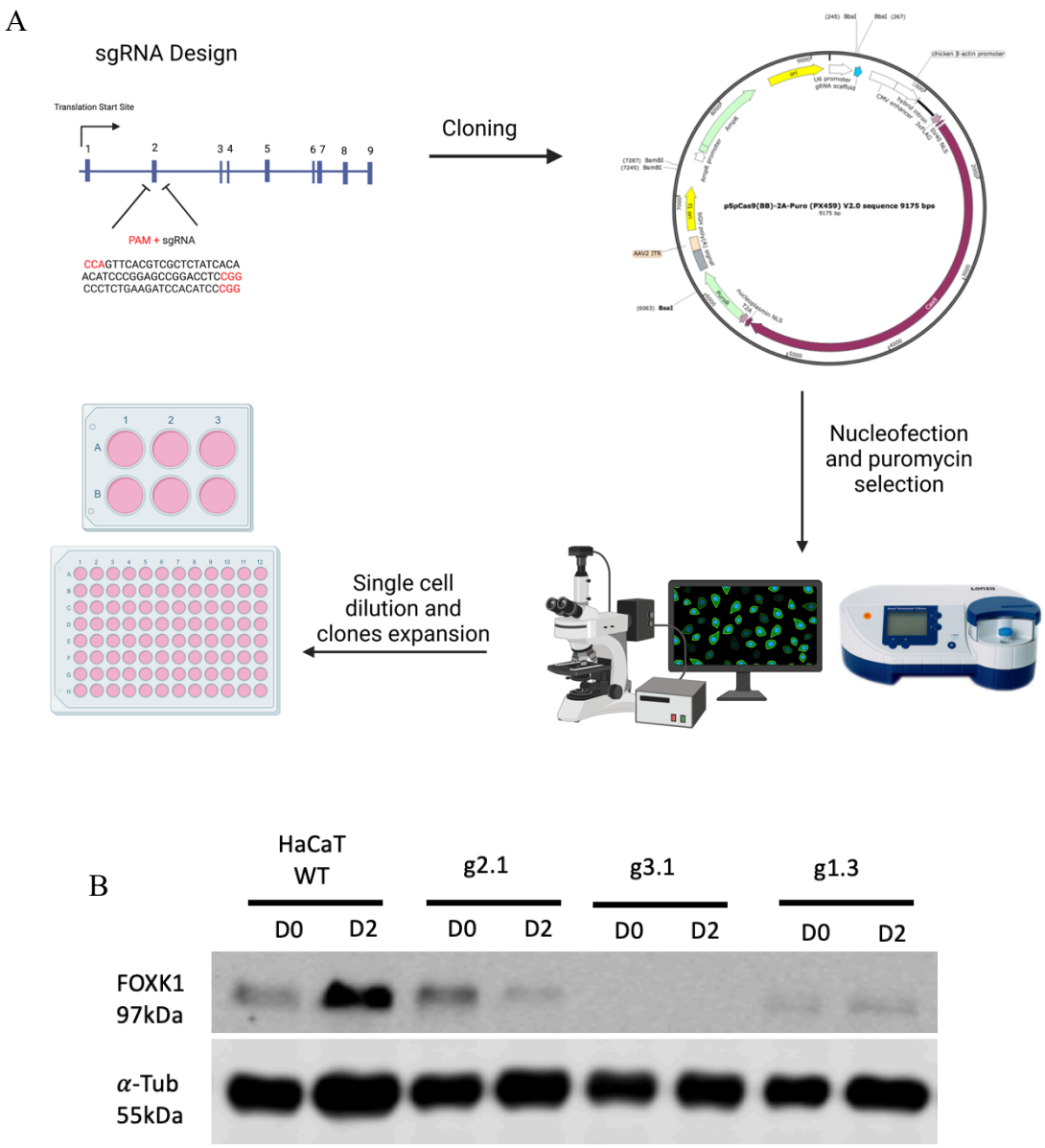


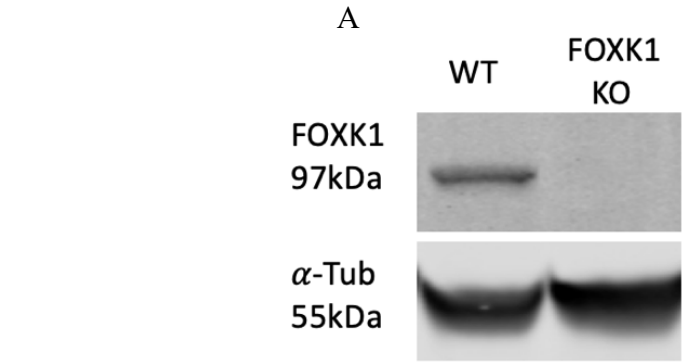
Figure 4.1 Generation of FOXK1 knockout using CRISPR-Cas9

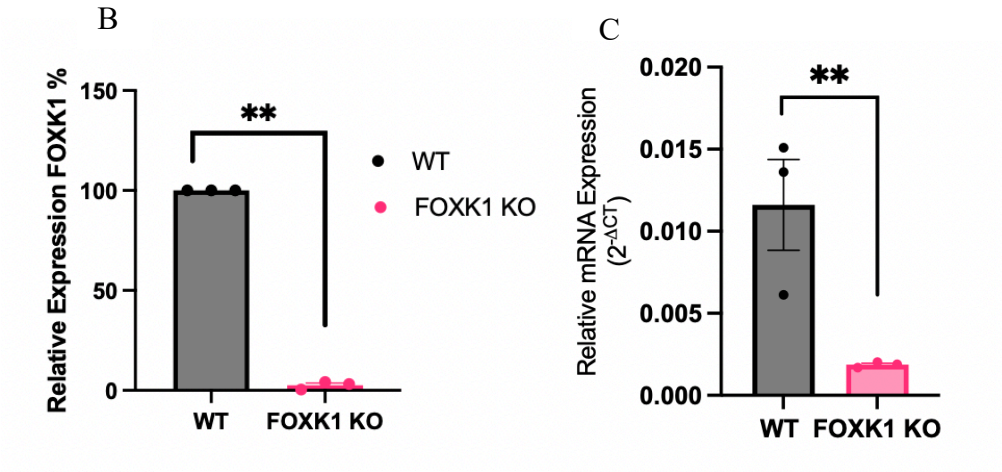
(A) Experimental workflow for CRISPR-Cas9-mediated gene editing. sgRNA sequences were designed to *Foxk1* and cloned into pX459 then transfected into HaCaT keratinocytes by means of nucleofection. Cells were selected using puromycin and serially diluted to single cells and left to expand. **(B)** Western blotting using a FOXK1-specific antibody was used to assess FOXK1 expression in the cultured cell lines.

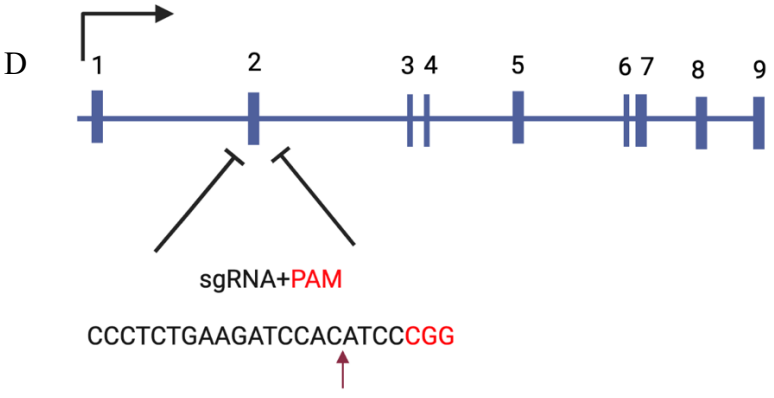
4.3.2 CRISPR gene editing results in loss of FOXK1 expression

Following the procedure of generating FOXK1 knockout cell lines (as described in Figure 4.1), a FOXK1 KO cell line was generated. FOXK1 expression in KO and WT cells was measured via both western blotting and qRT-PCR. The results reveal that FOXK1 mRNA and protein levels were significantly reduced in the KO in comparison with WT HaCaT keratinocytes (Figure 4.2 A–C).

To identify the mutations that caused the FOXK1 knockout, genotype screening was performed, as explained in the Methods (Chapter 2, section 2.4.8). In CRISPR-Cas9, sgRNA directs Cas9 nuclease to the target sequence in the *Foxk1* gene and cuts DNA in a specific location (Figure 4.2 D). Genotyping of genomic DNA extracted from twelve bacterial colonies confirmed that Cas9 cut upstream of the PAM sequence in FOXK1 in the FOXK1 KO clone, which is produced from sgRNA3, causing one bp deletion in elven of the twelve sequenced colonies and two bp deletions in one of the twelve sequenced colonies. These deletions caused a frameshift in the FOXK1 reading frame and introducing the premature stop codon resulted in an inactive gene. Interestingly, the two bp deletion was found in only one sequenced colony and may be a heterozygous mutation whereby Cas9 caused two different mutations in both alleles and coincidently, this different allele in only one colony was sequenced. It is also possible that there was a sequencing error in colony identification, leading to the observed two bp deletion in this one colony, which is the most likely in this case.







E

WT 5' AGATCTC**CCCTCTGAAGATCCACATCCCGG**AGCCGG 3'

KO 5' AGATCTC**CCCTCTGAAGATCCAC—TCCCGG**AGCCGG 3' -1bp (11 of 12 colonies)

KO 5' AGATCTC**CCCTCTGAAGATCC——ATCCCGG**AGCCGG 3' -2bp (1 of 12 colonies)

Caption on next page

Figure 4.2 CRISPR-Cas9-mediated deletion of FOXK1

(A–B) Western blotting and quantitative data showing the expression of the FOXK1 KO cell line in comparison with WT. Densitometric quantification of FOXK1 was conducted using Image Studio Lite. Data were analysed using a t-test (**P<0.01). **(C)** qRT-PCR was used for relative mRNA expression of FOXK1 in WT and in the FOXK1 KO cell line. Data were analysed using a t-test (**P<0.01). Error bars are presented as the mean±SE. **(D)** A diagram showing the location of the sgRNA sequence in *Foxk1* loci. PAM (in red) sequence, and the arrows show the double-strand cleavage position in which Cas9 nuclease cleaved the FOXK1 gene. **(E)** Genotype sequencing for FOXK1 KO clones shows one bp deletion in eleven of sequenced clones and two bp deletions in one of sequenced clones.

4.3.3 Loss expression in FOXK1 CRISPR clone did not block differentiation

To investigate the effect of knocking out FOXK1 in HaCaT cells, a differentiation assay was carried out on the FOXK1 KO clone and compared to WT HaCaT cells. Whole-cell lysates were collected on days 0, 1 and 3 after inducing differentiation, and FOXK1 and differentiation marker IVL were analysed by western blotting. Western blotting shows FOXK1 expression in HaCaT WT as a control and the FOXK1 KO clone, respectively. Western blotting showed loss expression of the FOXK1 protein in the KO clone in comparison to HaCaT WT cells (Figure 4.3 A). Furthermore, increased expression of involucrin (IVL) in WT and KO cells was detected after switching to high Ca^{2+}. Similar to the results observed in the FOXK1 knockdown experiment, decreased expression of FOXK1 did not block differentiation.

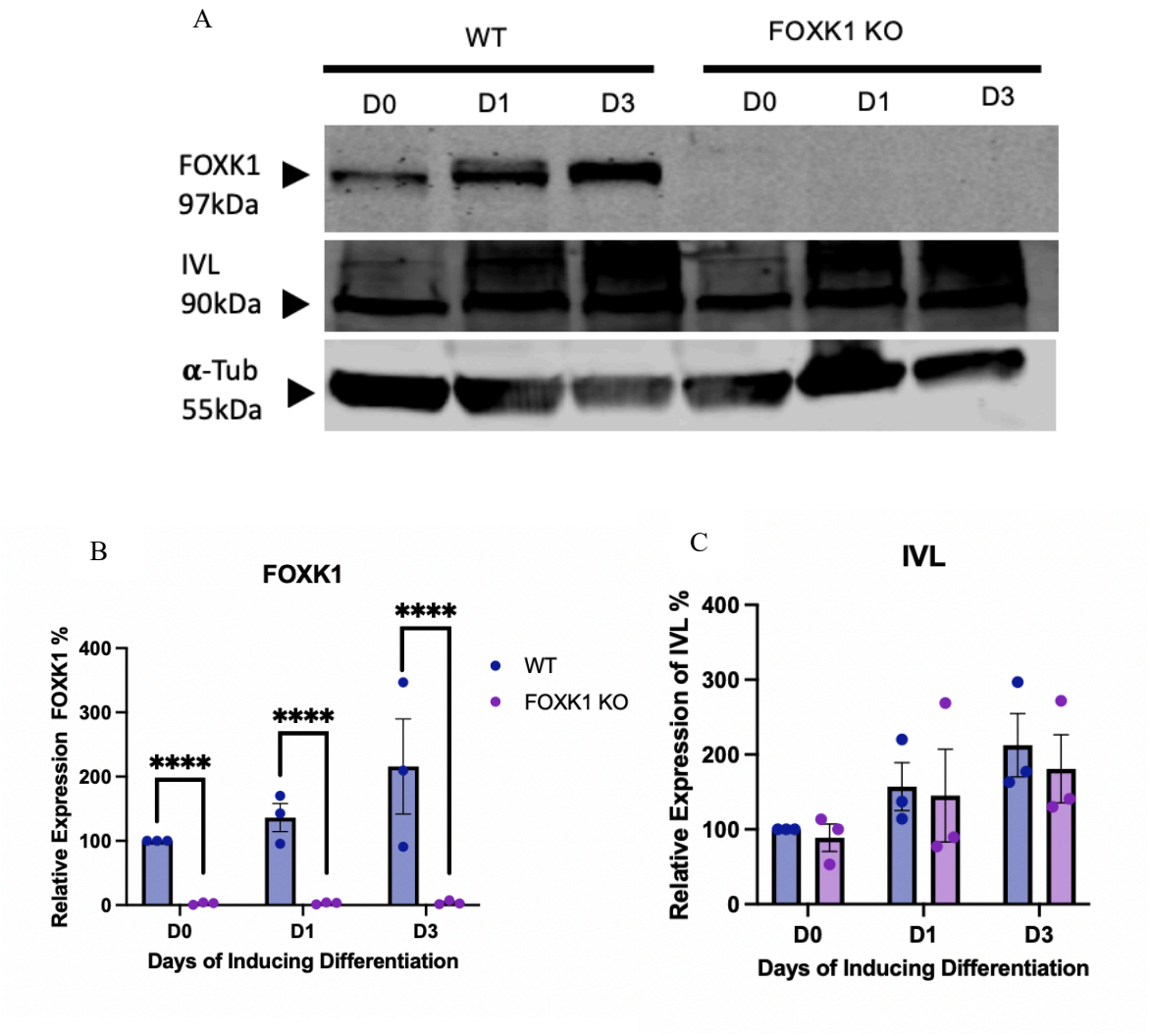


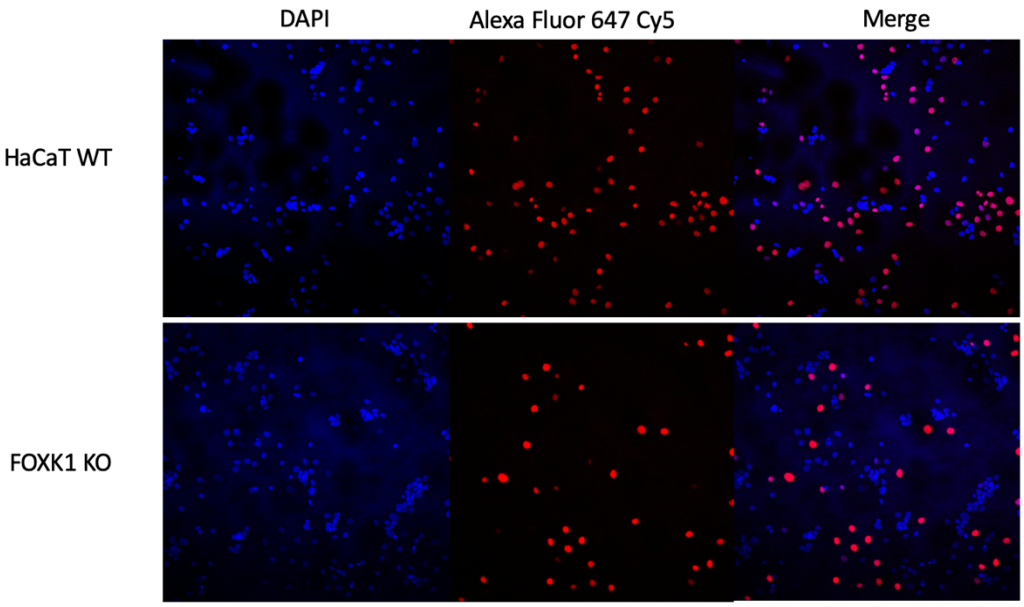
Figure 4.3 FOXK1 CRISPR cells showed loss of expression of FOXK1 and that loss of expression did not block differentiation

FOXK1 knockout cells were induced to undergo differentiation by switching from low-calcium to high-calcium medium. Whole-cell lysates were obtained on days 0, 1 and 3 after differentiation induction. **(A)** Western blotting analysis confirmed a loss of expression of FOXK1 in KO clones in comparison to HaCaT WT. Involucrin expression was increased within 1 day of inducing differentiation in all cells, which suggests that FOXK1 is not critical for keratinocyte differentiation. **(B–C)** Multiple comparisons were calculated using a two-way ANOVA (FOXK1 ****P<0.0001). Densitometric quantification was conducted using Image Studio Lite. These data are from three separate experiments (n=3). Error bars are presented as the mean±SE.

4.3.4 FOXK1 knockout affected cell proliferation in HaCaT keratinocytes

It was reported that the knockout of FOXK1 in muscle stem cells caused a decrease in muscle stem cell proliferation and impaired muscle regeneration, whereby suggesting a role in regulating the cell cycle and proliferation (Garry et al., 2000; Hawke et al., 2003). It was speculated that knocking out FOXK1 might have similar effects on keratinocyte proliferation. Cell proliferation was analysed in HaCaT WT and FOXK1 KO cells by an EdU assay. 5-ethynyl-2′-deoxyuridine (EdU) and fluorescent dye were used to stain DNA during DNA synthesis. EdU is incorporated into newly synthesised DNA; therefore, it can be used to measure DNA proliferation. As presented in Figure 4.4, HaCaT WT and FOXK1 KO cells were incubated with EdU and then stained and viewed. The reddish-purple colour (Figure 4.4 A) represents cells in which EdU has been incorporated into DNA. Immunofluorescence analysis (Figure 4.4 B) showed a significant decrease of about 50% in DNA synthesis in FOXK1 KO cells in comparison to HaCaT WT cells, which suggests that FOXK1 is involved in cell proliferation.

A



B

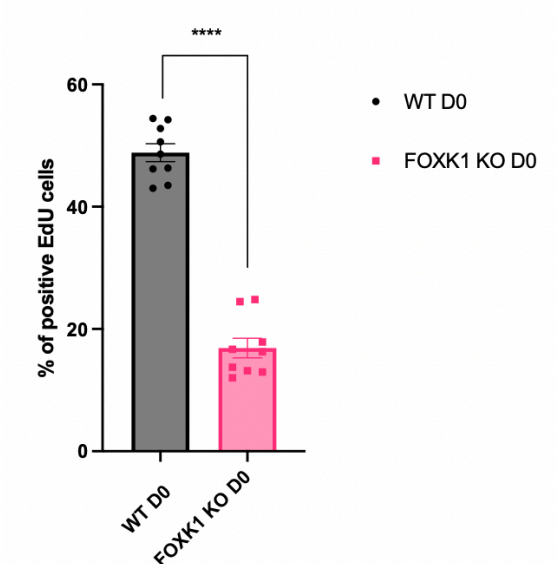


Figure 4.4 EdU labelling showed fewer actively dividing cells in FOXK1 KO cells

HaCaT WT and FOXK1 KO cells were incubated in low Ca^{2+} DMEM with EdU overnight and then stained for EdU. **(A)** This is a representative EdU immunofluorescence image showing the total number of cells stained with a nuclear dye (DAPI) and positive EdU cells (red). **(B)** This is a quantification analysis of three independent experiments (n=3) showing the percentage of positive EdU cells which were quantified to the total number of cells. Statistical analysis was conducted using a t-test, and the p-value is as follows: ****P<0.0001.

4.3.5 Knocking out FOXK1 did not affect cell viability and cytotoxicity but may cause an increase in metabolic activity

Research on muscle cells showed that FOXK1 regulates proliferation and cell death. To investigate whether knocking out FOXK1 might affect keratinocyte viability and cell death, the RealTime-Glo MT Cell Viability Assay and the CellTox Green Cytotoxicity Assay (Promega) were utilised on HaCaT WT and FOXK1 KO cells. RealTime-Glo luminescence reading and percentage change of the viable cell number for HaCaT WT and FOXK1 KO cells (Figure 4.5 A and B). CellTox fluorescence reading and percentage change of the number of dead cells for both cell lines (Figure 4.5 C and D). It was hypothesised that there will be an increase in cell viability in FOXK1 KO cells compared to the WT because FOXK1 KO cells have a slower proliferation rate than the WT cells (Figure 4.4). However, Figure 4.5 shows that there were no significant differences between the two cell lines regarding viability and death. It is of note that the RealTime-Glo MT the luminescence signal is correlated with the number of metabolically active cells; thus, KO cells might be metabolically more active than WT cells. However, there was no significant difference in the rate of cell death between FOXK1 KO and WT cells.

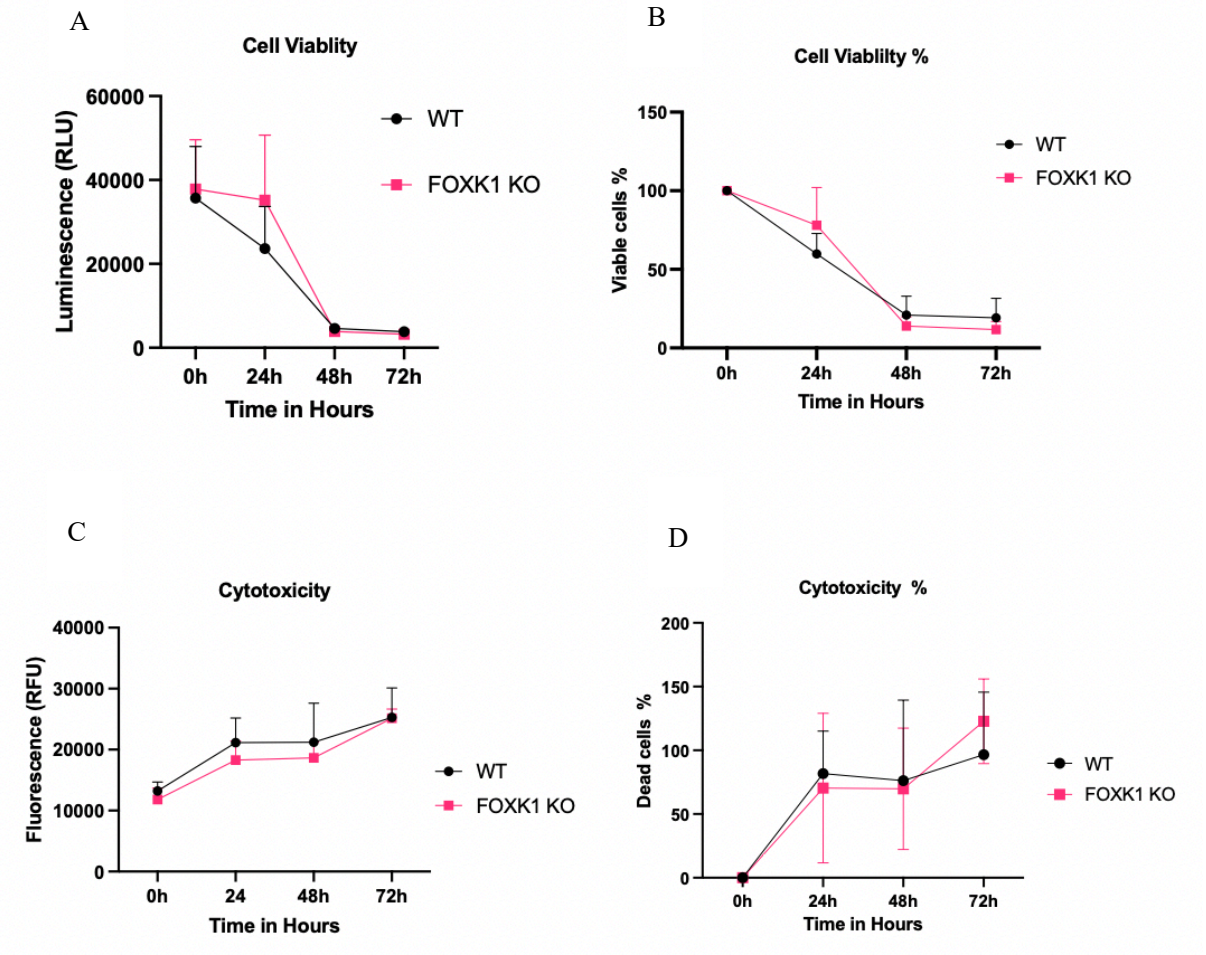


Figure 4.5 Cell viability and cytotoxicity assays of KO cells in comparison to HaCaT WT showed no difference between the cells

WT and FOXK1 KO HaCaT keratinocytes were cultured in low Ca^{2+} medium. Cell viability and cell death were determined using the RealTime-Glo MT Cell Viability Assay and the CellTox Green Cytotoxicity Assay (Promega) over 72 hours. **(A)** Cell viability luminescence signal for WT and KO. **(B)** % of viable WT and KO cells. **(C)** Cell cytotoxicity fluorescence signal for WT and KO. **(D)** % dead WT and KO cells over time. Statistical significance was calculated using a t-test (ns $P>0.05$) (n=3) and data are presented as the mean±SE.

4.3.6 Knocking out FOXK1 has no effect on cell migration in keratinocytes

A scratch assay is a method commonly used to investigate cell motility. It is a very simple technique in which a "wound" or scratch is produced on a sheet of confluent cells grown on coverslips or culture plates. The wound closure is monitored over time by microscopic imaging. Using this technique, a single-cell movement and/or cell migration of a group of cells can be studied (Bindschadler et al., 2007; La Porta and Zapperi, 2020). To investigate whether knocking out FOXK1 would affect cell migration, a wound-healing scratch assay was performed on WT HaCaT and FOXK1 knockout cell lines. The rate of cell migration was analysed by measuring the gap area after 0, 4, 8, 14 and 24 hours via Fiji software. It was shown that knocking out FOXK1 in muscle cells resulted in a delayed wound closure (Garrey et al., 2000), thus suggesting that knocking out FOXK1 in keratinocytes would affect cell migration. However, Figure 4.6 shows no significant difference in the rate of wound closure between HaCaT WT and FOXK1 KO cells, whereby suggesting that knocking out FOXK1 did not affect cell migration.

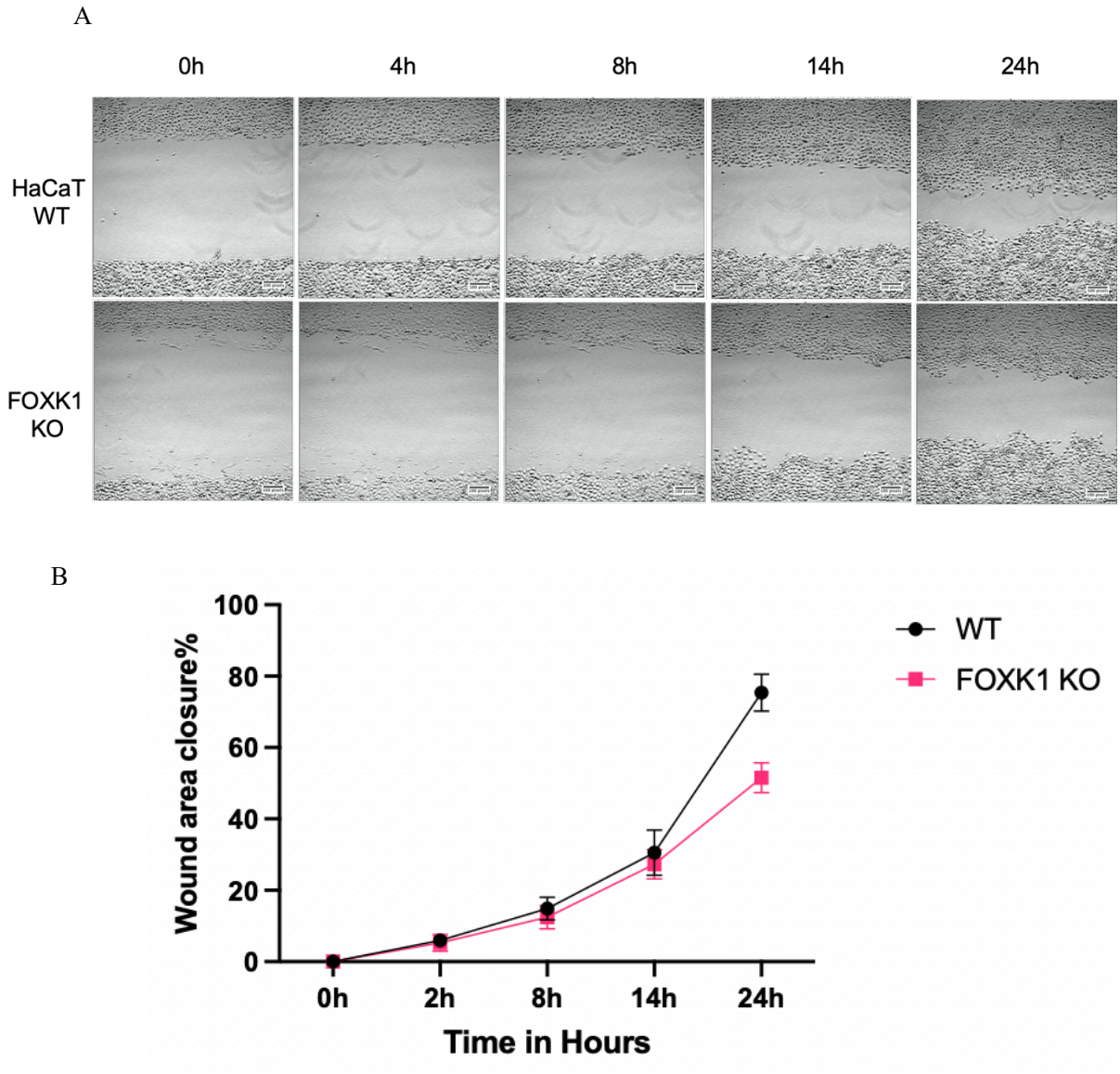


Figure 4.7 Knocking out FOXK1 in keratinocytes did not affect wound closure in the scratch assay

Scratch assay for WT and FOXK1 KO in low Ca^{2+} medium. **(A)** Representative images of wounds for HaCaT WT and FOXK1 cells 0, 4, 8, 14 and 24 hours after wounding. **(B)** % of the wound area closure of WT and FOXK1 KO cells. A t-test analysis was performed to compare the % of wound area closure over time (ns $P>0.05$) (n=3). Gap areas were measured via Fiji software. Error bars are presented as the mean±SE.

4.4 Discussion

The aim of this chapter is to produce FOXK1 KO keratinocyte cell lines and investigate the effect of knocking out FOXK1 upon keratinocyte differentiation. The CRISPR-Cas9 technique has been successfully used with HaCaT cells (Dahlhoff et al., 2017; Gao et al., 2017; Al-Raawi and Kanhere, 2020). Therefore, CRISPR-Cas9 gene editing was performed to produce FOXK1 knockout cells. Despite repeated attempts, however, I was able to generate only one FOXK1 KO cell line from sgRNA3. To produce cell lines, a single cell must be isolated and grown to produce a colony, but the majority of colonies die in the first stages of single-cell isolation. The exact procedure was used to produce several JARID2 KO in HaCaT cells (Al-Raawi and Kanhere, 2020); therefore, the reason of the unsuccessful attempts might be that FOXK1 is critical for the survival of keratinocytes. Moreover, sgRNA1 and sgRNA2 were designed to target the FOXK1 sequence at exon 2 similarly to sgRNA3; however, both were closer to the start of the exon, which could be that the two sgRNA sequences produced mutations which were toxic to the cell and might have caused cell death early in the single-cell isolation step.

Western blotting of whole-cell lysates for HaCaT wild-type and FOXK1 CRISPR knockout (FOXK1 KO) cells during differentiation (Figure 4.3) showed a similar observed finding that FOXK1 expression increased after differentiation induction, while FOXK1 was not detected in KO cells, whereby indicating the successful knockout of FOXK1. Involucrin (IVL) expression increased in all cells, which indicates the induction of differentiation. These findings support the RNAi knockdown data that FOXK1 is not critical for inducing differentiation. However, increased expression of FOXK1 during differentiation suggests that it might be involved in regulating other events in later stages of terminal differentiation.

Knocking out FOXK1 in HaCaT keratinocytes did not affect differentiation. However, what are the other processes that might be affected? Regarding the aforementioned question, I wanted to investigate the effect of an FOXK1 knockout upon cell viability, cell death, proliferation, and division in HaCaT keratinocytes.

5-ethynyl-2′-deoxyuridine (EdU) staining showed an interesting finding. The statistical analysis in Figure 4.4 B indicates a significant decrease (about 50%) in cell division in FOXK1 KO keratinocytes. As mentioned previously, most research on FOXK1 has been conducted on muscle cells, and the majority of findings have shown how FOXK1 is critical for muscle stem cell proliferation and cell cycle progression. Knocking out FOXK1 in mice resulted in impaired stem cell proliferation function and caused severe muscle atrophy (Garry et al., 2000). One study showed that BrdU staining demonstrated a 50% decrease in the FOXK1 KO muscle progenitor population as well (Hawke et al., 2003), and another study showed a lower number of cells in the S phase and a higher number in the G_0/G_1 phase in FOXK1 KO muscle progenitors due to increased expression of $p21^{CIP}$ (Li et al., 2017). It was shown that FOXK1 is involved in chromatin remodelling and histone modification through binding between the FHA domain of FOXK1 and the phospho-Thr49 of Sds3. This interaction recruits Sin3b to form a repression complex. FOXK1/2 was coupled with Sin3A and its core components (HDAC1, HDAC2, Sap30, Sds3, Rbbp4 and Rbbp7) (Shi and Garry, 2012; Shi et al., 2012). For that, it appears that FOXK1 has a similar function in regulating cell proliferation in keratinocytes, as in muscle stem cells. Thus, it would be interesting to investigate the effect of knocking out FOXK1 upon the cell cycle and chromatin remodelling in keratinocytes as well.

Interestingly, although the EdU assay showed a significant decrease in cell proliferation in KO cells, cell viability and cell death assays showed that there were no significant differences

between FOXK1 KO and WT cells (Figure 4.5). Knocking out FOXK1 has no significant effect on the rate of cell death in comparison with HaCaT WT, but it would be beneficial to examine apoptosis through caspase assays to investigate the effect of knocking out FOXK1 upon apoptosis. Because this particular cytotoxicity assay measures cell death based on cell membrane integrity, it does not differentiate between different types of cell death (apoptosis and necrosis). Thus, it would be more beneficial to combine it with apoptosis assays to examine the effect of knocking out FOXK1 in keratinocytes.

Nonetheless, when trying to explain the results presented in Figure 4.5 A and B, it is important to distinguish between cell viability and proliferation. While they are usually used interchangeably as a term that refers to live cells, they hold different meanings. Cell viability refers to cellular metabolic activity, its growth in mass, and its overall health, while cellular proliferation is concerned with the growth rate of the cell population through division (Su et al., 1998; Khan, 2019). Here, there are no significant differences in cell viability between the two cell lines. However, it is shown from previous data in Figure 4.4 that after overnight incubation with EdU, there was about 50% less cell proliferation in FOXK1 KO in comparison to WT. The principle of the RealTime Glo MT Cell Viability Assay is that the metabolically active cell reduces prosubstrate to substrate. The NanoLuc luciferase enzyme rapidly degrades substrate and provides luminescence; thus, there is no accumulation of a luminescence signal over time and the signal detected at that specific time is correlated with the number of metabolically active viable cells. Therefore, there should be less of a viability signal in FOXK1 KO unless the FOXK1 KO cells are metabolically more active than the WT. Research on FOXK1 showed that it has a function in the regulation of metabolism (He et al., 2018; Sakaguchi et al., 2019; Wang et al., 2019). The knockdown of FOXK1 and FOXK2 showed

upregulation in mitochondrial oxidative phosphorylation and ATP synthesis genes. Interestingly, the knockdown caused an alteration in the mitochondrial size and morphology; moreover, it caused an increase in the copy number of the mitochondrial genes mt-Nd1 and mt-Nd6 — part of the NADH dehydrogenase 1 enzyme (which is involved in ATP synthesis) (Sakaguchi et al., 2019). Therefore, it can be speculated that knocking out FOXK1 in HaCaT caused upregulation in mitochondrial oxidative phosphorylation and ATP synthesis genes as well. An investigation into the effect of FOXK1 knockout upon the mitochondrial morphology of HaCaT cells and activity is recommended so as to gain more detailed data on the role of FOXK1 in keratinocyte metabolism.

However, another explanation regarding these findings could be an effect of the in vitro experiment design itself. Reproducibility research showed that replicating metabolic activity results in vitro depends on strictly optimised parameters such as the nutrient concentrations, O_2, pH, and cell density in cell culture (Wright et al., 2018; Hirscha and Schildknecht, 2019; Chacko and Eliceiri, 2019). Here, cells were seeded at $5X10^3$ cells per well and then left for 24 hours, at which point the first reading was taken. It could be that at the 24-hour reading mark (42 hours after seeding), WT cells had reached the maximum proliferation capacity, while the FOXK1 KO cells still had more room with which to divide. ATP-based approaches do not give clear data on the state of cells (e.g. whether cells divide slowly or are quiescent) (Adan et al., 2016). Furthermore, the cells were kept in the same medium for over 72 hours. Confluency, the reduction of nutrients, and the accumulation of cellular waste inhibit certain cellular activities or become toxic to cells (Wright et al., 2018; Chacko and Eliceiri, 2019). Such a difference in conditions was shown to affect toxicology and drug testing in cancer research (Chacko and Eliceiri, 2019; Niepel et al., 2019). Another explanation is the difference between

the technical and biological repeats caused by the variability of experimental results. The variability of results could be due to a change in one or more of experimental parameters such as pipetting, cell handling, and plate reader performance (Hirscha and Schildknecht, 2019). During cell culturing, FOXK1 KO cells stick together more than do WT and are more difficult to count under a microscope because of their attachment to one another; therefore, it might be that there were more KO cells seeded than expected, which caused the results shown.

To conclude, more optimisation of the viability assay are recommended. Growth curve optimisation for multiple seeding densities for HaCaT WT and FOXK1 KO cells is recommended where cells reach confluency after the 72-hour seeding time point. Furthermore, it would be interesting to follow up the viability assay with testing the effect of confluency of the rate of cell division though single-cell monitoring and real-time imaging, as well as to examine cellular quiescence in both cell lines.

Cell migration is a critical process in biology. Cells move from one location to another during embryogenesis and development as well as during the immune response and wound repair following an injury; moreover, cancer cells move from one location to another through metastasis as well, and understanding this process is very important for cancer prognosis (Bindschadler et al., 2007; La Porta and Zapperi, 2019). Garry et al. (2000) reported that mice undergoing a FOXK1 KO suffered from severe muscle atrophy and that the wound-healing process following muscle injury was dysregulated and disrupted. Therefore, I wanted to examine whether a reduction in the proliferative ability of FOXK1 KO cells would affect cell migration in keratinocytes. I chose the wound-healing scratch assay to test migration between HaCaT WT and FOXK1 KO cells (Figure 4.6). The results indicated no significant differences in the rate of wound closure between the WT control and the FOXK1 KO cells; as presented

in Figure 4.6, however, it was found that the rate of cell division decreased in FOXK1 KO keratinocytes. One possibility with which to explain this finding is the nature of the "wound scratch assay" being a procedure that is performed on a monolayer sheet of cells, differing from the natural organisation of normal cells, which contain multiple layers of cells with varying degrees of cellular differentiation, as well as other biological and biochemical components that maintain the skin as an organ. In typical skin injury, blood vessels provide the wounded area with platelets and inflammatory cells, which produce growth factors, cytokines, and other biochemicals important for the healing process. Proliferation and re-epithelialisation are where cell division and migration are the main processes of healing, wherein it could take days and weeks for the wound to heal completely (Demidova-Rice et al., 2021; Dekoninck and Blanpain, 2019). Being grown in a laboratory and in a monolayer might limit the potential of some of these steps; for that, one might argue that this method does not equate to an actual wound. However, this method monitors cellular movement in a well-defined condition (Grada et al., 2017). Other parameters might affect cell migration in the cell culture environment, such as adhesion to the culture plate surface, cell–cell adhesion, and cell density (Tlili et al., 2018; La Porta and Zapperi, 2020). It was found that cells sensed their proximity and coordinated cell proliferation, migration velocity, and distance accordingly (Bindschadler et al., 2007; La Porta and Zapperi, 2020). In the initial phases after wounding, cells at the wound edge will spread so as to fill the empty space. The speed and distance of cellular movement could be affected by cell–cell adhesion. The process of proliferation increases as well in order to compensate for the loss of cells after wounding. However, it was found that as the space decreases as the density of the cell population increases, cell division and migration decrease because confluent cells lose motility due to jamming. Therefore, the results from Figure 4.6 could be explained as follows: it is possible that cell migration in HaCaT WT was slower due to the rate of division

in comparison to the slower division in FOXK1 KO cells. The HaCaT WT population increases at a faster rate than that of FOXK1 KO cells, and this increase limits the space for cellular movement. On the contrary, FOXK1 cells have a slower rate of proliferation, but cells might compensate for this by spreading and moving as single cells or as sheets. However, the factors affecting cellular migration, distance, velocity, and direction have yet to be fully understood (Tlili et al., 2018; La Porta and Zapperi, 2020). Therefore, it would be interesting to investigate the role of FOXK1 in cell motility, migration and adhesion in keratinocytes. It might be informative to examine cell migration by means of other assays such as live single-cell tracking and adhesion assays to obtain clearer data on FOXK1 KO in keratinocytes.

In summary, knocking out FOXK1 in keratinocytes did not affect the induction of differentiation, nor did it affect the rate of cell death. However, FOXK1 KO cells were found to have a significantly slower rate of proliferation than did the wild-type cells. The slower rate of proliferation did not affect cell migration in the wound scratch assay. Whether knocking out FOXK1 in keratinocytes affected cellular metabolic activity has yet to be confirmed.

Chapter 5

5 Analysis of Gene Expression in FOXK1 Knockout Keratinocytes During Differentiation

5.1 Introduction

In the previous chapter it was shown that FOXK1 is not critical for inducing differentiation in keratinocytes (Figure 4.3). However, FOXK1 knockdown caused a slower rate of proliferation in undifferentiated keratinocytes (Figure 4.4). This finding is consistent with findings in FOXK1 research on muscle cells that reduced FOXK1 expression caused a slower rate of proliferation in muscle progenitor cells and that knocking out FOXK1 caused muscle atrophy and impaired the muscle-healing process (Garry et al., 2000; Hawke et al., 2003; Li et al., 2017). FOXK1-mediated gene expression regulation during keratinocyte proliferation and differentiation have yet to be investigated, so this experiment aimed to identify the genome-wide effect of a FOXK1 knockout upon gene expression in keratinocytes. Genome-wide RNA sequencing in HaCaT WT and FOXK1 KO cells can help to identify the role of FOXK1 in gene expression regulation in keratinocytes. Therefore, next-generation RNA sequencing (RNA-seq) was performed to identify FOXK1-regulated genes in HaCaT cells. However, in this chapter, the main focus of the data analysis was to analyse the genes which were differentially regulated by FOXK1 during differentiation. This analysis showed that FOXK1 caused the upregulation of keratinocyte differentiation genes and the downregulation of cell proliferation and cell division genes.

5.1.1 Genes and proteins used for validation experiments

In this chapter, some genes were chosen to validate RNA-seq data by means of and RT-qPCR and western blotting. CDK1, CDC45, cyclinD1, ΔNP63, proliferating cell nuclear antigen (PCNA), phospho-histone 3 (PHH3), cytokeratin 19 (KRT19), and keratin5 (KRT5) are used in this chapter as cell cycle, cell proliferation, and basal stem markers. Keratin1 (KRT1), keratin10 (KRT10), involucrin (IVL), and transglutaminase1 (TGM1) are used in this chapter as differentiation markers.

5.2 Results

5.2.1 RNA-Seq of HaCaT WT and FOXK1 KO cells in basal and differentiated states

RNA-Seq is an experimental technique which uses deep sequencing to measure the expression of all genes in the genome (Wand et al., 2009; Kukurba and Stephen, 2015; Stark, et al., 2019). RNA-Seq is widely used because it can provide data on the entire transcriptomes of the cell (including mRNA, pre-mRNA, and non-coding RNA). It is a very sensitive method that generates data (even with nanograms of RNA) and is highly reproducible (Wang et al., 2009; Kukurba and Stephen, 2015). In general, an RNA-Seq experiment is carried out in three main steps: pure RNA extraction, synthesis of cDNA library, and then sequencing. Thereafter, the data are processed computationally by mapping the short reads produced against a reference genome or transcriptome. The results then provide a quantitative understanding of the changes in gene expression between the biological samples or the different conditions (Figure 5.1) (Wand et al., 2009; Kukurba and Stephen, 2015; Stark et al., 2019). For that, RNA-Seq was performed on RNA samples which were extracted from HaCaT WT and FOXK1 KO cells on days 0 and 3 of differentiation, as mentioned in Materials and Methods (section 2.6) and summarised (Figure 5.2).

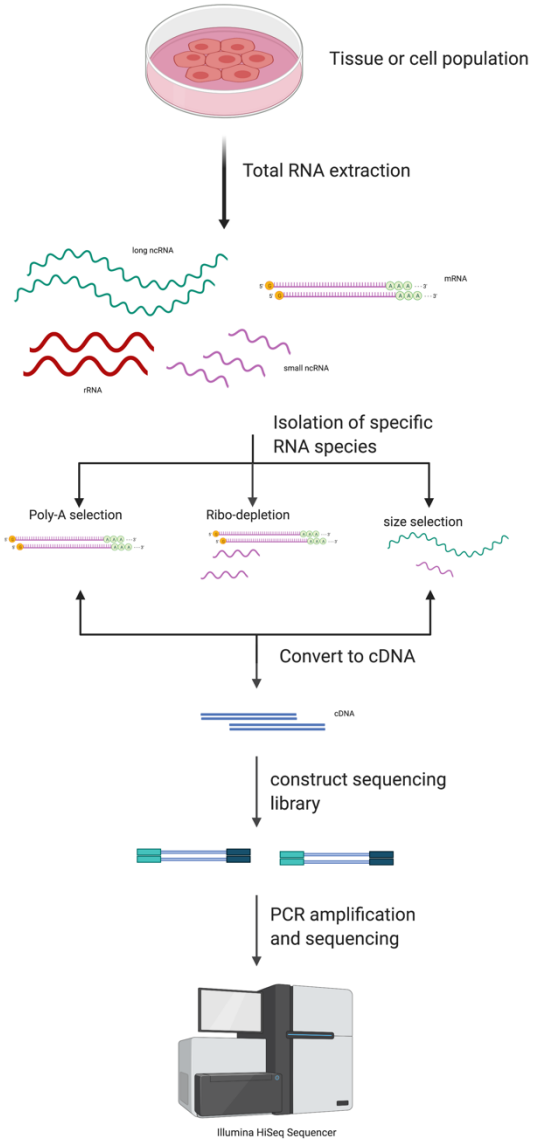


Figure 5.1 Overview of RNA-Seq experiment workflow

Firstly, total RNA is isolated from the sample of choice. Secondly, specific RNA species are isolated, such as choosing to enrich for polyadenylated transcripts. Next, RNA is converted to cDNA through reverse transcriptase with the addition of sequence adaptors at the end of the cDNA fragments. Finally, PCR amplification and sequencing are carried out (adapted from Kukurba and Stephen, 2015).

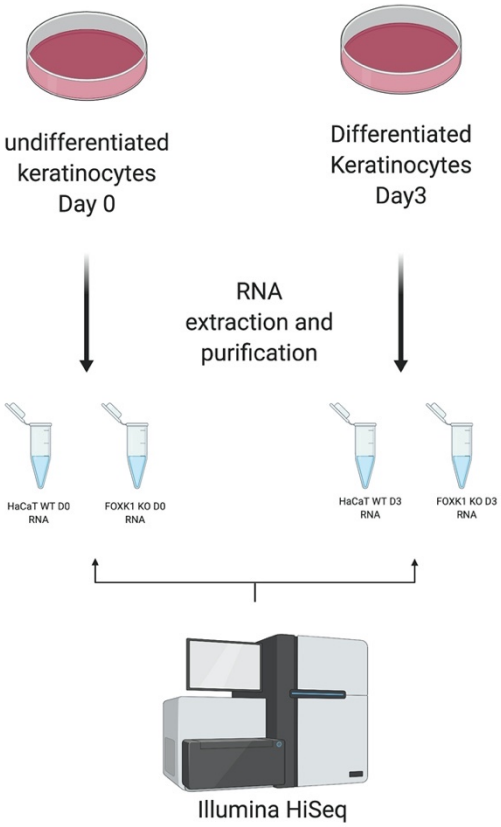


Figure 5.2 An overview of RNA-Seq sample preparation

Genome-wide RNA sequencing was performed on HaCaT WT and FOXK1 KO keratinocytes on days 0 and 3 of differentiation. RNA-Seq libraries were prepared using the TrueSeq method and sequenced on an Illumina HiSeq 2500 (University of Birmingham).

5.2.2 RNA-Seq analysis of HaCaT WT and FOXK1 KO cells showed upregulation of differentiation genes and downregulation of proliferation genes

First, RNA-Seq data were analysed to identify the wide effect of a FOXK1 knockout upon gene expression in keratinocytes from day 0 to day 3 after inducing differentiation in HaCaT WT as well as FOXK1 KO cells. To begin with, analysis for gene expression changes in WT during differentiation was performed to have a baseline understanding of gene expression in Ca^{+2}-induced differentiation in HaCaT wild type, then compared to the effect of FOXK1 KO on gene expression during differentiation. A total of 2920 genes changed in expression in WT during differentiation. The total number of genes which were upregulated during differentiation in WT was 1429, while the total number of downregulated genes in WT during differentiation was 1489 (Figure 5.3 A). As for the FOXK1 KO, 3204 genes changed in expression during differentiation. The total number of genes which were upregulated during differentiation in KO was 1598, while the total number of downregulated genes in KO cells during differentiation was 1606 (Figure 5.3 B). A Venn diagram (Figure 5.4) was used to map the gene lists to identify the number of overlapping and non-overlapping genes between the WT and KO cell lines. Out of the 1429 upregulated genes of WT, 992 genes overlapped with the 1598 KO upregulated genes. As for the 1498 genes which were downregulated in WT cells during differentiation, 1051 genes overlapped with 1606 KO downregulated genes during differentiation (Figure 5.4 A).

The DAVID functional annotation tool was used to determine which biological pathways were affected by differentiation and whether there are differences in these pathways between the WT and FOXK1KO keratinocytes on days 0 and 3 of differentiation. The Database for

Annotation, Visualization and Integrated Discovery (DAVID) is a web-based bioinformatic tool which helps researchers in the interpretation of big data gene and protein lists to annotate functional groups (Jiao et al., 2012). As expected, epidermal differentiation and keratinisation genes were upregulated while cell division and proliferation genes were downregulated in day 3 differentiated WT cells in comparison to day 0 WT undifferentiated cells (Figure 5.4 B and C). Interestingly, similar findings were shown when examining functional annotation of genes from the FOXK1 KO cells. In KO cells, cell division and cell cycle genes were downregulated, while epidermal differentiation and keratinisation genes were upregulated (Figure 5.4 D and E). In conclusion, a high Ca^{2+} assay indeed induced differentiation in keratinocyte cells, and knocking out FOXK1 did not block differentiation.

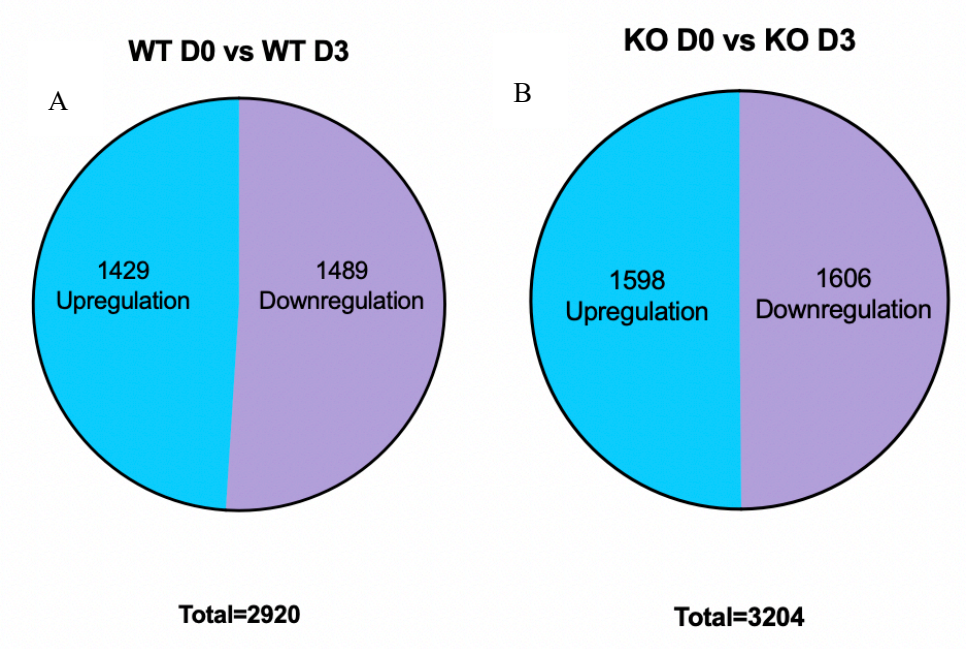
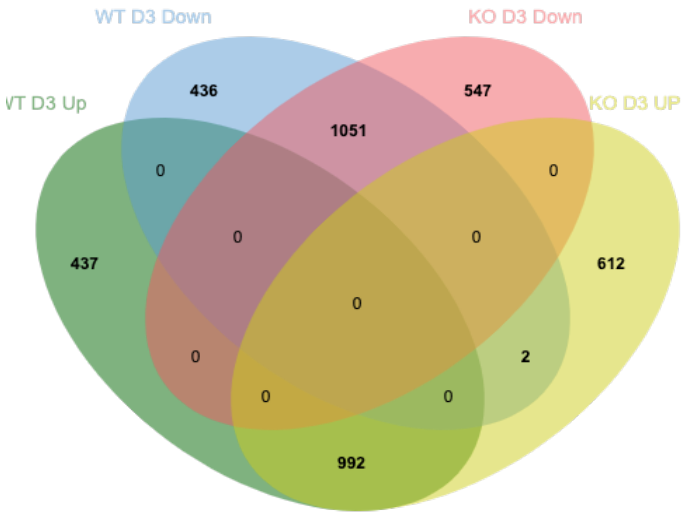


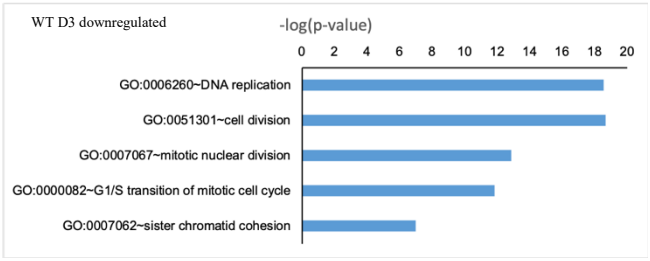
Figure 5.3 Changes in gene expression in HaCaT WT compared to FOXK1 KO during differentiation

(A) A pie chart presenting the total gene expression changes in HaCaT WT during differentiation. Upregulated genes in blue and downregulated genes in violet. **(B)** A pie chart presenting the total gene expression changes in FOXK1 KO cells during differentiation (gene lists were generated by Dr Aditi Kanhere).

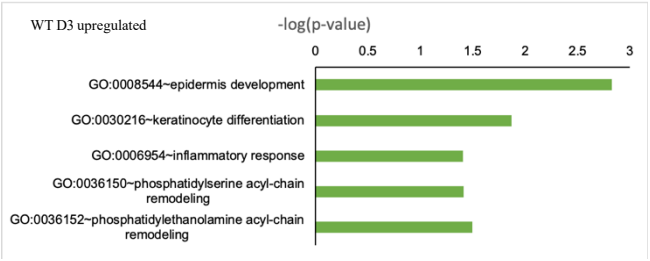
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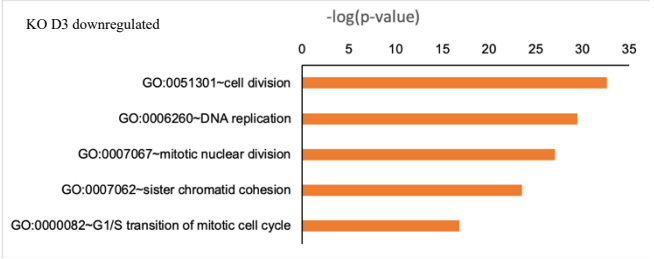
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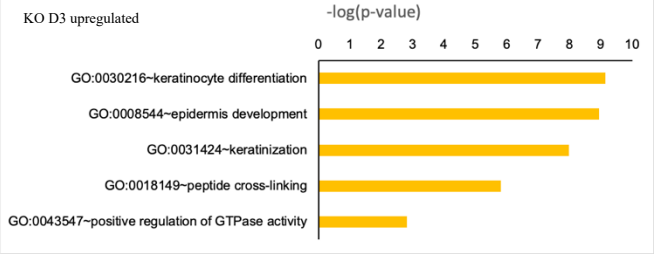
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Figure 5.6 Functional annotation of the gene expression changes in HaCaT WT and FOXK1 KO keratinocytes during differentiation

Summary of the number of significantly upregulated and downregulated genes in WT and FOXK1 KO on days 0 and 3 of differentiation. **(A)** A Venn diagram of genes which were upregulated and downregulated in WT and KO cells during differentiation. The Venn diagram shows the number of overlapping and non-overlapping genes between WT and KO during differentiation. Using the DAVID, the functional categories of the top genes which were statistically significant were obtained. **(B)** This shows the most statistically significant genes which were downregulated in WT cells during differentiation. **(C)** This shows the most statistically significant genes which were upregulated in WT cells. **(D)** This shows the most statistically significant genes which were downregulated in FOXK1 KO cells during differentiation. **(E)** This shows the most statistically significant genes which were upregulated in FOXK1 KO cells during differentiation (gene lists and functional annotation were performed by Dr Aditi Kanhere).

5.2.3 FOXK1 might be required for the upregulation of cell proliferation genes and downregulation of cell differentiation genes

In this section, the main aim was to analyse genes which were differentially regulated by FOXK1 during differentiation. RNA-Seq data were further analysed to profile the gene expression differences between FOXK1 KO and HaCaT WT on days 0 and 3 of differentiation. On day 0, 732 genes showed more than a twofold change in the KO in comparison with WT (Figure 5.5 A). Among these, 260 were upregulated genes and 462 were downregulated genes. RNA-Seq data on day 3 of differentiation showed that 932 genes displayed more than a twofold change in expression. Among these, 464 were upregulated genes and 468 were downregulated genes (Figure 5.5 B). A detailed analysis of common and unique genes between the two time points in FOXK1 KO cells in comparison with WT cells is presented with the help of a Venn diagram (Figure 5.6 A).

Further analysis by DAVID of the differentially regulated genes by FOXK1 during differentiation revealed that cell division and proliferation genes were downregulated in FOXK1 KO cells in an undifferentiated state (day 0) (Figure 5.4 B), while genes associated with development were upregulated in FOXK1 KO cells (Figure 5.4 B). Subsequently, the functional annotation grouping for differentiated cells (day 3) showed a more prominent effect of knocking out FOXK1 in keratinocytes. It was identified that genes associated with cell cycle transition, DNA replication, and cell division, such as CDC45, CDK1 and cyclin B1, were remarkably downregulated in FOXK1 KO cells (Figure 5.5 A). Strikingly, genes associated with differentiation, keratinisation, and protein cross-linking, such as involucrin, keratin 9, and transglutaminase, were among the most significantly upregulated genes in FOXK1 KO cells (Figure 5.5 B). To summarise, the changes in gene expression after knocking out FOXK1 show

that it might have an important role in upregulating cell proliferation genes and downregulating differentiation genes in keratinocytes.

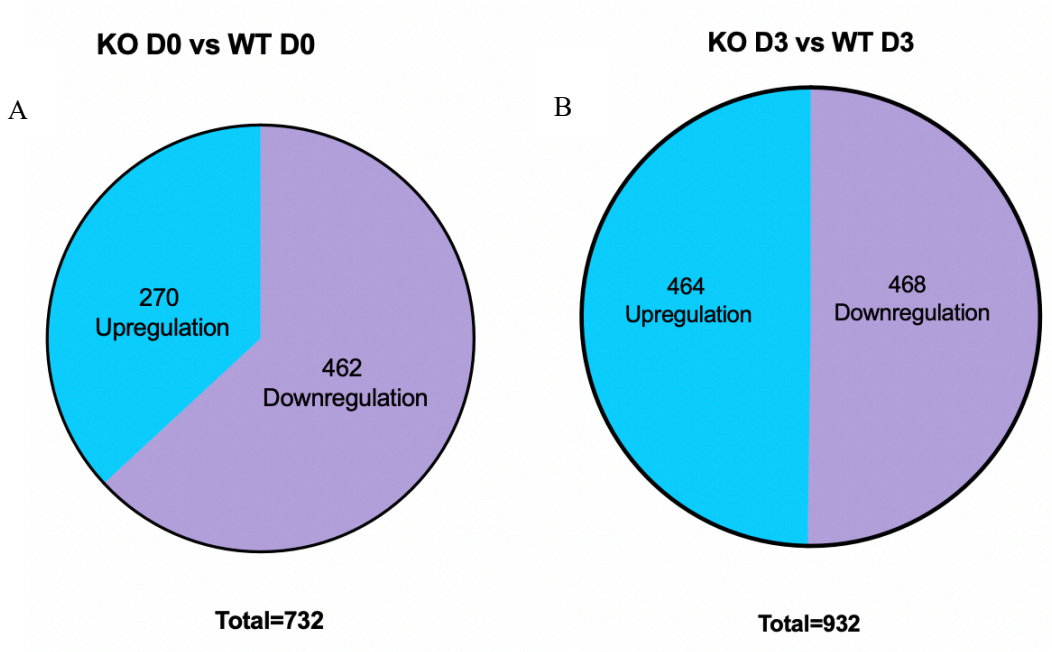
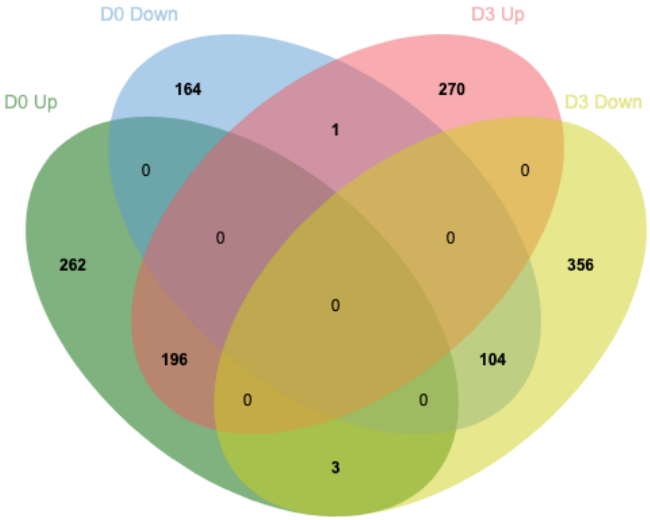


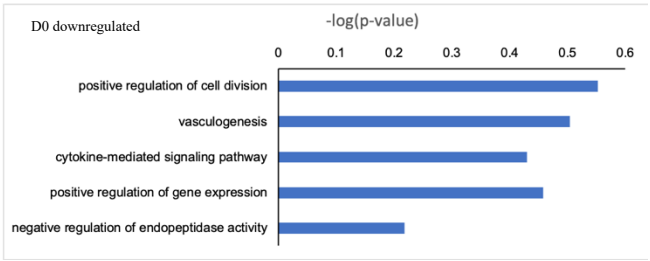
Figure 5.9 Changes in gene expression in FOXK1 KO compared to HaCaT WT

(A) A pie chart presenting the gene in which there were more than twofold changes between FOXK1 KO and HaCaT WT in an undifferentiated state (day 0). **(B)** A pie chart presenting the gene in which there were more than twofold changes between FOXK1 KO and HaCaT WT in a differentiated state (day 3) (gene lists were generated by Dr. Aditi Kanhere).

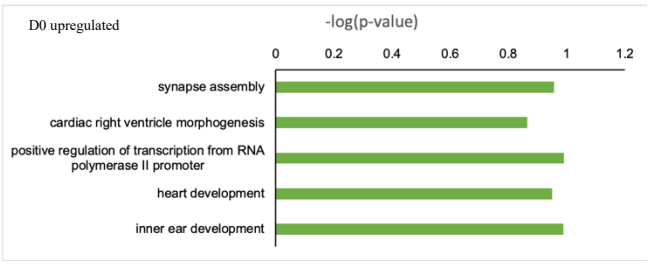
A



B



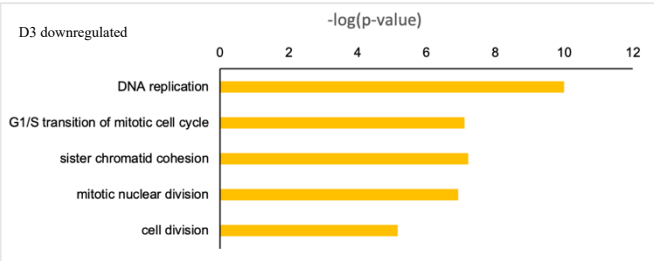
C



D



E



Caption is on the next page

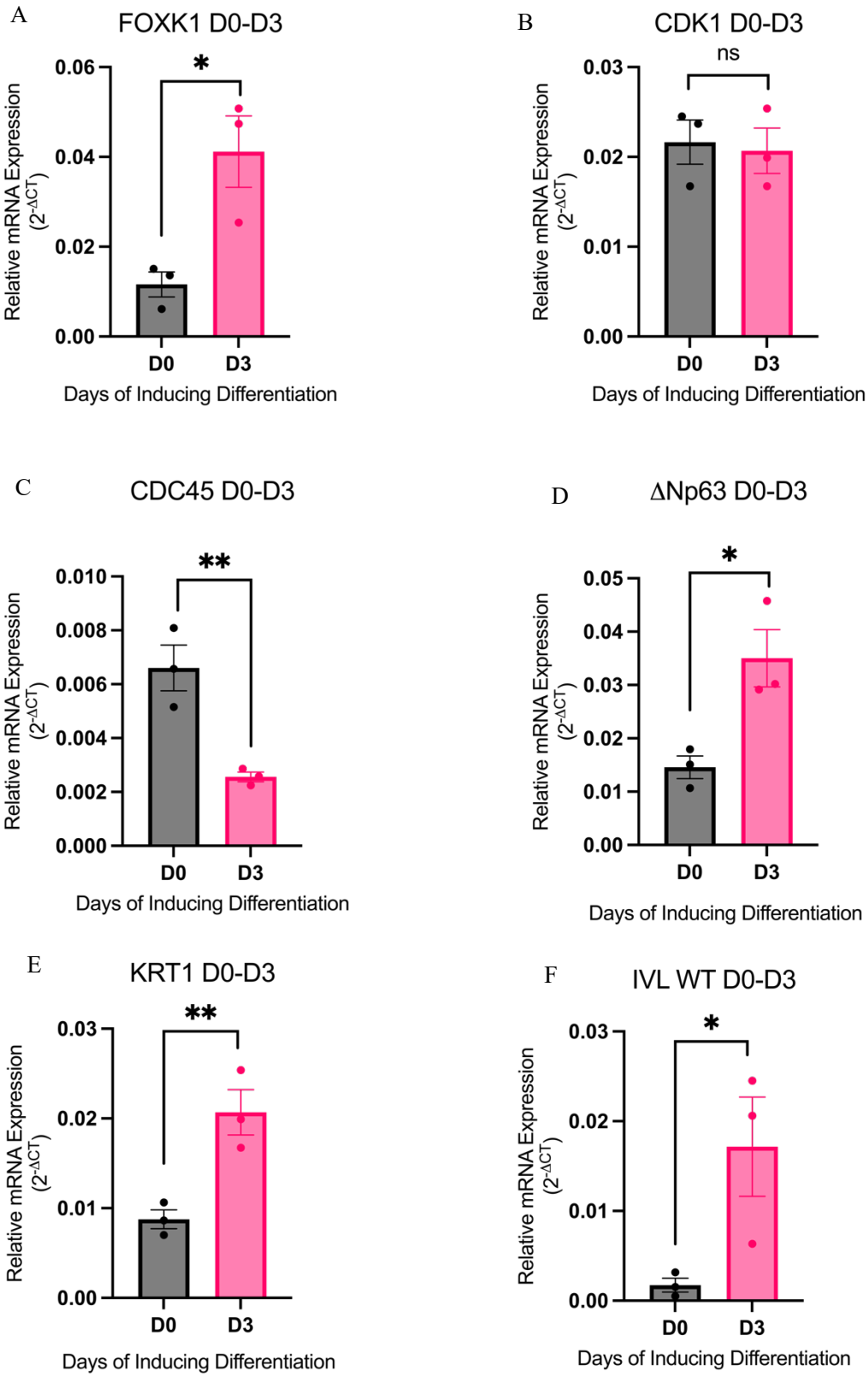
Figure 5.12 Functional annotation for gene expression changes between HaCaT WT and FOXK1 KO keratinocytes during differentiation

A summary of the significantly downregulated and upregulated genes in FOXK1 KO cells in comparison to WT on day 0 and day 3 of differentiation. **(A)** A Venn diagram of genes which were upregulated and downregulated in FOXK1 KO in comparison with WT cells during differentiation. The Venn diagram showed the number of overlapping and non-overlapping genes between WT and KO during differentiation. Using the DAVID, the functional categories of the top genes which were statistically significant were optioned. **(B)** This shows the most statistically significant genes which were downregulated in FOXK1 KO cells in an undifferentiated state (day 0). **(C)** This shows the most statistically significant genes which were upregulated in FOXK1 KO cells in an undifferentiated state (day 0). **(D)** This shows the most statistically significant genes which were upregulated in FOXK1 KO cells during differentiation (day 3). **(E)** This shows the most statistically significant genes which were downregulated in FOXK1 KO cells during differentiation (day 3) (gene lists and functional annotation were performed by Dr. Aditi Kanhere).

5.2.4 Cell proliferation genes were downregulated in HaCaT WT during differentiation while differentiation genes were upregulated

To validate the RNA-Seq results, RT-qPCR was used to measure RNA expression for FOXK1 and the cell cycle and cell division genes CDK1 and CDC45, as well as for ΔNP63 as a basal cell marker and the genes of involucrin (IVL) and keratin1 (KRT1) as differentiation markers. Figure 5.7 shows the relative mRNA expression of the selected genes in WT cells on day 0 in comparison with day 3 of differentiation induction. As expected, FOXK1 mRNA expression was induced in WT on day 3 in comparison to day 0 (Figure 5.7 A). CDK1 promotes cell cycle progression from the G_2 phase to the M phase by binding between CDK1 and cyclinB1 (Malumbres, 2020). CDC45 is a member of the CDC45–MCM–GINS complex (CMG) that is essential for DNA unwinding at the replication fork (Simon et al., 2016). RT-qPCR showed no difference in CDK1 expression during differentiation; however, CDC45 mRNA expression showed a significant (**P<0.01) decrease on day 3 of differentiation. The level of CDK1 mRNA expression on day 0 and day 3 could indicate that it has regulatory roles other than cell cycle regulation. It was shown that CDK1 regulated cell morphology, which is an important factor in cell differentiation. Additionally, CDK1 regulates translation and is involved in DNA damage repair processes (Kalous et al., 2020). Nevertheless, it would be interesting to validate whether CDK1 protein levels are constant during differentiation as well. However, the cell division marker CDC45 showed decreased mRNA levels on day 3 in comparison to day 0. CDC45 decreased expression could indicate a reduction of cell division and that cells exited proliferation and entered terminal differentiation. Interestingly, the ΔNP63 mRNA levels in Figure 5.7 D present a significant increase during differentiation, although ΔNP63 is known to be a basal stem marker for keratinocytes and its expression decreases during terminal

differentiation (Truong et al., 2006). However, an increased mRNA level of transcription factor does not necessarily mean increased expression of its target genes. It would be interesting to examine ΔNP63 protein levels as well. As expected, IVL and KRT1 differentiation markers showed significant induction on day 3 in comparison to day 0, whereby supporting previously observed results that switching to a high Ca^{2+} medium induces keratinocyte differentiation. In conclusion, switching to high Ca^{2+} medium promoted cell cycle exit and induced terminal differentiation in HaCaT keratinocytes.



Caption is on the next page

Figure 5.15 Expression of FOXK1, cell cycle, basal, and differentiation marker genes in HaCaT WT cells during differentiation

FOXK1, cell cycle, basal, and differentiation marker mRNA levels were compared in WT between days 0 and 3 of differentiation by means of RT-qPCR. **(A)** The relative mRNA level of FOXK1. **(B–D)** Relative mRNA levels for CDK1, CDC45, and ΔNP63 as cell cycle, cell division, and basal markers, respectively. **(E–F)** Relative mRNA levels for IVL and KRT1 as differentiation markers. GAPDH mRNA was used as a housekeeping gene control. Data from the three separate experiments (n=3) are represented as the mean±SE, and multiple comparisons were performed using Student's t-test (**P<0.01, *P<0.05 and ns P>0.05).

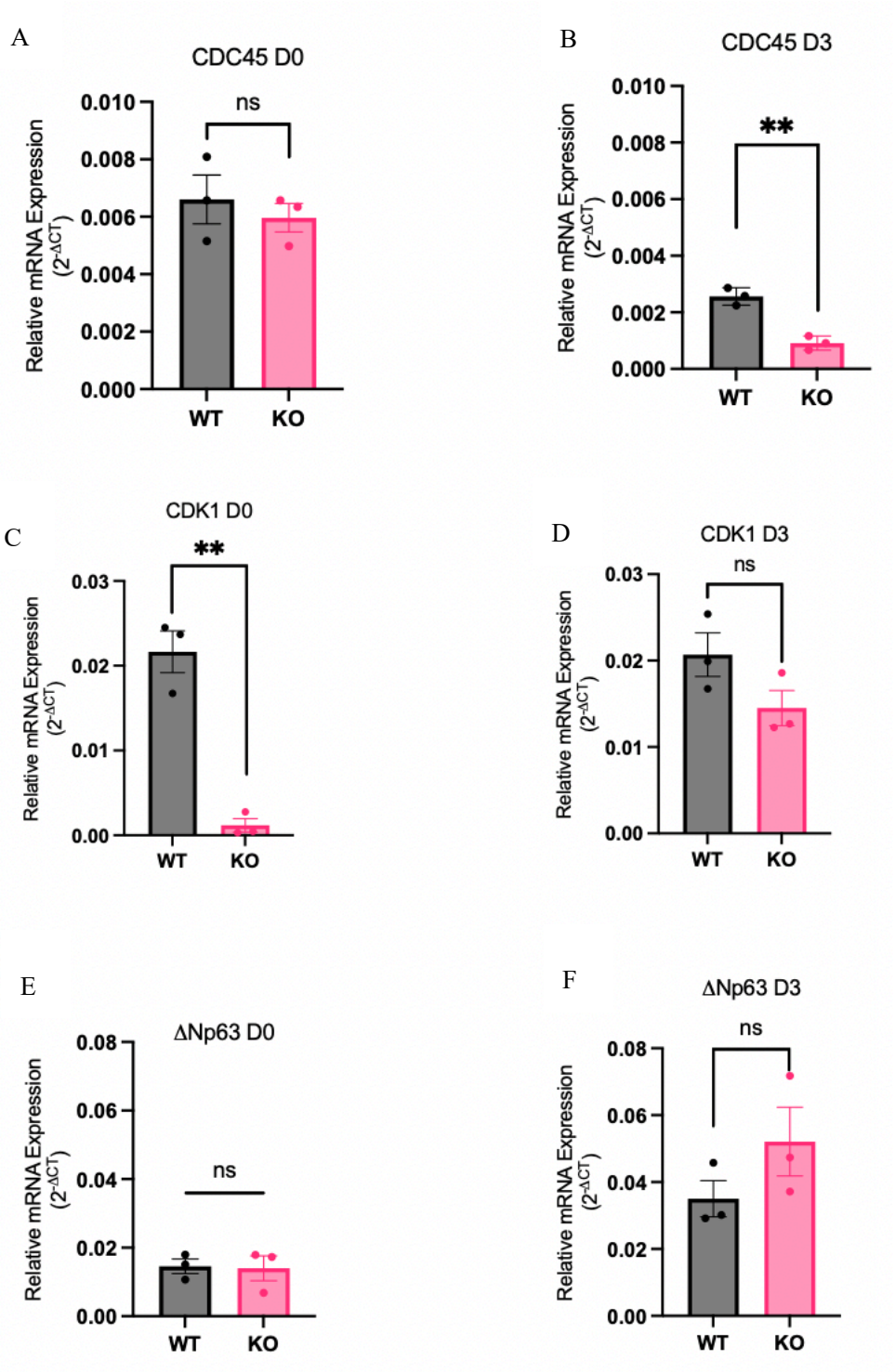
5.2.5 FOXK1 is involved in regulating keratinocyte proliferation and differentiation

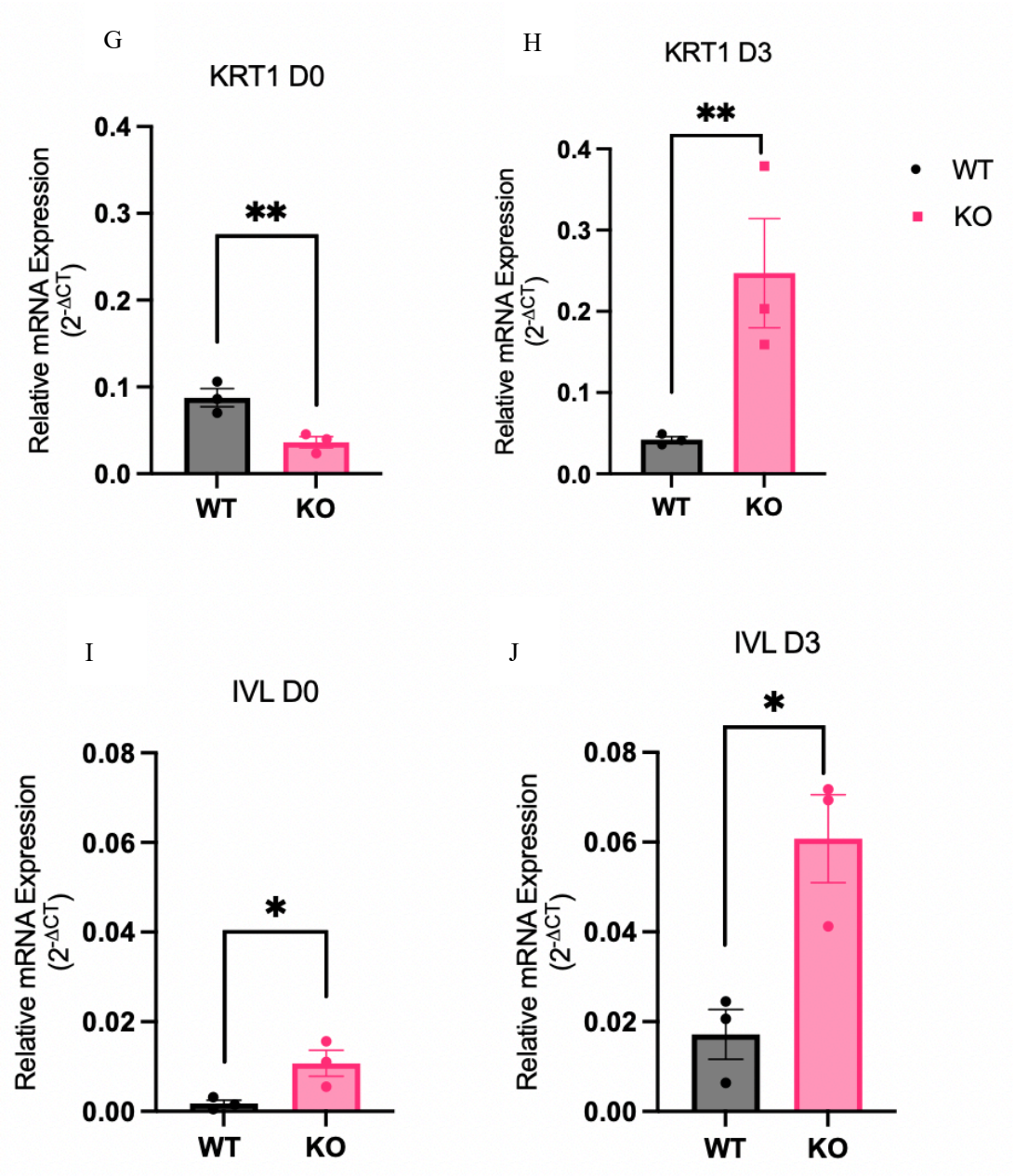
FOXK1 regulates the cell cycle and cell differentiation in muscle cells (Garry et al., 2000; Hawke et al., 2003; Freddie et al., 2007; Shi et al., 2012; Wencong et al., 2020), therefore, it was speculated that FOXK1 might have a similar role in keratinocytes. RNA-Seq functional annotation data showed that cell cycle and cell division genes were downregulated while epidermal differentiation genes were upregulated in FOXK1 KO in comparison to HaCaT WT cells (Figure 5.6 B–E). To validate these findings, the expression of cell cycle and cell division genes CDK1 and CDC45, as well as ΔNP63 as a basal cell marker in FOXK1 KO cells versus HaCaT WT was analysed using RT-qPCR. In addition to these genes, the differentiation marker genes involucrin (IVL) and keratin1 (KRT1) in FOXK1 KO cells versus HaCaT WT were analysed by means of RT-qPCR.

Figure 5.8 shows the relative fold change in mRNA levels of KO genes versus WT genes on day 0 and day 3 of differentiation. The measurements showed no significant change in mRNA levels of CDC45 between WT and KO in an undifferentiated state. However, the significant downregulation of mRNA levels in KO cells in comparison to WT on day 3 of differentiation suggested decreased cell division after differentiation induction. In contrast, CDK1 showed a significant decrease in mRNA expression on day 0 in KO cells in comparison to WT, which suggests that the KO cells had a disturbed cell cycle in comparison to WT; meanwhile, there was no significant reduction in mRNA expression on day 3 of differentiation in KO in comparison to WT cells (Figure 5.8 C and D). ΔNP63 mRNA levels were similar between the two cell lines on day 0 and day 3 of differentiation (Figure 5.8 E and F), which suggests that FOXK1 had no effect on keratinocyte basal markers; however, it would be interesting to

examine the effect of knocking out FOXK1 upon more basal markers such as keratin 5 and keratin 14, integrins, and cadherins. Interestingly, the expression levels of KRT1 and IVL mRNA significantly increased in KO in comparison to WT cells on both day 0 and day 3 of differentiation, whereby suggesting that FOXK1 might regulate differentiation genes (Figure 5.8 G–J).

Taken together, these observations from Figure 5.8 A–J. FOXK1 might be involved in regulating some of the cell cycle components promoting cell proliferation and regulating keratinisation in keratinocytes.


A
CDC45 D0
ns
Relative mRNA Expression
(2-ΔCT)
0.010
0.008
0.006
0.004
0.002
0.000
WT
KO
B
CDC45 D3
**
C
CDK1 D0
**
0.03
0.02
0.01
0.00
D
CDK1 D3
ns
E
ΔNp63 D0
ns
0.08
0.06
0.04
0.02
F
ΔNp63 D3
ns



Caption is on the next page

Figure 5.18 Expression of cell cycle, basal, and differentiation marker genes in undifferentiated (day 0) and differentiated (day 3) states in FOXK1 KO versus WT cells

Cell cycle, basal, and differentiation marker mRNA levels were compared in FOXK1 versus WT on days 0 and 3 of differentiation by means of RT-qPCR. **(A–B)** The relative mRNA level of CDC45 as a cell division marker on day 0 and day 3 in KO versus WT. **(B–D)** Relative mRNA levels for CDK1 as a cell cycle marker on days 0 and 3 of differentiation in KO versus WT. **(E–F)** Relative mRNA levels for ΔNP63 as a basal marker on days 0 and 3 of differentiation in KO versus WT. **(G–J)** Relative mRNA levels for IVL and KRT1 as differentiation markers on days 0 and 3 in KO versus WT. GAPDH mRNA was used as a housekeeping gene control. Data from the three separate experiments (n=3) are represented as the mean±SE, and statistical analysis was conducted using Student's t-test (**P<0.01, *P<0.05 and ns P>0.05).

5.2.6 Effect of knocking out FOXK1 on cell proliferation, basal, and differentiation marker protein expression

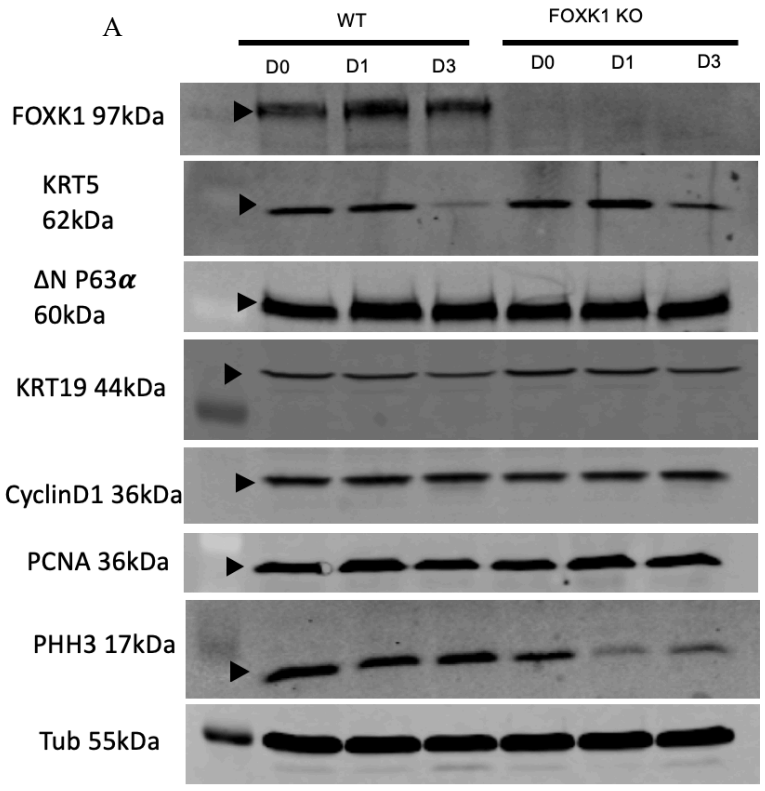
The RNA-Seq data indicated that knocking out FOXK1 resulted in the upregulation of differentiation gene expression and downregulation of cell cycle and cell division gene expression, therefore, it was speculated that knocking out FOXK1 has an effect on cell cycle, proliferation, basal, and differentiation proteins as well. For that, several proteins were chosen to validate whether gene expression affected protein levels by means of western blotting. Western blotting was carried out on HaCaT WT and FOXK1 KO cells during Ca^{2+}-induced differentiation on day 0 and day 3 (Figure 5.9 A and B).

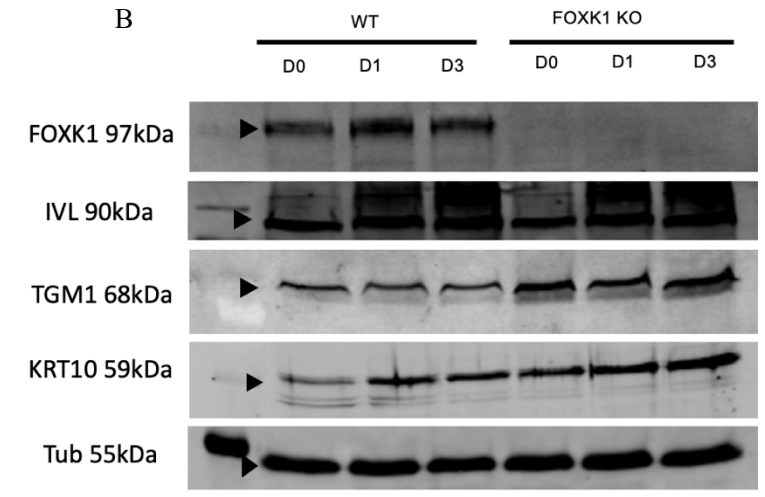
ΔNP63, cyclinD1, KRT19 and KRT5 protein expression were analysed as basal markers, and PCNA and PHH3 protein expression were analysed as proliferation markers via western blotting (Figure 5.9 A and C–H). CyclinD1 is a well-known cell cycle progression marker, and studies have shown that it plays a role in promoting cell cycle progression and proliferation in keratinocytes and that cyclinD1 expression decreases during differentiation (Nishi et al., 2009; Woods et al., 2010; Fernández-Hernández et al., 2013). KRT19 is also a well-known keratinocyte stem marker. It is expressed mainly in the basal layer cells of adult human skin (Khani et al., 2018). Studies on FOXK1 in muscle stem cells showed that FOXK1 regulated stem cell proliferation and blocked differentiation and that knocking down FOXK1 caused cell cycle arrest in the G_1/S phase (Hawke et al., 2003; Li et al., 2017). Protein expression for ΔNP63, KRT5 and KRT19 showed no significant differences in expression in FOXK1 KO cells in comparison to WT (Figure 5.9 A). However, densitometric analysis for cyclinD1 showed a significant (*$P<0.05$) decrease in protein expression on day 0 in FOXK1 KO in comparison to WT but not on day 3 of differentiation. In Figure 5.9 A, it looks like there is

decreased expression of KRT5 during differentiation, which is expected because it is known that different keratins are expressed in different stages of keratinocyte states: KRT5/K14 is expressed in basal cells and its expression decreases with terminal differentiation, while KRT1/K10 is the predominant keratin expressed in differentiated keratinocytes (Deyrieux and Wilson, 2007; Alam et al., 2011). However, KRT5 decreased expression was not statistically significant. It would be more informative to have more biological repeats to obtain more confident results on basal marker expression. Moreover, ΔNP63, cyclinD1 and KRT19 protein expression did not change during differentiation in WT and KO cells, although they were shown to be basal markers in keratinocytes and their expression decreased with terminal differentiation (Nishi et al., 2009; Alam et al., 2011; Soares and Huiqing, 2018; Khani et al., 2018). This might indicate that FOXK1 is involved in regulating some proliferation genes in specific steps during the cell cycle and cell differentiation in keratinocytes. However, protein expression of proliferating cell nuclear antigen (PCNA) and phospho-histone 3 (PHH3) in WT and FOXK1 KO cells showed no significant change during differentiation as well (Figure 5.9 A and G–H).

Keratin10 (KRT10), IVL and TGM1 protein expressions were analysed as differentiation markers via western blotting (Figure 5.9 B and I–K). Although it was previously shown that differentiation marker genes were significantly upregulated in KO cells versus WT, here, only TGM1 on day 0 was significantly upregulated in FOXK1 KO. This may be because the samples were frozen and thawed multiple times which caused protein degradation and caused this observed variability in western blotting. Furthermore, there were no significant differences in differentiation markers KRT10 and IVL protein expression between WT and KO cells on day 0 and day 3, suggests that knocking out FOXK1 affects gene expression but not protein

expression. Therefore, it would be interesting to examine the differences in posttranscriptional and posttranslational processes in WT versus KO cells.


A
WT
FOXK1 KO
D0
D1
D3
D0
D1
D3
FOXK1 97kDa
KRT5
62kDa
ΔN P63α
60kDa
KRT19 44kDa
CyclinD1 36kDa
PCNA 36kDa
PHH3 17kDa
Tub 55kDa


B
WT
FOXK1 KO
D0
D1
D3
D0
D1
D3
FOXK1 97kDa
IVL 90kDa
TGM1 68kDa
KRT10 59kDa
Tub 55kDa

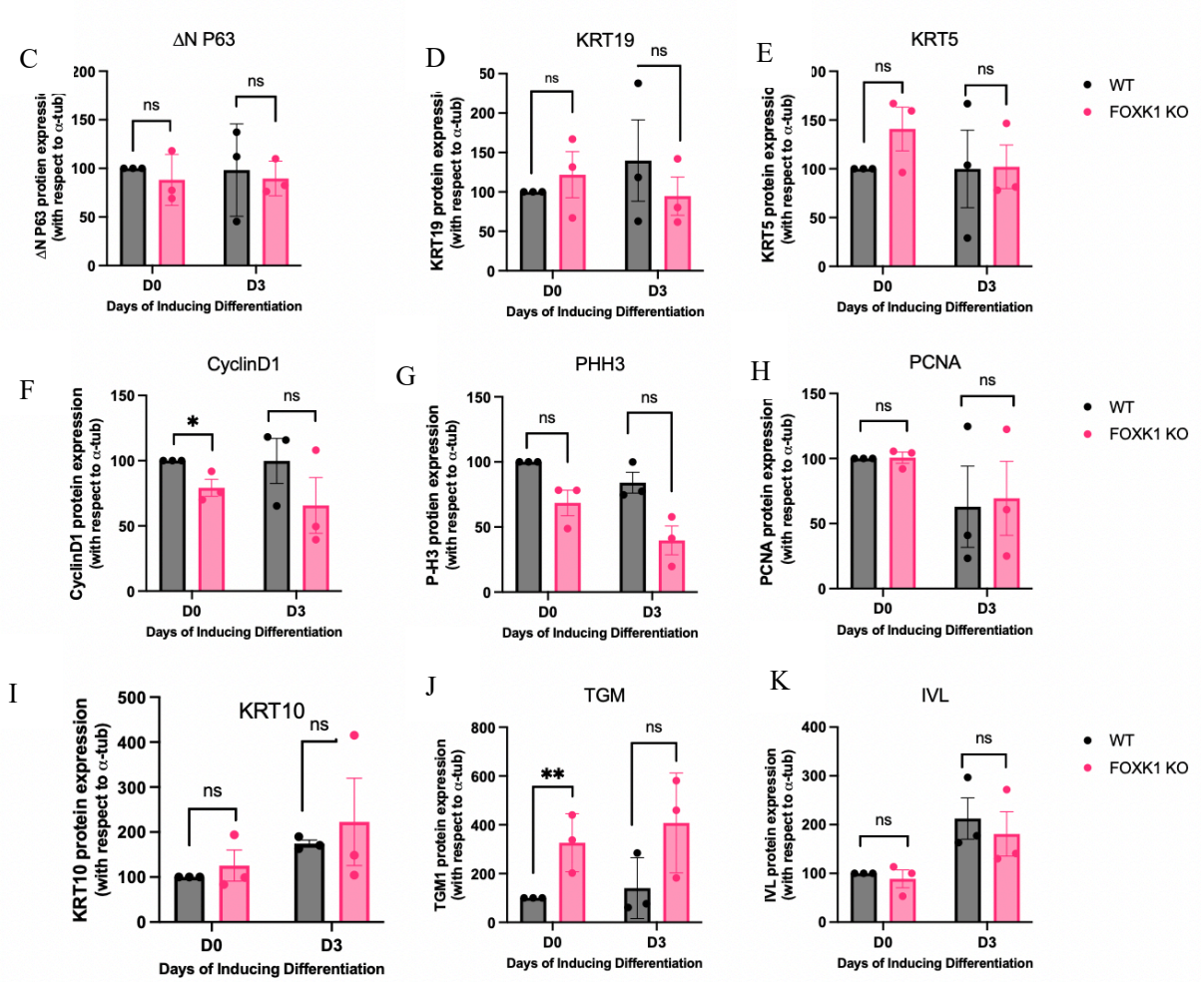


Figure 5.21 The effect of knocking out FOXK1 on cell proliferation, basal, and differentiation marker protein expression

HaCaT WT and FOXK1 KO cells were cultured in low Ca^{+2} and then differentiation was induced by switching to high Ca^{+2}. Whole-protein lysates were obtained for days 0, 1 and 3 following differentiation induction and analysed using western blotting. **(A)** Western blotting analysis of protein expression for PCNA and PHH3 as proliferation markers, cyclinD1 as a cell cycle marker, and ΔNP63, KRT19 and KRT5 as basal markers. **(B)** Western blotting for TGM1, IVL and KRT10 as differentiation markers. Expression of α-tubulin (α-tub) was used as a loading control. **(C–K)** Densitometric quantification of protein expression on days 0 and 3 of differentiation for WT and FOXK1 KO. Densitometric quantification was performed using Image Studio Lite. These data are from three separate experiments (n=3). Error bars are presented as the mean±SE. Statistical analysis was conducted using Student's t-test (**P<0.01, *P<0.05 and ns P>0.05).

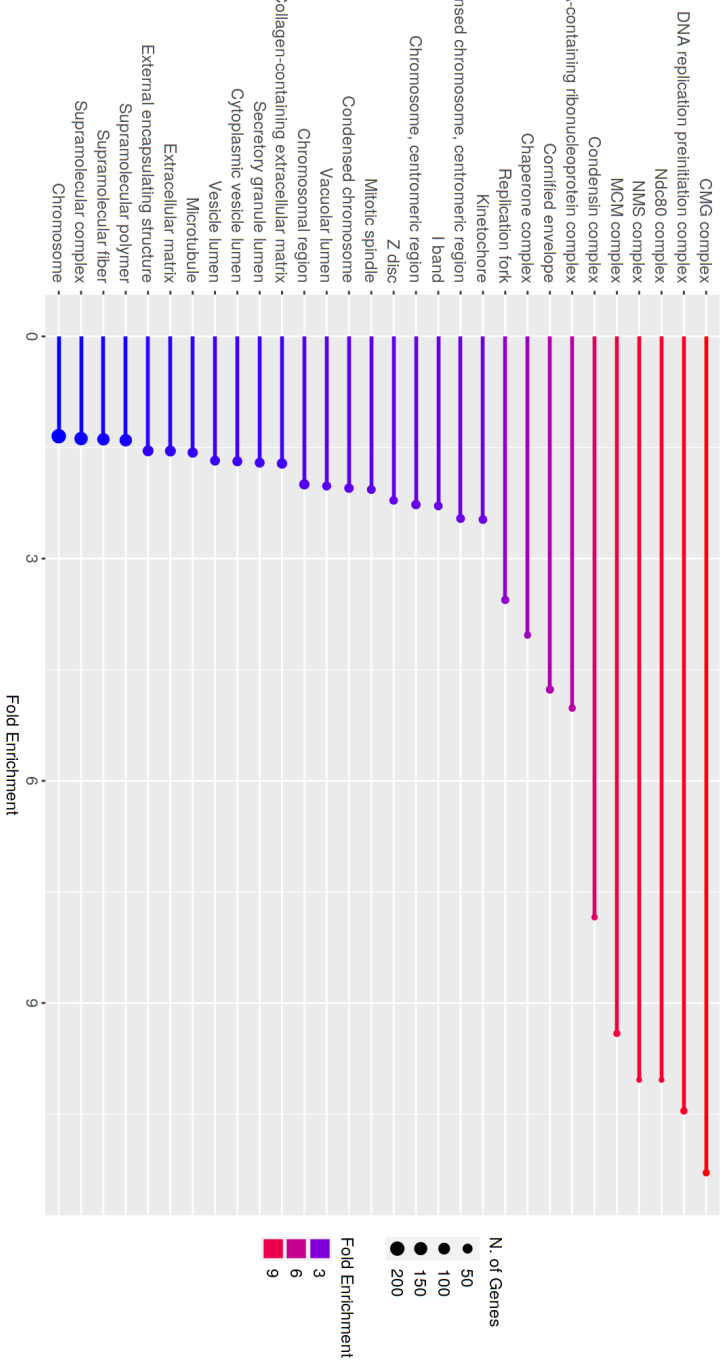
5.2.7 Functional association prediction and pathway analysis of upregulated and downregulated genes in WT and FOXK1

It has been shown previously in this chapter that the most significant differentially altered genes in keratinocytes after FOXK1 KO were genes involved in the cell cycle, proliferation and differentiation. However, it would be more informative to further understand the functional association of these genes to investigate the cellular components that they are involved with as well are their location in the cell signalling pathways. Therefore, genes which were differentially upregulated and downregulated by FOXK1 on days 0 and 3 of differentiation were submitted to the ShinyGO database for graphical enrichment analysis. Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analysis is a pool for biological analysis of genes through the mapping of signalling pathways (Kanehisa et al., 2017). ShinyGo has access to KEGG and other databases, wherein it allows one to graphically visualise enrichment, pathway, gene and protein characteristics and interactions (Ge et al., 2020). KEGG was used to highlight the positions of proteins of the differentially expressed genes within the enriched signalling pathways. It could be inferred also from these observations which signalling pathway could be validated to understand FOXK1 target genes, and whether there is feedback loop regulation of FOXK1 regarding proliferation and differentiation.

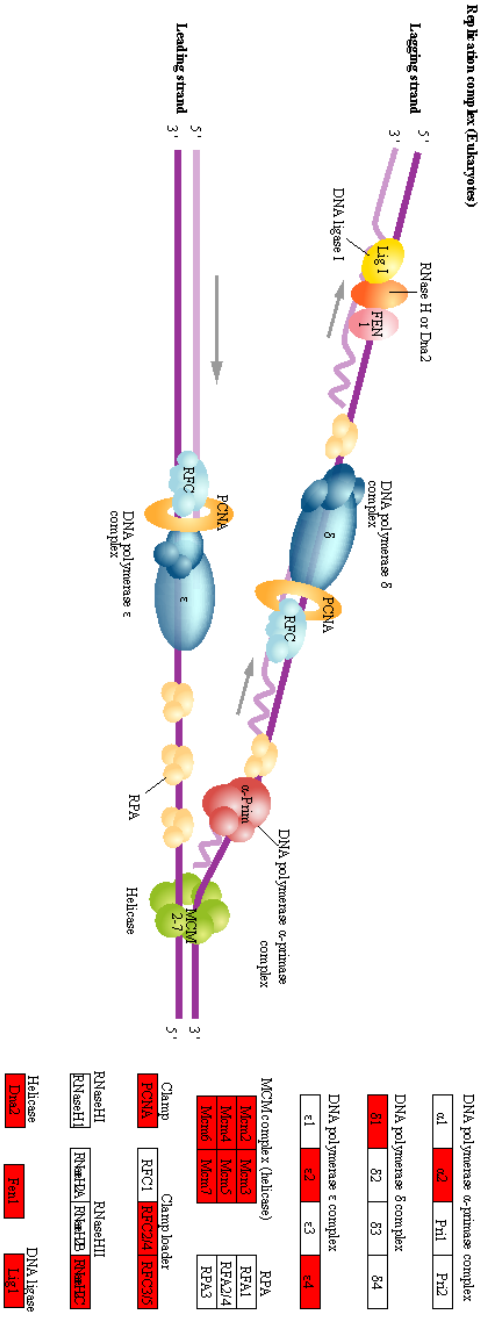
Figure 5.10 A shows the proteins lists generated from genes which were differentially upregulated and downregulated by FOXK1 on days 0 and 3 of differentiation. The ShinyGO cellular component tools showed that these proteins were enriched as part of the “human replicative helicase complex” including the CMG complex, MCM complex and Ndc80 complex, which are crucial for DNA replication, spindle attachment, and kinetochore formation (Lei and Ming, 2005; Remus and Diffley, 2009; Moiseeva and Bakkenist, 2018;

Helgeson et al., 2018). Moreover, using ShinyGo to access the KEGG and highlighting the positions of these proteins in the signalling pathways, KEGG analysis showed enrichment ($P<0.05$) for DNA replication, cell cycle, and FOXO signalling pathways (Figure 5.10 B–D). From these data, it could be speculated that FOXK1 has an important role in regulating the cell cycle and cell division in keratinocytes and that knocking out FOXK1 might have indirectly affected keratinocyte differentiation.

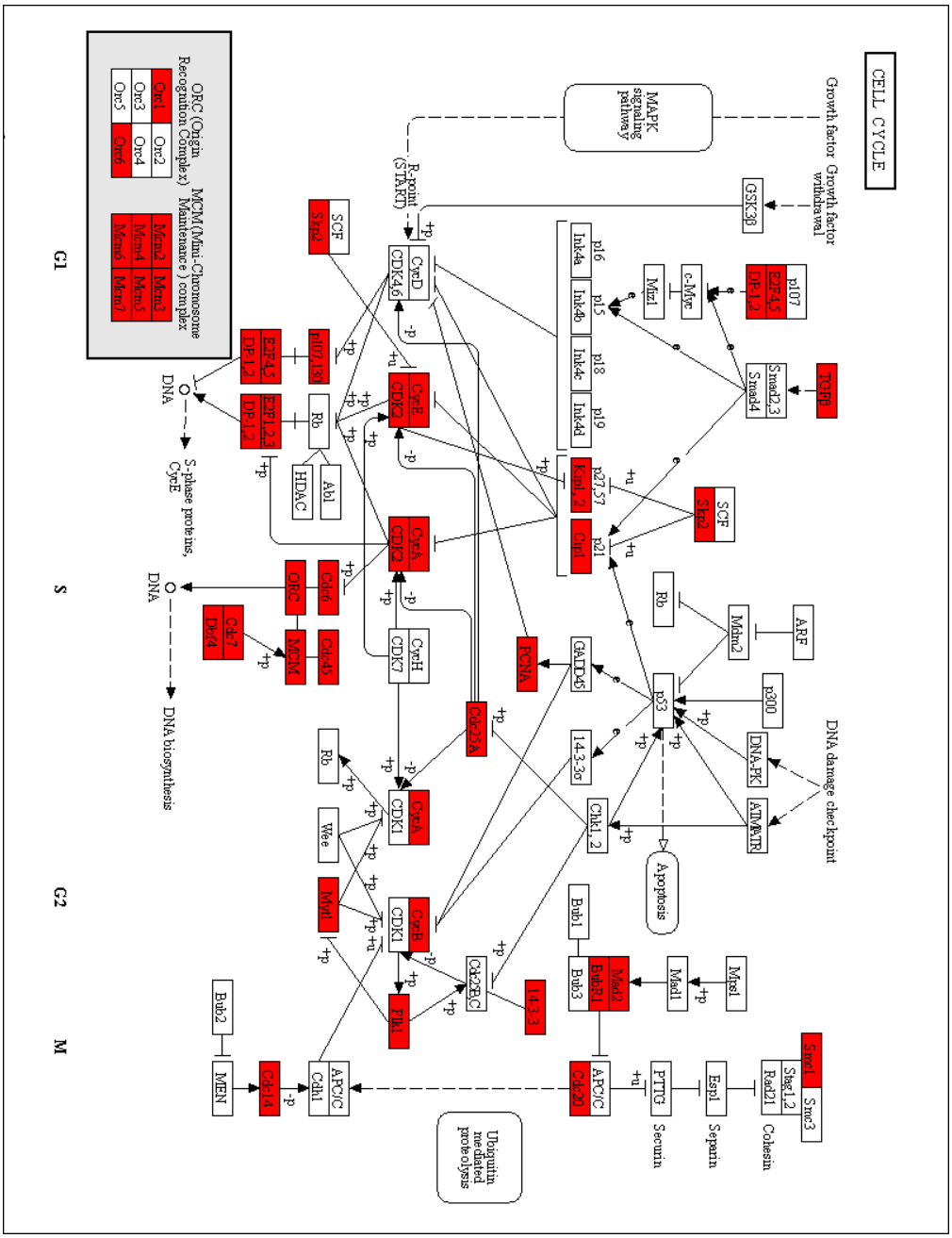
A. ShinyGo cellular component enrichments for upregulated and downregulated genes



B. KEGG pathway for DNA replication



C. KEGG pathway for cell cycle



D. KEGG for the FOXO signaling pathway

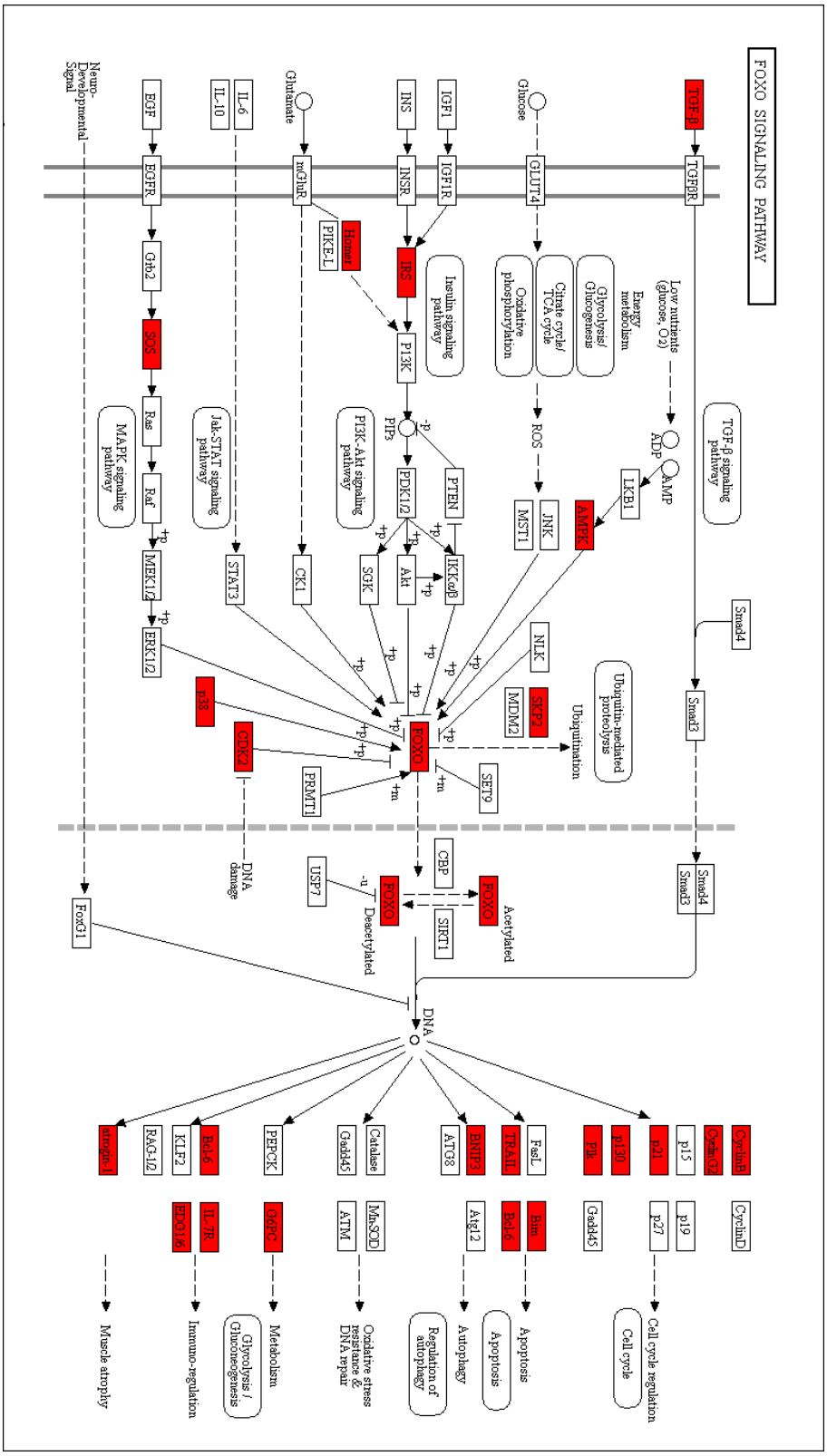


Figure 5.23 Functional associations and KEGG pathway enrichment analysis of upregulated and downregulated genes in FOXK1 KO compared to WT during differentiation

Protein lists generated from the differentially upregulated and downregulated genes by FOXK1 were submitted to ShinyGO and KEGG for functional association and pathway enrichment analysis. **(A)** ShinyGO cellular component enrichment for upregulated and downregulated genes. **(B)** KEGG pathway enrichment analysis highlighting gene transcripts (in red) from the gene list in the DNA replication pathway, **(C)** in the cell cycle pathway, and **(D)** in the FOXO signalling pathway (graphs and pathways were obtained from ShinyGO and KEGG).

5.3 Discussion

This chapter aimed to identify the genome-wide effect of an FOXK1 knockout upon gene expression in keratinocytes by means of next-generation sequencing (RNA-Seq). However, in this chapter, the main focus of data analysis was to analyse the genes which were differentially regulated by FOXK1 during differentiation. Switching keratinocytes from low to high Ca^{2+} medium is a well-established method with which to induce keratinocyte differentiation (Wilson, 2013; Bikle et al., 2021; Gutowska-Owsiak et al., 2020). The DAVID functional annotation of RNA-Seq data from HaCaT WT and FOXK1 KO cells showed in both cell lines an upregulation of differentiation and keratinisation genes and a downregulation of DNA replication and cell division genes (Figure 5.3 B–E). This was consistent with the conclusion drawn from Chapter 4 that knocking out FOXK1 did not block differentiation and, therefore, that FOXK1 is not critical for inducing differentiation.

In Chapter 3, it was established that FOXK1 expression increases during Ca^{2+}-induced differentiation, whereby suggesting that it has a role in regulating genes required during keratinocyte differentiation (Figure 3.1). However, reducing or knocking out FOXK1 expression, using RNAi or CRISPR-Cas9 respectively, did not inhibit keratinocyte differentiation (Figures 3.4 and 4.3). Here, FOXK1 mRNA levels were analysed and demonstrated a significant increase during differentiation, consistent with data from Chapters 3 and 4 which showed that FOXK1 protein expression increases during keratinocyte differentiation (Figure 5.7 A). However, the significance of this increase in expression has yet to be established. It is known that FOXK1 overexpression blocks myogenic stem cell differentiation (Freddie et al., 2007; Shi et al., 2012; Wencong et al., 2020), but FOXK1 expression during muscle cell differentiation has not been analysed and it would be interesting

to examine the expression of FOXK1 during muscle cell differentiation and in other cell types. Moreover, it would be interesting to overexpress FOXK1 in keratinocyte cells and determine whether FOXK1 overexpression in basal undifferentiated keratinocytes affects the induction of differentiation.

Although the expression of keratinocyte differentiation markers increased at both the protein level and the mRNA level during differentiation in both wild-type and FOXK1 KO cells (Figure 5.8 and Figure 5.9 I–K), cyclinD1, ΔNP63 and KRT19 protein expression did not change. This was surprising because they are known basal keratinocyte markers and their expression is known to decrease with terminal differentiation (Nishi et al., 2009; Alam et al., 2011; Soares and Huiqing, 2018; Khani et al., 2018). It is possible that this was a consequence of the type of differentiation assay used; previous work showed that the calcium switch method used in this thesis did not cause any change in β1 integrin, cyclinD1 or KRT19 expression, even though the expression of all three is reduced during differentiation (Michel et al., 1996; Belso et al., 2008; Nishi et al., 2009; Yang et al., 2016; Gursoy et al., 2016).

FOXK1 has been shown to have a role in regulating the cell cycle and cell proliferation in myogenic progenitor cells (Garry et al., 2000; Hawke et al., 2003; Freddie et al., 2007; Wang et al., 2015). FOXK1 is transiently expressed in many tissues during embryonic development, including the myotome, some parts of the brain, the heart tube, and the neuroretina (Garry et al., 2000; Fernandes et al., 2007; Shi et al., 2012; Sel et al., 2013). The loss of FOXK1 expression in the embryonic stage in some cases resulted in death soon after birth due to muscle atrophy and cell cycle dysregulation (Garry et al., 2000; Fernandes et al., 2007; Shi et al., 2012; Sel et al., 2013). Consistent with a role in regulating proliferation, RNA-Seq data showed that several cell division and proliferation were downregulated in FOXK1 KO keratinocytes on day

0 and day 3 (Figure 5.6 B and D), which is consistent with the reduced proliferation observed in FOXK1 KO cells. These data support findings which showed a 50% decrease in muscle progenitor cells in FOXK1 KO (Hawke et al., 2003). Additionally, a large number of genes involved in chromatin remodelling and G_1/S transition were downregulated, again consistent with the decrease in proliferation. In muscle stem cells, FOXK1 binds to the snail transcription factor (Sin3) of the Sin3–HDAC complex (Shi et al., 2012) and it is possible that FOXK1 might be similarly involved in chromatin remodelling in keratinocytes. Unlike at the mRNA level, protein expression of proliferating cell nuclear antigen (PCNA) and phospho-histone 3 (PHH3) in WT and FOXK1 KO cells showed no significant change during differentiation (Figure 5.9 A and G–H). However, the state of chromatin remodelling and DNA preparation for replication in FOXK1 KO could be the reason as to why FOXK1 KO cells have slower proliferation as well. It was shown that phosphorylation of histone 3 in ser10 is an important factor for opening chromatin in the late G_2 phase for DNA replication (Elmaci et al., 2018). Chromosome duplication must be controlled so as to occur once per cell cycle; thus, the DNA replication initiation process is divided into two distinct steps that take place at different times in the cell cycle (Alberts, 2017), the first of which is referred to as DNA licensing, which occurs from late mitosis to the early G_1 phase, wherein the ORC–CDC6–CDT1 complex then recruits the inactive DNA helicase MCM hexamer (MCM2-7). The second step is in the S phase through CDK-cyclin regulation, which activates MCM2-7 that unwinds the DNA and then facilitates the pre-replicative helicase complex (CMG) assembly which consists of the CDC45, Mcm2-7 and GINS complex. Then, CDC45 facilitates PCNA binding, which recruits DNA polymerase at the replication fork (Lei and Ming, 2005; Remus and Diffley, 2009; Moiseeva and Bakkenist, 2018). From RNA-Seq data, several genes involved in DNA licensing and DNA replication are among the significantly downregulated genes in KO cells, hence disturbing the cell cycle

and cell proliferation. Although western blotting is a reliable approach to investigating protein expression, it would be more useful to conduct a more global examination of protein expression via deep proteomic analysis such as tandem mass tag (TMT) mass spectrometry.

It was found that FOXK1 promotes stem cell proliferation and blocks differentiation in C2C12 muscle cells by repressing Mef2 and FOXO4, which are known to promote differentiation through the upregulation of CKI $p21^{CIP}$ (Shi et al., 2012). In another study, FOXK1 regulated muscle differentiation by repressing SRF transcription factor. The binding of FOXK1 to the MADS domain of SRF caused the repression of SM α-actin and PPGB genes involved for smooth, cardiac, and skeletal muscle differentiation (Freddie et al., 2007). In the RNA-Seq results, strikingly, genes associated with differentiation, keratinisation, and protein cross-linking were among the most significantly upregulated genes in KO cells in comparison to WT cells (Figure 5.5 E and Figure 5.6 D). RT-qPCR for KRT1 and IVL mRNA levels validated these findings; however, the change in mRNA levels did not affect protein levels. Nonetheless, protein expression for the basal markers ΔNP63, KRT19 and KRT5 showed no significant differences in expression in FOXK1 KO cells in comparison to WT (Figure 5.9 A). However, there was decreased expression of cyclinD1, whereby supporting the hypothesis that FOXK1 has an important role in the cell cycle. As FOXK1 expression was shown to increase during keratinocyte differentiation, it might be that FOXK1 does not directly regulate basal and differentiation genes, but rather regulates the cell cycle and proliferation.

The functional association analysis presented in Figure 5.10 A showed relationships between protein products of genes from RNA-Seq data that could help to support the previously mentioned hypotheses. These genes were enriched under several cellular components such as “CMG complex”, “MCM complex”, and “Ndc80 complex”, which supports the

aforementioned theory that FOXK1 might be regulating gene replication and gene division directly, which could be indirectly affecting cell differentiation genes. Furthermore, KEGG pathway enrichment highlighted the most significant pathways in which proteins of upregulated and downregulated genes are located (Figure 5.10 B–D). It could be that FOXK1 directly affects proliferation and indirectly regulates differentiation through the FOXO signalling pathway. Three members of the FOXO subfamily (FOXO1, FOXO4 and FOXO6) were shown to be among the upregulated genes in the FOXK1 KO day 0 RNA-Seq gene list. FOXO4 was shown to promote muscle progenitor differentiation. FOXK1 physically interacts with FOXO4 and supresses muscle differentiation and promotes cell cycle progression. Knocking down FOXK1 caused cell cycle arrest through the induction of the p21 cell cycle inhibitor and promoted muscle differentiation (Hawke et al., 2003; Shi et al., 2010; Shi et al., 2012; Li et al., 2017). It was shown that FOXK1 represses FOXO4 functions through the insulin signalling pathway as well (Sakaguchi et al., 2019). It would be interesting to investigate FOXO transcription factor expression in HaCaT WT during differentiation and compare it to its expression in FOXK1 KO cells during differentiation.

Then again, it is still unknown how the coupling between proliferation and differentiation regulation is achieved (Boward et al., 2016). Terminal differentiation is a complex process that involves interaction between several distinct processes that are regulated at multiple levels. Numerous studies have been conducted to elucidate the coupling between the cell cycle and cell differentiation. However, the processes of regulation and the cell fate decision-making factors of proliferation and differentiation remain unknown due to the complexity of these biological processes (Musacchio and Helin, 2013; Boward et al., 2016; Soufi and Dalton, 2016). It was shown that the length of the cell cycle may affect the cell fate. The cell cycle

length was shown to influence the spindle orientation and the type of division (symmetrical and asymmetrical), which in turn dictates the cell fate (Calegari and Huttner, 2003; Calegari et al., 2005; Barui and Datta, 2019). From our RNA-Seq data, FOXK1 KO cells demonstrated downregulation in G_1/S phase transitioning genes as well as in multiple DNA replication, G_2/M phase regulation, and chromosome cohesion genes. The slow rate of proliferation of the EdU data from Chapter 4 (Figure 4.4) could be explained as the length of the cell cycle perhaps influencing the cell terminal differentiation decision in FOXK1 KO keratinocytes. It would be interesting to examine the cell cycle length at multiple stages and its effect on keratinocyte differentiation as well as on FOXK1 overexpression in a similar way.

To summarise the most important findings in this chapter, knocking out FOXK1 in keratinocytes resulted in the upregulation of genes associated with differentiation and the downregulation of genes required for DNA replication and cell division. The next step would be to combine the RNA-Seq data with ChIP-Seq analysis to identify target genes for FOXK1 during differentiation.

Chapter 6

6 General discussion and future directions

Cell differentiation is an extremely important biological process in cellular growth, development and regeneration. The epidermis is structured as multiple layers of cells with varying degrees of terminal differentiation (Eckhart et al., 2013). The outer layer of the skin is continuously shed due to physical or chemical affecters; therefore, lost cells have to be replaced for the skin to accomplish its function (Liu et al., 2013; Watt, 2015). Terminal differentiation is a complex process that involves interaction between several distinct processes that are regulated at multiple levels. It involves the time-dependent coordination of gene expression changes and intracellular and intercellular changes in response to a wide range of cell stimuli. This tight regulation maintains that the epidermis is constantly intact in the correct structure and that terminal differentiation only occurs when epidermal regeneration is necessary. Thus, the balance between cell proliferation and differentiation is critical for tissue health and integrity. This balance becomes exceedingly important in tissues that constantly need to be regulated, like the skin. The regulation between proliferation and differentiation is governed by different factors inside of the cell or through the outer environment; however, the regulation mechanism has yet to be fully understood (Watt and Huck, 2013; Reichenbach et al., 2018). Recently, a connection between the Rnd3 GTPase protein and the FOXK1 transcription factor in the regulation of keratinocyte differentiation was discovered (Begum, 2017). It was shown that Rnd3-depleted HaCaT cells displayed stemness characteristics such as a smaller cell size, increased adhesion to ECM, and clustering in tight colonies. What is more, they showed increased expression of the basal stem marker p63, increased desmoplakin expression, and reduced differentiation (Begum, 2017). As part of this work, SILAC mass spectrometry was

used to identify proteins that were altered in expression when Rnd3 was depleted by means of RNAi. A list of proteins that were significantly upregulated and downregulated is presented in Table 1.4. Further analysis of these proteins identified that some of them are involved in regulating the transcription of differentiation genes, including the forkhead box (FOX) transcription factor FOXK1 (Begum, 2017). As FOXK1 was shown to be a critical regulator in muscle progenitor differentiation (Garry et al., 2000; Freddie et al., 2007; Shi et al., 2010; Li et al., 2017; Li et al., 2022), it was speculated that a similar function of FOXK1 is true for keratinocyte differentiation.

The FOXK1 transcription factor is one of two members of the FOXK subfamily of FOX transcription factors (Hofmann and Bucher, 1995). FOXK1 is mainly studied in muscle cells, wherein it was shown to be involved in regulating muscle stem cell proliferation, the cell cycle, and cell differentiation (Garry et al., 2000; Freddie et al., 2007; Shi et al., 2010; Li et al., 2017; Li et al., 2022). It was shown that FOXK1 and FOXK2 were involved in signal transduction and metabolism, where both were found to be downstream targets for mTOR, Wnt/β-catenin, and TGF-β pathways (Wang et al., 2015; Peng et al., 2016; Nakatsumi et al., 2017). Until writing this thesis, there were no data on the role of FOXK1 in keratinocytes; thus, the work undertaken here is to investigate the role of FOXK1 in keratinocyte differentiation.

Here, it was shown that knocking down and knocking out FOXK1 did not block differentiation in keratinocytes. Cells still expressed differentiation markers such as involucrin; transglutaminase1 (Chapter 3, Figure 3.4 and Chapter 4, Figure 4.3). Therefore, it was concluded that FOXK1 is not critical for inducing keratinocyte differentiation and might have other important roles in cells. FOXK1 expression was shown to increase during differentiation; therefore, this increase is intriguing (Chapter 3, Figure 3.1). It would be interesting to see

whether FOXK1 expression in muscle progenitor cells is similar to its expression in keratinocytes. Moreover, it would be interesting to find the reason for this increase during differentiation in keratinocytes by uncovering the upstream signal transduction pathways of FOXK1. Furthermore, it would be interesting to find the connection between Rnd3 and FOXK1 signalling pathways by means of experiments to study cell signalling such as pathway inhibition and activation.

Studies on FOXK1 in mice showed that it is critical for muscle stem cell proliferation and cell cycle progression. Knocking out FOXK1 in mice resulted in impaired stem cell proliferation function and caused severe muscle atrophy (Garry et al., 2000). EdU staining (Chapter 4, Figure 4.4) showed a significant decrease (about 50%) in cell division in FOXK1 KO keratinocytes, which is consistent with BrdU data on FOXK1 in muscle progenitor cells in which it was shown that knocking out FOXK1 resulted in a 50% decrease in the rate of muscle progenitor cell proliferation (Hawke et al., 2003). It was shown in another study that knocking out FOXK1 expression resulted in a lower number of cells in the S phase and a higher number in the G_0/G_1 phase in FOXK1 KO muscle progenitors due to increased expression of $p21^{CIP}$ (Li et al., 2017). However, the wound-healing scratch assay showed no significant differences in the rate of wound closure between KO and WT cells (Chapter 4, Figure 4.6). Thus, it was concluded from this finding that FOXK1 might be involved in the cell cycle, cell migration, and motility regulation. Nevertheless, further investigation into KO cells is needed because cell migration and motility are critical processes in cell biology and cancer. FOXK1 was shown to be overexpressed in several types of cancer and FOXK1 overexpression resulted in poor prognosis and increased metastasis (Peng et al., 2016; Wu et al., 2016; Wang et al., 2018; Wang et al., 2020; Yang et al., 2020; Xu et al., 2021). Therefore, utilising other methods such as

single-cell tracking, cell migration, and 3D cell culture models would give more insight into the role of FOXK1 in these cellular processes.

Interestingly, there were no significant differences in cell viability and cell death in FOXK1 KO keratinocytes in comparison to the WT (Chapter 4, Figure 4.5). However, Figure 4.4 shows less cell proliferation in KO in comparison to WT; therefore, it could be that there is an increase in the metabolic activity of KO cells in comparison to WT. Research into FOXK1 showed that it has a function in metabolism regulation, wherein the knockdown of FOXK1 and FOXK2 showed upregulation of mitochondrial oxidative phosphorylation and ATP synthesis genes (He et al., 2018; Sakaguchi et al., 2019; Wang et al., 2019). The knockdown caused an alteration in the mitochondrial size and morphology; moreover, it caused an increase in the copy number of the mitochondrial genes mt-Nd1 and mt-Nd6 — part of the NADH dehydrogenase 1 enzyme, which is involved in ATP synthesis (Sakaguchi et al., 2019). Therefore, it can be speculated that knocking out FOXK1 in HaCaT caused upregulation of mitochondrial oxidative phosphorylation and ATP synthesis. Yet, it would be interesting to examine the effect of knocking out FOXK1 upon keratinocyte metabolism through the use of specialised procedures such as the Seahorse Bioscience XFp Extracellular Flux Analyzer (which is a powerful tool that simultaneously measures mitochondrial respiration and glycolysis). It would be interesting to use mass spectrometry for metabolite analysis as well. Furthermore, it would be interesting examine apoptosis by means of caspase assays to investigate the effect of knocking out FOXK1 upon cell death.

The most significant findings on FOXK1 and its role in keratinocytes were obtained from the RNA-Seq experiment. RNA-Seq was used to investigate the genome-wide effect of knocking out FOXK1 upon gene expression in keratinocytes. Downregulated and upregulated gene lists

from FOXK1 KO and WT keratinocytes during differentiation were generated (Chapter 5). This chapter found that FOXK1 KO cells have significant upregulation in the gene expression of differentiation, protein cross-linking, and keratinisation genes in comparison to WT cells. Moreover, it was shown that genes involved in DNA replication, cell division, G_1/S transition, and sister chromatid cohesion were significantly downregulated (Figure 5.6). ShinyGo functional association (Figure 5.10) demonstrated a comprehensive relationship between gene transcripts from RNA-Seq data, which showed enrichment under several cellular components involved in cell division and the cell cycle, consistent with findings in the literature. FOXK1 was shown to be involved in cell cycle regulation and chromatin remodelling. Furthermore, FOXK1 was shown to bind to the snail transcription factor (Sin3) of the Sin3–HDAC complex through the interaction between the FHA domain of FOXK1 and phospho-Thr49 of Sds3 (Shi et al., 2012). Additionally, co-immunoprecipitation resulted in pulling down FOXK1, FOXK2 and Sin3A, which were coupled with core components such as HDAC1, HDAC2 and Sap30. FOXK1 and FOXK2 were found to be involved in the repression of autophagy genes through histone deacetylation for H4 (Bowman et al., 2014).

However, it is still not confirmed whether knocking out FOXK1 affected protein levels for some of these genes; thus, it would be significant to explore protein expression changes in FOXK1 KO cells on a larger scale via deep proteomic analysis such as tandem mass tag (TMT) mass spectrometry. It would be a significant tool for RNA-Seq data validation as well as a method with which to examine post-translational modifications and their effect on protein turnover and localisation.

Furthermore, KEGG pathway enrichment highlighted the most significant pathways in which upregulated and downregulated genes are located (Chapter 5, Figure 5.10). As it was shown

that one of the significantly affected pathways in KO cells was the FOXO pathway (Chapter 5, Figure 5.10 D), it is speculated that FOXK1 directly regulates the cell cycle and proliferation in keratinocytes and regulates differentiation genes indirectly through pathways such as the FOXO pathway. FOXK1 physically interacts with FOXO4 and supresses muscle differentiation and promotes cell cycle progression. Knocking down FOXK1 promoted muscle differentiation (Hawke et al., 2003; Shi et al., 2010; Shi et al., 2012; Li et al., 2017). It would be interesting to investigate FOXO transcription factor expression in keratinocytes during differentiation and compare it to its expression in FOXK1 KO cells. It would be interesting to see whether there are connections between FOXK1 and other known regulators of keratinocyte differentiation. Additionally, it would be interesting to examine biopsies obtained from patients with skin diseases and see whether FOXK1 is involved in causing these diseases.

FOXK1 and FOXK2 are two members of the FOXK subfamily of FOX transcription factors. They share almost 50% of sequence identity and were found to have similar functions in some cell lines. Here, FOXK2 expression decreased during differentiation (Chapter 3, Figure 3.3). What is more, there are no data on FOXK2 function in keratinocytes; thus, it would be interesting to investigate the role of FOXK2 along with FOXK1. Producing a stable FOXK2 knockout and an FOXK1/2 double knockout as well as FOXK1/2 overexpressed keratinocyte cell lines would be needed for further insight into the role of these transcription factors. Performing a differentiation assay, proliferation, apoptosis, and RNA sequencing experiments and comparing the results of all these conditions in relation to keratinocytes would be interesting.

An attempt to investigate FOXK1 protein–DNA interaction through ChIP-Seq was made. The technique is performed by cross-linking proteins to DNA via formaldehyde and then chopping

chromatins into short fragments using sonication or restriction enzymes. Thereafter, a specific antibody against that particular protein is used to immunoprecipitate only protein-bound chromatin fragments, following which, proteinase K is used to reverse cross-linked protein from DNA. The purified DNA is then processed further to generate DNA libraries and sequenced (Kaufmann et al., 2010). ChIP for WT and FOXK1 KO keratinocytes on days 1 and 3 of differentiation was performed. However, the DNA samples generated in the laboratory were very low in concentration. They were sent to ChIP-Seq facilities to generate libraries and for sequencing; however, reports came that the samples were very low in concentration and quality for successful downstream processing. It could be that the antibody used was not the best for ChIP; therefore, experimenting with other antibodies available for FOXK1 is recommended. FOXK1 expression in HaCaT cells is not abundant. Almost 30 μg of whole-cell lysates needs to be loaded into the gel when performing western blotting for FOXK1 to be detected; therefore, more time for ChIP optimisation, such as sample sizes and replicates, would generate a better quality and volume of samples. ChIP-Seq data on FOXK1 would be supportive of RNA-Seq data in investigating gene expression regulation pathways. It would be beneficial to see DNA binding sites, enhancers, and promoters and identify other interacting proteins such as activators and repressors.

For future work, it would be interesting to further analyse the role of FOXK1 in keratinocytes in animal models via procedures such as a conditional knockout to inactivate FOXK1 expression in animal skin without affecting FOXK1 in other tissues. It would be interesting to see whether a loss of FOXK1 function in animal epidermis would affect its proliferation, differentiation, would healing, and metabolism.

In conclusion, this study has expanded the knowledge on the role of the forkhead box (FOX) transcription factor FOXK1 in keratinocytes. FOXK1 is not critical for inducing keratinocyte differentiation; however, the findings suggest the possibility that FOXK1 is important for regulating the cell cycle and proliferation, which could be indirectly affecting keratinocyte differentiation. Further studies are needed in order to confirm these results.

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Appendix 5. 1 List of the differentially upregulated genes by FOXK1 at day 0 of differentiation

Biological Process	Number of Genes	Genes List
synapse assembly	9	- SLIT and NTRK like family member 6(SLITRK6) - SPARC/osteonectin, cwcv and kazal like domains proteoglycan 2(SPOCK2) - Wnt family member 5A(WNT5A) - acetylcholinesterase (Cartwright blood group)(ACHE) - fibronectin leucine rich transmembrane protein 3(FLRT3) - piccolo presynaptic cytomatrix protein(PCLO) - plexin D1(PLXND1) - protocadherin beta 13(PCDHB13) - sidekick cell adhesion molecule 1(SDK1)
cardiac right ventricle morphogenesis	5	- GATA binding protein 3(GATA3) - SRY-box 4(SOX4) - bone morphogenetic protein 4(BMP4) - semaphorin 3C(SEMA3C) - transforming growth factor beta 2(TGFB2)
positive regulation of transcription from RNA polymerase II promoter	42	- AT-rich interaction domain 4A(ARID4A) - B-cell CLL/lymphoma 11A(BCL11A) - BARX homeobox 2(BARX2) - BCAS3, microtubule associated cell migration factor(BCAS3) - Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2(CITED2) - ETS variant 1(ETV1) - GATA binding protein 3(GATA3) - MAF bZIP transcription factor A(MAFA) - MAF bZIP transcription factor B(MAFB) - MAF bZIP transcription facto r(MAF)

		- MYB proto-oncogene, transcription factor (MYB) - PLAG1 zinc finger (PLAG1) - RAR related orphan receptor A(RORA) - SIX homeobox 2(SIX2) - SRY-box 4 (SOX4) - Wnt family member 5A(WNT5A) - amyloid beta precursor protein binding family B member 1(APBB1) - ankyrin repeat domain 1(ANKRD1) - bone morphogenetic protein 2(BMP2) - bone morphogenetic protein 4(BMP4) - bone morphogenetic protein receptor type 1B(BMPR1B) - calcium binding and coiled-coil domain 1(CALCOCO1) - cyclin dependent kinase inhibitor 2A(CDKN2A) - cyclin dependent kinase inhibitor 2B(CDKN2B) - distal-less homeobox 3(DLX3) - engrailed homeobox 1(EN1) - forkhead box O4(FOXO4) - hes family bHLH transcription factor 5(HES5) - histone deacetylase 5(HDAC5) - homeobox C13(HOXC13) - inhibitor of DNA binding 4, HLH protein (ID4) - nitric oxide synthase 1(NOS1) - purinergic receptor P2Y1(P2RY1) - regulator of cell cycle (RGCC) - serpin family F member 2(SERPINF2) - toll like receptor 4(TLR4) - transcription factor Dp-2(TFDP2) - transcription factor Dp-2(TFDP2) - transcriptional repressor GATA binding 1(TRPS1) - vestigial like family member 1(VGLL1) - vestigial like family member 2(VGLL2) - zinc finger protein 350(ZNF350)
heart development	14	- Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2(CITED2)

		- FRAS1 related extracellular matrix protein 2(FREM2) - GATA binding protein 3(GATA3) - SRY-box 4(SOX4) - blood vessel epicardial substance(BVES) - bone morphogenetic protein 2(BMP2) - cysteine rich protein 1(CRIP1) - fibronectin leucine rich transmembrane protein 3(FLRT3) - myoglobin(MB) - phospholipase C epsilon 1(PLCE1) - secreted protein acidic and cysteine rich(SPARC) - transcription factor Dp-2(TFDP2) - transcriptional repressor GATA binding 1(TRPS1) - transforming growth factor beta 2(TGFB2)
inner ear development	7	- heart development 14 Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2(CITED2) - FRAS1 related extracellular matrix protein 2(FREM2) - GATA binding protein 3(GATA3) - SRY-box 4(SOX4) - blood vessel epicardial substance(BVES) - bone morphogenetic protein 2(BMP2) - cysteine rich protein 1(CRIP1) - fibronectin leucine rich transmembrane protein 3(FLRT3) - myoglobin(MB) - phospholipase C epsilon 1(PLCE1) - secreted protein acidic and cysteine rich(SPARC) - transcription factor Dp-2(TFDP2) - transcriptional repressor GATA binding 1(TRPS1) - transforming growth factor beta 2(TGFB2)
cell adhesion	21	- ADAM metallopeptidase domain 22(ADAM22) - BOC cell adhesion associated, oncogene regulated (BOC) - FRAS1 related extracellular matrix protein 2(FREM2) - L1 cell adhesion molecule(L1CAM)

		- NUAK family kinase 1(NUAK1) - Thy-1 cell surface antigen(THY1) - acetylcholinesterase (Cartwright blood group)(ACHE) - amelotin(AMTN) - cadherin 11(CDH11) - collagen type V alpha 1 chain(COL5A1) - hes family bHLH transcription factor 5(HES5) - insulin like growth factor binding protein 7(IGFBP7) - integrin subunit beta 2(ITGB2) - laminin subunit alpha 4(LAMA4) - neogenin 1(NEO1) - neural precursor cell expressed, developmentally down-regulated 9(NEDD9) - neurotrimin(NTM) - olfactomedin 4(OLFM4) - protein tyrosine phosphatase, receptor type S(PTPRS) - protocadherin 18(PCDH18) - sorbin and SH3 domain containing 1(SORBS1)

Appendix 5. 2 List of the differentially downregulated genes by FOXK1 at day 0 of differentiation

Biological Process	Numbers of Genes	Genes List
positive regulation of cell division	6	- epiregulin(EREG) - interleukin 1 alpha(IL1A) - interleukin 1 beta(IL1B) - placental growth factor(PGF) - platelet derived growth factor C(PDGFC) - vascular endothelial growth factor A(VEGFA)
vasculogenesis	6	- EGF like domain multiple 7(EGFL7) - atypical chemokine receptor 3(ACKR3) - hyaluronan synthase 2(HAS2) - neurotrophic receptor tyrosine kinase 2(NTRK2) - troponin I3, cardiac type(TNNI3) - vascular endothelial growth factor A(VEGFA)
cytokine-mediated signaling pathway	8	- Janus kinase 2(JAK2) - epiregulin(EREG) - interferon regulatory factor 5(IRF5) - interleukin 1 alpha(IL1A) - interleukin 1 beta(IL1B) - interleukin 20 receptor subunit alpha(IL20RA) - interleukin 6(IL6) - proteolipid protein 2(PLP2)
positive regulation of gene expression	11	- colony stimulating factor 2(CSF2) - cytochrome P450 family 26 subfamily B member 1(CYP26B1) - fibroblast growth factor receptor 4(FGFR4) - interleukin 1 alpha(IL1A) - interleukin 1 beta(IL1B) - interleukin 6(IL6) - myocyte enhancer factor 2C(MEF2C) - neurotrophic receptor tyrosine kinase 2(NTRK2) - olfactomedin 1(OLFM1) - ribosomal protein S6 kinase A2(RPS6KA2) - vascular endothelial growth factor A(VEGFA)

negative regulation of endopeptidase activity	7	- SPARC/osteonectin, cwcv and kazal like domains proteoglycan 1(SPOCK1) - TIMP metallopeptidase inhibitor 1(TIMP1) - furin, paired basic amino acid cleaving enzyme(FURIN) - serpin family B member 2(SERPINB2) - serpin family E member 1(SERPINE1) - serpin family E member 2(SERPINE2) - tissue factor pathway inhibitor 2(TFPI2)

Appendix 5. 3 List of differentially upregulated genes by FOXK1 at day 3 of differentiation

Biological Process	Numbers of Genes	Genes List
keratinocyte differentiation	15	- POU class 3 homeobox 1(POU3F1) - Wnt family member 5A(WNT5A) - chromosome 1 open reading frame 68(C1orf68) - desmoglein 4(DSG4) - involucrin(IVL) - keratin 16(KRT16) - late cornified envelope 1B(LCE1B) - late cornified envelope 1C(LCE1C) - late cornified envelope 1D(LCE1D) - late cornified envelope 1E(LCE1E) - late cornified envelope 1F(LCE1F) - late cornified envelope 3D(LCE3D) - late cornified envelope 3E(LCE3E) - proline rich 9(PRR9) - small proline rich protein 1B(SPRR1B)
keratinization	12	- involucrin(IVL) - keratin 16(KRT16) - keratin 17(KRT17) - late cornified envelope 1B(LCE1B) - late cornified envelope 1C(LCE1C) - late cornified envelope 1D(LCE1D) - late cornified envelope 1E(LCE1E) - late cornified envelope 1F(LCE1F) - late cornified envelope 3D(LCE3D) - late cornified envelope 3E(LCE3E) - small proline rich protein 1B(SPRR1B) - trichohyalin(TCHH)
peptide cross-linking	12	- chromosome 1 open reading frame 68(C1orf68) - involucrin(IVL) - late cornified envelope 1B(LCE1B) - late cornified envelope 1C(LCE1C) - late cornified envelope 1D(LCE1D) - late cornified envelope 1E(LCE1E) - late cornified envelope 1F(LCE1F) - late cornified envelope 3D(LCE3D) - late cornified envelope 3E(LCE3E) - proline rich 9(PRR9) - small proline rich protein 1B(SPRR1B) - transglutaminase 5(TGM5)

epidermis development	14	- calmodulin like 5(CALML5) - chromosome 1 open reading frame 68(C1orf68) - connective tissue growth factor(CTGF) - cystatin E/M(CST6) - keratin 14(KRT14) - keratin 16(KRT16) - keratin 17(KRT17) - keratin 9(KRT9) - late cornified envelope 1C(LCE1C) - late cornified envelope 1E(LCE1E) - late cornified envelope 3D(LCE3D) - parathyroid hormone like hormone(PTHLH) - small proline rich protein 1B(SPRR1B) - transglutaminase 5(TGM5)
positive regulation of transcription from RNA polymerase II promoter	45	- B-cell CLL/lymphoma 11A(BCL11A) - B-cell CLL/lymphoma 11B(BCL11B) - BARX homeobox 2(BARX2) - Jun proto-oncogene, AP-1 transcription factor subunit(JUN) - LIM homeobox transcription factor 1 beta(LMX1B) - MAF bZIP transcription factor A(MAFA) - MAF bZIP transcription factor B(MAFB) - MAF bZIP transcription factor(MAF) - MYB proto-oncogene, transcription factor(MYB) - Meis homeobox 3(MEIS3) - NK2 homeobox 1(NKX2-1) - PLAG1 zinc finger(PLAG1) - POU class 3 homeobox 1(POU3F1) - RAR related orphan receptor A(RORA) - SIX homeobox 2(SIX2) - SMAD family member 7(SMAD7) - Wnt family member 5A(WNT5A) - amyloid beta precursor protein binding family B member 1(APBB1) - ankyrin repeat domain 1(ANKRD1) - bone morphogenetic protein 2(BMP2) - bone morphogenetic protein 4(BMP4) - cyclin dependent kinase inhibitor 2B(CDKN2B) - distal-less homeobox 2(DLX2) - distal-less homeobox 3(DLX3) - endothelin 1(EDN1) - engrailed homeobox 1(EN1) - forkhead box I1(FOXI1) - forkhead box J1(FOXJ1) - heart and neural crest derivatives expressed 1(HAND1)

		- hes family bHLH transcription factor 5(HES5) - hes related family bHLH transcription factor with YRPW motif 2(HEY2) - indian hedgehog(IHH) - interleukin 23 subunit alpha(IL23A) - mesenchyme homeobox 1(MEOX1) - nuclear receptor subfamily 4 group A member 2(NR4A2) - paired box 8(PAX8) - phosphotyrosine interaction domain containing 1(PID1) - regulator of cell cycle(RGCC) - serpin family F member 2(SERPINF2) - solute carrier family 11 member 1(SLC11A1) - spalt like transcription factor 4(SALL4) - toll like receptor 4(TLR4) - transcription factor AP-2 epsilon(TFAP2E) - transcriptional repressor GATA binding 1(TRPS1) - vestigial like family member 1(VGLL1)

Appendix 5. 4 List of the differentially downregulated genes by FOXK1 KO at day 3 of differentiation

Biological Process	Number of Genes	Genes List
DNA replication	25	- BRCA1 interacting protein C-terminal helicase 1(BRIP1) - BRCA1, DNA repair associated(BRCA1) - Bloom syndrome RecQ like helicase(BLM) - DNA polymerase alpha 1, catalytic subunit(POLA1) - DNA polymerase alpha 2, accessory subunit(POLA2) - DNA replication and sister chromatid cohesion 1(DSCC1) - KIAA0101(KIAA0101) - RB binding protein 7, chromatin remodeling factor(RBBP7) - TIMELESS interacting protein(TIPIN) - TOPBP1 interacting checkpoint and replication regulator(TICRR) - Cell division cycle 25A(CDC25A) - Cell division cycle 45(CDC45) - Cell division cycle 6(CDC6) - Chromatin assembly factor 1 subunit B(CHAF1B) - Claspin(CLSPN) - Cyclin dependent kinase 1(CDK1) - Denticleless E3 ubiquitin protein ligase homolog(DTL) - Deoxyuridine triphosphatase(DUT) - Exonuclease 1(EXO1) - Minichromosome maintenance 10 replication initiation factor(MCM10) - Minichromosome maintenance complex component 3(MCM3) - Minichromosome maintenance complex component 6(MCM6) - Origin recognition complex subunit 1(ORC1) - Replication factor C subunit 3(RFC3) - Ribonucleotide reductase regulatory subunit M2(RRM2)

G1/S transition of mitotic cell cycle	18	- DNA polymerase alpha 1, catalytic subunit(POLA1) - DNA polymerase alpha 2, accessory subunit(POLA2) - F-box protein 5(FBXO5) - IQ motif containing GTPase activating protein 3(IQGAP3) - Cell division cycle 25A(CDC25A) - Cell division cycle 45(CDC45) - Cell division cycle 6 (CDC6) - Cell division cycle associated 5(CDCA5) - Cyclin dependent kinase 1(CDK1) - Cyclin dependent kinase inhibitor 2C(CDKN2C) - Cyclin dependent kinase inhibitor 3(CDKN3) - Minichromosome maintenance 10 replication initiation factor (MCM10) - Minichromosome maintenance complex component 3(MCM3) - Minichromosome maintenance complex component 6(MCM6) - Origin recognition complex subunit 1(ORC1) - Protein kinase, membrane associated tyrosine/threonine 1(PKMYT1) - Ribonucleotide reductase regulatory subunit M2(RRM2) - Thymidylate synthetase (TYMS)
Sister chromatid cohesion	18	- BUB1 mitotic checkpoint serine/threonine kinase(BUB1) - MAD2 mitotic arrest deficient-like 1 (yeast)(MAD2L1) - NUF2, NDC80 kinetochore complex component(NUF2) - Aurora kinase B(AURKB) - Baculoviral IAP repeat containing 5(BIRC5) - Cell division cycle associated 5(CDCA5) - Cell division cycle associated 8(CDCA8) - Centromere protein E(CENPE) - Centromere protein F(CENPF) - Centromere protein H(CENPH)

		- Centromere protein I(CENPI) - Centromere protein K(CENPK) - Centromere protein M(CENPM) - Centromere protein U(CENPU) - Kinesin family member 18A(KIF18A) - Kinetochore scaffold 1(KNL1) - Spindle and kinetochore associated complex subunit 1(SKA1) - Structural maintenance of chromosomes 1A(SMC1A)
Mitotic nuclear division	26	- BUB1 mitotic checkpoint serine/threonine kinase(BUB1) - F-box protein 5(FBXO5) - NIMA related kinase 2(NEK2) - NUF2, NDC80 kinetochore complex component(NUF2) - PDZ binding kinase(PBK) - TIMELESS interacting protein(TIPIN) - abnormal spindle microtubule assembly(ASPM) - anillin actin binding protein(ANLN) - aurora kinase B(AURKB) - baculoviral IAP repeat containing 5(BIRC5) - cell division cycle 25A(CDC25A) - cell division cycle 6(CDC6) - cell division cycle associated 3(CDCA3) - cell division cycle associated 5(CDCA5) - centromere protein F(CENPF) - centrosomal protein 55(CEP55) - cyclin A2(CCNA2) - cyclin B2(CCNB2) - cyclin dependent kinase 1(CDK1) - family with sequence similarity 83 member D(FAM83D) - kinesin family member 15(KIF15) - kinetochore scaffold 1(KNL1) - non-SMC condensin II complex subunit G2(NCAPG2) - protein kinase, membrane associated tyrosine/threonine 1(PKMYT1) - spindle and kinetochore associated complex subunit 1(SKA1) - spindle and kinetochore associated complex subunit 3(SKA3)

cell division	28	- BUB1 mitotic checkpoint serine/threonine kinase(BUB1) - F-box protein 5(FBXO5) - MAD2 mitotic arrest deficient-like 1 (yeast)(MAD2L1) - NIMA related kinase 2(NEK2) - NUF2, NDC80 kinetochore complex component(NUF2) - TIMELESS interacting protein(TIPIN) - baculoviral IAP repeat containing 5(BIRC5) - cell division cycle 25A(CDC25A) - cell division cycle 6(CDC6) - cell division cycle associated 3(CDCA3) - cell division cycle associated 5(CDCA5) - cell division cycle associated 7(CDCA7) - cell division cycle associated 8(CDCA8) - centromere protein E(CENPE) - centromere protein F(CENPF) - cyclin A2(CCNA2) - cyclin B1(CCNB1) - cyclin B2(CCNB2) - cyclin dependent kinase 1(CDK1) - family with sequence similarity 83 member D(FAM83D) - kinetochore localized astrin/SPAG5 binding protein(KNSTRN) - kinetochore scaffold 1(KNL1) - non-SMC condensin I complex subunit G(NCAPG) - non-SMC condensin I complex subunit H(NCAPH) - non-SMC condensin II complex subunit G2(NCAPG2) - spindle and kinetochore associated complex subunit 1(SKA1) - spindle and kinetochore associated complex subunit 3(SKA3) - structural maintenance of chromosomes 1A(SMC1A)