The role of Akt1 in skin barrier formation

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Declaration

‘I, Aishath Shanaz Naeem, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.’

Abstract

Atopic Dermatitis (AD) is a chronic inflammatory disease characterised by pruritus, hyperkeratosis, parakeratosis and dry skin. Association of filaggrin mutations with AD has been reported, however not all AD patients have filaggrin mutations suggesting other mechanisms give rise to the barrier defect present in AD. Akt1 activity is essential for cornified envelope formation and correct profilaggrin processing, although the functional role of Akt1 in these processes remains unclear. The aim of this study was to investigate the role of downstream targets of Akt1 signaling in profilaggrin processing and cornified envelope formation. Using shRNA to knock down Akt1 activity in rat epidermal keratinocyte cell lines, an *in vitro* organotypic model was created that phenocopies AD displaying hyperkeratosis, parakeratosis and impaired filaggrin processing. Results show that reduced Akt activity led to decreased lamin A/C degradation disrupting nuclear disintegration process giving rise to parakeratosis. HspB1 is reported to interact with filaggrin and also involved in filaggrin processing. Inhibition of Akt activity demonstrated a switch between HspB1-filaggrin to HspB1-actin interaction in the upper epidermis, which may interfere with filaggrin processing. Cathepsin H (Ctsh) was down regulated 4-fold in our Akt1 knockdown cultures and its expression co-localized with filaggrin in the granular layer of human epidermis. Filaggrin processing was impaired in Ctsh shRNA knockdown cell lines, and both $Ctsh^{+/-}$ and $Ctsh^{-/-}$ mice displayed hyperkeratosis and reduced filaggrin expression. Inhibition of RAPTOR (a component of mTORC1) has previously been reported to increase Akt1 activity. Raptor overexpression in REKs showed a decrease in Akt phosphorylation, Ctsh and filaggrin processing, and treatment with Rapamycin, an mTORC1 inhibitor, reverses these effects. In uninvolved AD skin, increase in RAPTOR correlated with decrease in both Akt phosphorylation and filaggrin expression. This thesis presents a novel mechanism where increase in RAPTOR can lead to a reduction in epidermal granular Akt1 phosphorylation leading to impaired filaggrin processing and barrier defects in AD.

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Dedicated

to

my father & mother

For your love, encouragement and continuous assurance

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Abbreviations

%	Percentage
°C	Degrees centigrade
aa	Amino acid
AB	Antibiotic
ACTB	β-Actin gene
ACTL9	Actin like protein 9
AD	Atopic dermatitis
Akt	V-akt murine thymoma viral oncogene homolog 1
AM	Antimycotic
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
AP-1	Activator protein 1
APS	Ammonium per sulphate
ATRA	All trans retinoic acid
au	Arbitrary units
BCA	Bicinchoninic acit
BMP	Bone morphogenic protein
bp	Base pairs
BSA	Bovine serum albumin
CAD	Caspase-activated Dnase
CD	Cluster of differentiation
cDNA	Complimentary dioxyribonucleic acid
cm	Centimetre
Co-IP	Co-Immunoprecipitation
CO_2	Carbondioxide
Conc.	Concentrated
CTSH	Cathepsin H
$CuSO_4$	Copper Sulphate
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic cells
DED	De-epidermalised dermis
dLNs	Draining lymph nodes
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase1L2	Dnase-1-Like-2
ECL	Enhanced chemiluminescence
EDC	Epidermal differentiation complex
EDMD	Emery-Dreifuss muscular dystrophy
EDTA	Ethylene-dinitrilo tetraacetic acid

EGF	Epidermal growth factor
ESI-QTOF	ElectroSpray Ionisation-Time-of-Flight
F-actin	Filamentous actin
FBS	Foetal bovine serum
FLG	Filaggrin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	Genome wide association studies
H&E	Haematoxylin & eosin
HC	Hydrocortisone
HCl	Hydrochloric acid
HGPS	Hutchinson-Guilford Progeria Syndrome
HPLC	High Performance Liquid Chromatography
HRP	Hydrogen peroxide
Hsp27	Heat shock protein 27
HspB1	Heat shock protein beta-1
IFN-Y	Interferon-Y
Ig	Immunoglobin
IL	Interleukin
INM	Inner nuclear membrane
Inv	Involucrin
IPA	Ingenuity Pathway Analysis
IRS-1	Insulin receptor substrate 1
IV	Ichthyosis Vulgaris
K1	Keratin 1
K10	Keratin 10
K14	Keratin 14
K5	Keratin 5
kDa	Kilo dalton
L	Litre
LCs	Langerhans cells
LEKTI	Lymphoepithelial Kazal type related inhibitor
LINC	Linker of nucleoskeleton cytoskeleton
LMNA	Lamin gene
Lor	Loricrin
LXR	Liver X receptor
ml	Milli litre
mM	Milli molar
MMPs	Metalloproteinases
mRNA	messenger ribonucleic acid
MS	Mass spectrometry
mTORC	Mammalian target of rapamycin complex

NA	Numerical aperture
Na-Citrate	Sodium Citrate
NaCl	Sodium Hydroxide
NF-AT	Nuclear-factor for activated T-cells
NHEKs	Normal human epidermal keratinocytes
nm	nanometer
nM	nano molar
NMF	Natural moisturising factor
NS	Netherton Syndrome
OVOL1	OVO homologue-like 1
p	Probability
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen associated molecular patterns
PBS	Phosphatee-buffered saline
PBST	Phosphatee-buffered saline tween-20
PC	Proprotein convertase
PCA	Pyrrolidone carboxylic acid
PCR	Polymerase chain reaction
PDK1	3-phosphoinositide-dependent protein kinase 1
PEP1	Profilaggrin endopeptidase 1
PH domain	Pleckstrin homology domain
PI3K	Phosphoinositide-3-kinase
PIP2	Phosphatidylinositol-3, 4, 5-diphosphates
PIP3	Phosphatidylinositol-3, 4, 5-triphosphates
PLGS	ProteinLynx Global Server
PMSF	Phenylmethanesulfonyl Fluoride solution
PTEN	Phosphatase and tensin homolog
RAPTOR	Regulatory associated protein of mTOR
RAR	Retinoic acid receptor
REK	Rat epidermal keratinocyte
Rictor	Rapamycin insentitive companion of mTOR
RIPA	Radioimmuno precipitation buffer
RPTOR	Raptor gene
RTK	Receptor tyrosine kinase
RXR	Retinoid X receptor
Ser473	Serine 473
SCORAD	Scoring of Atopid Dermatitis
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
Ser301	Serine 301
Ser404	Serine 404
shRNA	short hairpin ribonucleic acid

SNPs	Single nucleotide polymorphism
SP	Serine protease
Sp-B	Surfactant protein B
SPINK5	SP inhibitor lymphoepithelial Kazal-type trypsin inhibitor
T308	Threonine 308
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween-20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TEWL	Transepidermal water loss
TGM1	Transglutaminase 1
Th	T helper
TLRs	Toll like receptors
TNF	Trypsin Neutralizing Factor
UCA	Urocanic acid
UCL	University College London
UV	Ultra violet
V	Voltage
v/v	volume by volume
w/v	weight by volume
µg	micro gram
µl	micro litre
µM	micro molar

Chapter 1 : Introduction

1.1 Skin: A protective barrier

1.1.1 Overview of skin

The skin is a vital organ of the human body consisting of organized and functionally distinct layers that form a tough and impermeable structure protecting the organism from the harsh environment (Fuchs & Raghavan, 2002). It is comprised of three layers, the epidermis, the dermis and the hypodermis. The epidermis, which is the outermost layer, mainly contributes to the physical barrier function. The dermis is predominantly composed of connective tissue matrix but also has specialized cells such as macrophages, mast cells, lymphocytes and fibroblasts. Finally the innermost layer, the hypodermis, which is attached to the dermis via collagen and elastin fibres, consists mainly of adipocytes acting as an energy reserve and heat insulator (Fig 1.1) (Fuchs, 1990; Fuchs & Raghavan, 2002). Skin primarily acts as a protective barrier and apart from its function in maintaining its structural integrity, the skin also plays important roles in temperature regulation, protection from UV radiation and the innate immune system (Mack *et al*., 2005; Reichrath, 2006). Maintenance of epidermal homeostasis and morphogenesis is important, and any disruptions can result in skin disorders, therefore it is important to understand fully the intricate pathways involved for designing better therapeutic approaches.

1.1.2 Structure and function of epidermis

The epidermis is a self-renewing stratified squamous epithelium that is predominantly composed of keratinocytes although specialized cells such as Merkel cells, melanocytes and Langerhans cells contribute to a small percentage of the total epidermal cell population (reviewed in Arwert *et al*., 2012; Koster & Roop, 2007). The epidermis is organized into four histologically distinct layers, which are the basal, spinous, granular, and the outermost cornified layer (stratum corneum), each at a histologically defined point along a continuum of keratinocyte differentiation (Fig 1.2). The efficiency of barrier function of the epidermis relies on the proper execution of the keratinocyte terminal differentiation program (Kypriotou *et al*., 2012; Hedge & Raghavan, 2013). The process is initiated in the basal layer, which is the innermost layer of the epidermis and comprises of a single layer of mostly mitotically active cells,

which produce all the suprabasal cells in the epidermis. In the presence of the appropriate stimuli, basal cells proliferate and give rise to daughter cells that lose the integrin mediated contacts allowing them to detach from the basement membrane and migrate towards the spinuous layer (Hall & Watt, 1989; Watt, 2002; Fuchs, 2007). As the cells move into the spinous layer, they undergo mitotic arrest, stop expressing the basal keratins K5 and K14 and begin expression of early differentiation markers K1 and K10, the presence of which is considered the most dependable sign that the keratinocytes have committed to differentiation (Fig 1.2) (Fuchs and Green, 1980; Fuchs, 2007). At this stage cell-cell contacts mediated by adherens and desmosomal junctions are established (Owens *et al.,* 2000). In the granular layer, named for the presence of the basophilic keratohyalin granules, the cornified envelope proteins such as loricrin and transglutaminases are expressed which are necessary for the formation of the main epidermal barrier structure, the cornified layer (Fuchs & Raghavan, 2002). Filaggrin, a filament aggregating protein, is expressed here in its precursor form, profilaggrin, which is the major component of the keratohyalin granules (Presland *et al.,* 1992). Filaggrin binds and collapses the keratin network in the upper granular layer promoting the formation of the flattened shape characteristic of the corneocyte (Mack *et al*., 1993; Presland *et al*., 2001; Kuechle *et al*., 2000). At the transitional stage, prior to cornified envelope formation, the nucleus and other cellular organelles get degraded, forming an enucleated corneocyte (Gan *et al.,* 1990; Presland *et al.,* 1992). This non-viable cornified layer is made of a covalently crosslinked multiprotein complex of key structural proteins such as involucrin, loricrin and filaggrin, which are heavily cross-linked by transglutaminases and surrounded by a lipid matrix providing an excellent barrier to the environment (Candi *et al.,* 2005). The cornified layer is eventually shed in a process called desquamation. In humans the whole process, starting from the undifferentiated basal cell ending with the dead squame, takes approximately 14 days (Subramanyan, 2004) .

1.1.3 Barrier function

A vital function of the skin is to provide a competent barrier between the organism and the environment (Reviewed in Proksch *et al*., 2008). The skin structure has been likened to a system of bricks and mortar, with the multiprotein complex of the cornified keratinocyte designated as bricks and the intercellular lipid layer representing the mortar (Nemes and Steinert, 1999), where defects in either of these compartments

can compromise the barrier function of the skin (reviewed in Fuchs and Cleveland, 1998; Jungersted *et al.,* 2008). The heavily cross-linked cornified envelope proteins such as filaggrin, involucrin and loricrin contribute greatly to the integrity of the skin barrier whereas the lipid component along with epidermal tight junctions play important roles in regulating the skin permeability (Kypriotou *et al*., 2012; Lopez *et al*., 2007; Furuse *et al*., 2002). The lipids, mainly comprising of ceramides, cholesterol and fatty acids, are synthesized in the granular layer and temporarily stored in lamellar bodies (LB) (Feingold, 2007; Wertz *et al*., 1984). During cornification the contents of LB are released into extracellular space and incorporated into the stratum corneum (Elias *et al*., 2014). Tight junctions in the granular layer help promote skin barrier by forming adhesion like contacts between cells allowing only selective passage of small molecules and preventing water loss (Kirshner *et al.,* 2010; Furuse *et al.,* 2002). Incorporated into the cornified layer are also specialized proteins such as plakin proteins and corneodesmosin, which strengthen adhesion in the stratum corneum (DiColandrea *et al.,* 2000; Lundstrom *et al.,* 1994).

1.1.4 Skin immune system

As the outer most organ of the body with constant exposure to potential pathogens, the skin is required to also act as an immune protective organ, a role fulfilled by a thorough immune surveillance system coordinated by a network of epithelial and immune cells residing in the skin (Nestle *et al*., 2009). Importance of well-coordinated skin immune system towards maintaining epidermal homeostasis is demonstrated by the incidence of skin infections in immune compromised individuals and in inflammatory disorders such as psoriasis and atopic dermatitis (Bonness and Bieber, 2007; Lowes *et al.,* 2007). The stratum corneum along with the tight junctions in the granular layer forms a formidable barrier against environmental insults (Candi *et al.,* 2005), however to reinforce this defense both the epidermis and dermis are populated with immune cells that work in concert to detect and initiate immune responses that successfully repair any breach to the barrier (Nestle *et al.,* 2009). Similar to cells of the immune system, keratinocytes also have an innate role in detecting pathogens by expressing germ line encoded pattern recognition receptors such as toll like receptors (TLRs) that recognise a wide range of pathogen components (Takeuchi *et al.,* 2002; Martinon *et al.,* 2009). Epidermal immune cells such as macrophages and epidermal dendritic cell (DC) populations called Langerhans cells (LCs) and inflammatory dendritic epidermal cells

(IDECs) play a central role in uptake of invading pathogens and presenting them to either Th1 or Th2 type cells (Nestle *et al.,* 2009). They can migrate to draining lymph nodes (dLNs), present the pathogen to T cells activating adaptive immune responses. Many immune cells such as CD4+, natural killer T cells, mast cells and DCs are also found in the dermis where traversing blood and lymphatic vessels facilitate their access and exit from the skin (Nestle *et al.,* 2009). Keratinocytes, LCs, dermal DCs and mast cells also release chemokines, cytokines and antimicrobial peptides (AMPs) that can also initiate an immune response (Murphy *et al.,* 2000; Yang *et al.,* 2001).

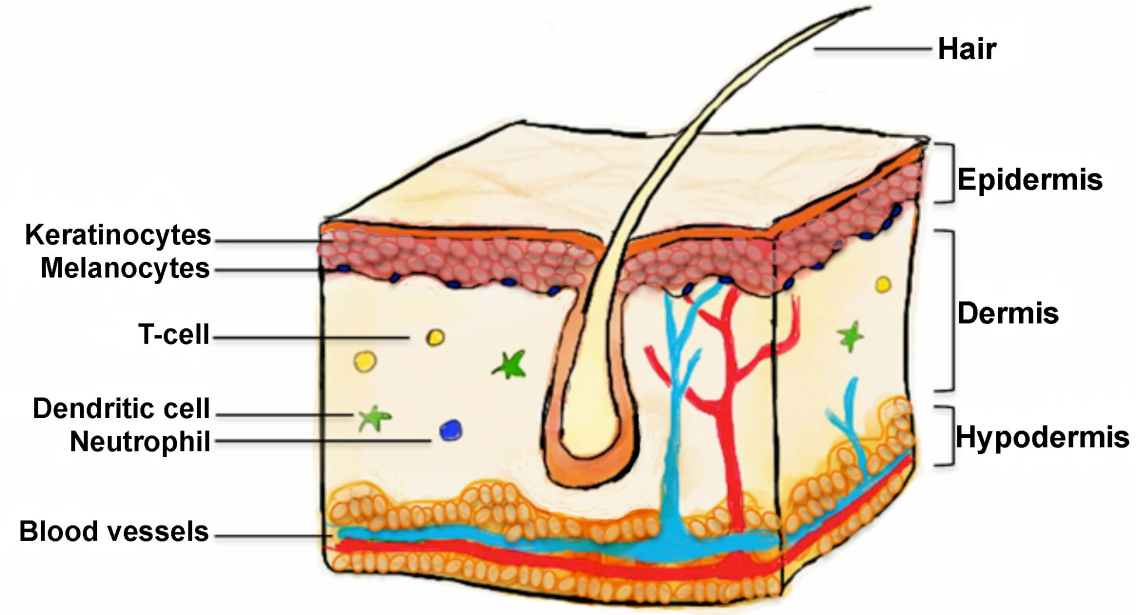

Figure 1.1 Schematic showing normal anatomy of human skin consisting of epidermis, dermis and hypodermis.

The outer layer epidermis is primarily composed of keratinocytes. The dermis is populated with various types of immune cells including neutrophils, mast cells and dermal dendritic cells. The hypodermis, mainly consists of adipocytes, is interspersed with blood and lymph vessels.

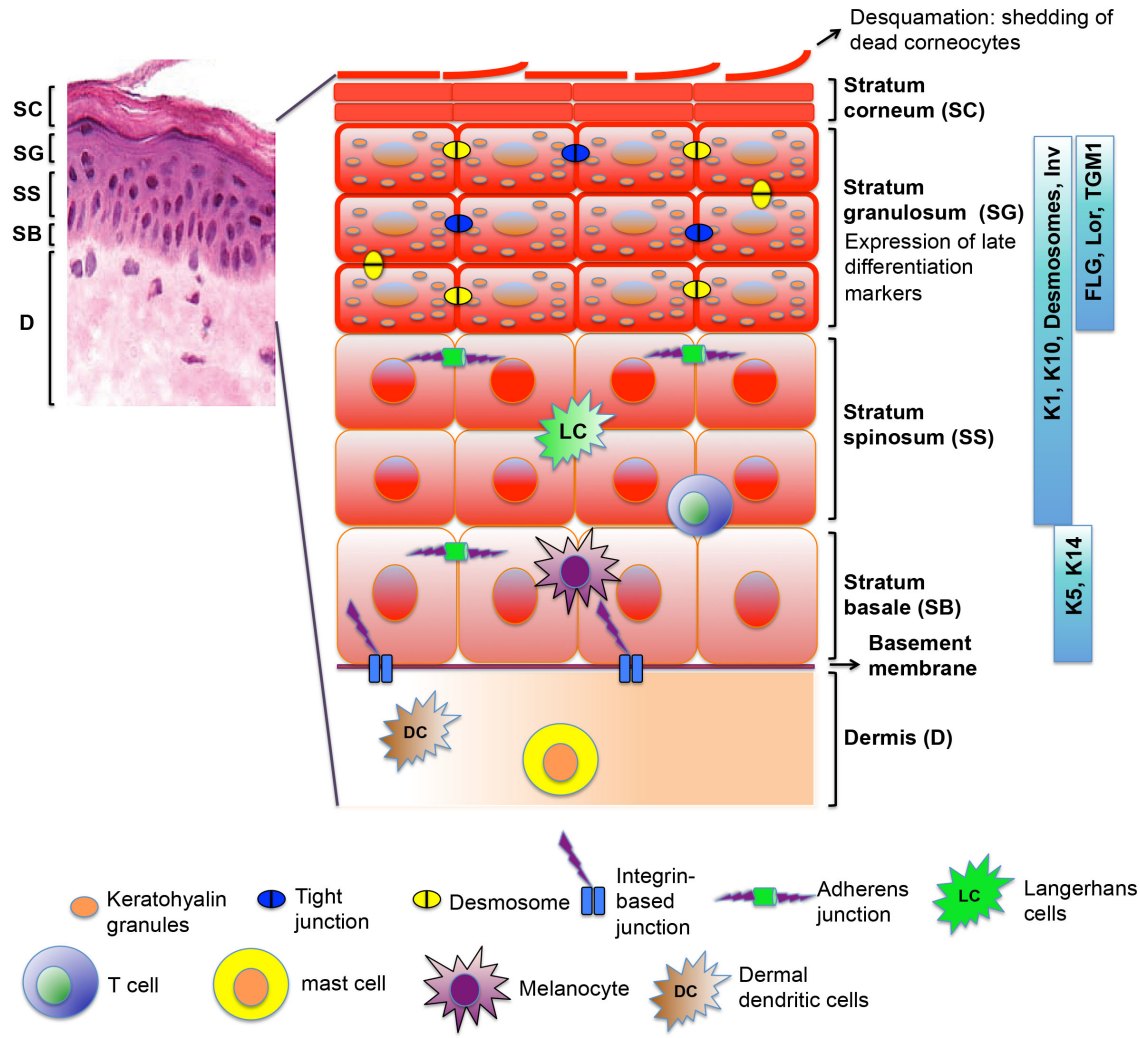

Figure 1.2 Illustration of the stratified epidermis.

H&E stained human skin sample (Guttman-Yassky et al., 2010) accompanied with a schematic of the four layers of the epidermis, stratum basale, spinosum, granulosum and corneum. The basal layer rests on the basement membrane via attachments facilitated by integrin based junctions and expresses keratins 5 & 14. Melanocytes are also visible in the basal layer. The early differentiation markers: Keratin 1 (K1), Keratin 10 (K10) and Involucrin (Inv) are expressed in the spinuous layer. The cornified envelope proteins, filaggrin (FLG) and loricrin (Lor) are expressed in the granular layer along with the crosslinking enzyme transglutaminase 1 (TGM 1). Filaggrin is found in the keratohyalin granules that are present in abundance in the granular layer. Tight junctions, desmosomes and adherens junctions assist in organizing the rigid structure of the epidermis. Skin resident immune cells such as Langerhans cells and T cells in the epidermis along with dendritic cells and mast cells in the dermis are part of the immune surveillance system in the skin. SC – Stratum corneum, SG – Stratum granulosum, SS – Stratum spinosum, SB – Stratum basale, D – Dermis.

1.2 Atopic Dermatitis

1.2.1 Atopic dermatitis: An overview

Atopic Dermatitis (AD) is a chronic inflammatory disease that affects up to 10-20 % of children and 1-3 % of adults worldwide. The etiology of AD is complex, resulting from interactions between genetic factors, dysfunctional skin barrier, immunological responses and environmental triggers (Elias & Schmuth, 2009; Barnes, 2009; Brandt & Sivaprasad, 2011). Despite the multifactorial nature of the disease, and varying clinical patterns with age, the common symptoms of AD are relapsing pruritus, chronic lesions, increased transepidermal water loss and lichenification (Leung and Bieber, 2003). AD is the first clinical manifestation in patients during the process of atopic march, a concept that characterizes subsequent progression from AD to other atopic disorders such as allergic rhinitis and asthma. In fact, almost 70 % of patients with severe AD develop asthma (Spergel, 2010). An underlying atopy is considered important in linking these diseases where atopy is defined as a personal or familial inclination to produce elevated levels of IgE antibodies and sensitization to environmental allergens (Leung and Bieber, 2003).

There is currently no cure for AD, and management of the disease is a clinical challenge due its complex pathophysiology and varying severity of clinical presentation (Simpson, 2010; Eichenfield *et al*., 2014). The current therapies that exist for AD involve anti-inflammatory drugs to target the inflammatory responses observed during the acute and chronic phase of disease, antibiotics to treat secondary skin infections and emollients to hydrate the skin and maintain skin barrier function (Elias *et al.,* 2014; Boguniewicz & Leung, 2013). Understanding the mechanisms involved in causing barrier disruption in eczema may therefore lead to more targeted therapies for treating this disease.

1.2.2 Epidemiology of AD

Prevalence of AD has increased 2-3 fold in the past three decades, mostly in industrialized countries and has become a national health burden (Bieber, 2010; Asher *et al.,* 2006). Studies have estimated that the annual cost of AD to the health services in the USA in 1993 was US $364 million (Lapidus *et al*., 1993). This study was only limited to children and excluding the costs to patient and family. Estimated annual cost

of AD to third parties in the United States from 1997 to 1998 ranged from US $0.9 billion to US$ 3.8 billion (Ellis *et al*., 2002). In the UK, a community study of AD in Scotland estimated annual cost of £465 million (Herd *et al*., 1996). Another UK study reported the annual cost owing to AD in children between the ages of 1 to 5 estimated to be £47 million in 1995 to 1996 (Emerson *et al*., 2001). In Canada, the total cost of AD was estimated to be Cdn $1.4 billion annually. Apart from financial burden, the disease can have a major impact on other aspects of life. Surveys have indicated that almost 60 % of AD patients have reported that the disease impaired the performance of their daily activities such as school performance, sports, leisure and social activities (Paller *et al*., 2002). AD prevalence varies extensively between countries from around 20 % in industrialised countries such as Australia, New Zealand, Urban Africa, England and Scandinavia to less than 5 % in regions such as China, Eastern Europe, rural Africa and central Asia (Leung and Bieber, 2003). Lifestyle and environment play a major part in the development of AD, in fact higher prevalences have been recorded in urban regions and in higher socioeconomic groups (Leung and Bieber, 2003; Bieber, 2010). AD usually emerges for the first time during early infancy, with 45 % of cases appearing during first 6 months of life and 85 % being showing symptoms before the age of 5. Though eczematous lesions often appear during early infancy, it may persist into or in some cases even start in adulthood (Leung and Bieber 2003).

1.2.3 Clinical manifestations and diagnosis of AD

AD typically occurs as a relapsing condition where the patients may experience periods of remission and exacerbations. The disease presents a diverse clinical spectrum ranging from pityriasis alba (dry depigmented patches) to severe eczematous lesions and excoriations (skin abrasions caused by repetitive scratching) (Leung and Bieber. 2003). The most common symptoms are pruritus (itching) and xerosis (dry skin). AD can occur in three age related stages that may be separated by periods of remission (Fig 1.3). During infancy (under the age of 2 years) the affected areas include the cheeks, neck, groin and extensor part of arms and legs. These affected areas display a more acute stage of AD and consist of highly pruritic, dry and scaly patches that can sometimes ooze and weep fluid. During childhood, (between age of 2 and 12 years), the most affected areas are usually the flexural areas especially in antecubital (creases of elbow) and popliteal fossae (back of the knee), with rashes also appearing in other areas such as ankles, wrists and necks (Rudikoff and Lebwohl, 1998). During this stage,

chronic lesions are observed characterized by thickened plaques that show lichenification caused by repeated scratching of skin. The lesions can also display papules (small pimple or swelling of the skin) and excoriation. It is rare for adults to get AD and when they do, symptoms are different from those in childhood AD. The lesions are more diffuse with prominent xerosis and underlying erythema (Banfield *et al.,* 2001). Amongst affected areas, facial involvement is common with possible lichenification present in flexural areas (Rudikoff and Lebwohl, 1998). Using the diagnostic criteria set down by Hanifin and Rajka as a basis, the clinical features listed in table 1-1 are often used as a criterion for a diagnosis of AD (Table 1-1). The most widely used method for diagnosing the severity of AD is the SCORAD (SCORing of Atopic Dermatitis) system. SCORAD is a clinical tool that assesses the extent and intensity of AD, which comprises scoring the affected areas as a percentage of whole body, and also assigning a representative area a score based on the clinical signs of the disease including itch and sleep loss (Oranje *et al.,* 2007).

Table 1-1 Diagnostic criteria of AD

Essential Features
Pruritus
Facial and extensor eczema in infants and children
Flexural eczema in adults
Chronic or relapsing dermatitis
Frequently associated features
Personal or family history of atopic disease
Xerosis
Cutaneous infections
Non-specific dermatitis of the hands or feet
Raised Serum IgE concentrations
Positive immediate-type allergy skin tests
Early age of onset
Other features
Ichthyosis, palmar hyperlinearity, keratosis pilaris
Pityriasis alba
Nipple eczema
White dermatographism and delayed blanch response
Anterior subcapsular cataracts, keratoconus
Dennie-Morgan infraorbital folds, orbital darkening
Facial erythema or pallor
Excluding conditions
Seborrheic dermatitis
Contact dermatitis
Ichthyosis
Psoriasis
Photosensitive dermatoses
Immune deficiency diseases
Erythroderma of other causes

Table based on Eichenfield *et al.*, 2014 and Yazganoglu & Ozkaya, 2011

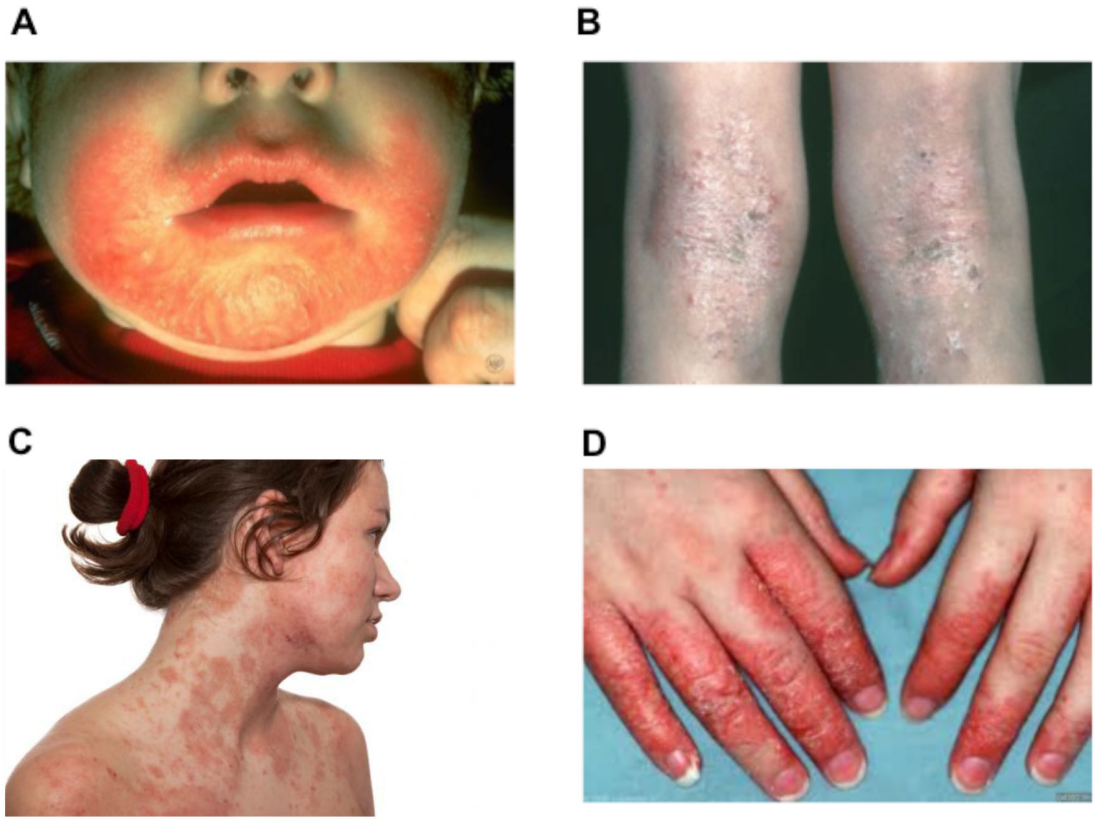

Figure 1.3 Clinical presentation of AD

(A) Typical facial eczematous rash on a child. (B) Lesions on the back of the knee appears lichenified and hyperpigmented. (C) Face and neck affected on an adult. (D) Hand eczema on an adult (Images adapted from http://www.aad.org/dermatology-a-to-z/diseases-and-treatments/a---d/atopic-dermatitis/signs-symptoms).

1.2.4 Pathophysiology of AD

Even clinically unaffected skin of AD patients tends to be dry with terminal differentiation defects and some immune abnormalities (Suarez *et al.,* 2011). The natural course of AD flares follows both an acute and chronic phase each with differing histologies and immunological processes (Allam & Novak, 2005). Acute skin lesions are characterized by marked intercellular oedema in the epidermis (spongiosis) and large number of antigen presenting cells such as Langerhans cells, inflammatory dendritic epidermal cells and macrophages binding IgE molecules on their surface. Marked dermal infiltration of CD4 activated T cells are also observed in acute lesions. Chronic lichenified lesions display thickening of the stratum corneum (hyperkeratosis), retention of nuclei in stratum corneum (parakeratosis) and thickening of the stratum basale and spinosum (acanthosis) and elongated rete ridges (reviewed in Leung *et al.,* 2013; Allam & Novak, 2005). Figure 1.4 demonstrates the common histological changes observed in AD skin. Increased numbers of IgE bearing antigen presenting cells are also observed in chronic lesions with majority of dermal infiltrate comprising of macrophages (Tanei *et al*., 2013). Additionally chronic lesions also contain eosinophils, a hallmark of end stage inflammatory response during allergic reactions. Eosinophils are thought to contribute to chronic inflammation by producing proinflammatory cytokines and eosinophilic toxic granule proteins that can cause tissue damage (Reviewed in Eyerich & Novak, 2013; Leung *et al.,* 2013).

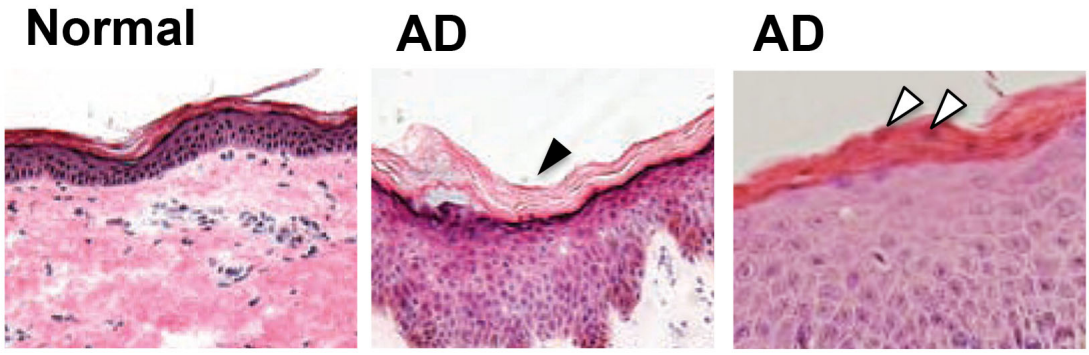

Figure 1.4 Comparison between normal and AD.

The epidermis in individuals with AD have a thickened cornified layer characteristic of AD (black arrow head, middle panel). Parakeratosis (retained nuclei in the cornified layer) is another feature sometimes observed in AD (white arrow heads, last panel) (adapted from Guttman-Yassky et al., 2011 ; Nakamura et al., 2011).

1.2.5 Immunological abnormalities in AD

AD has a strong immune component with perturbations to both innate and adaptive immune system. AD patients display a tendency to have frequent bacterial and viral skin infections, hyper reactivity to environmental elements and predisposition to IgE-mediated sensitization (Fig 1.5) (Eyerich & Novak, 2013). AMPs such as B-defensins produced by keratinocytes that have high antibiotic properties are reduced in AD patients. The skin of these patients is often prone to skin infections with over 90 % of patients affected by *Staphylococcus aureus*, a phenomenon that is attributed to reduced levels of B-defensins in the skin (Leung & Bieber, 2003). Most AD patients have elevated serum IgE levels and increased peripheral blood eosinophil count. These patients display a predominant Th2 immune response with increased frequency of allergen specific T cells producing the interleukins 4 (IL-4), 5 (IL-5) and 13 (IL-13) and decreased levels of interferon-γ (IFN-γ) in peripheral blood (Eyerich *et al.,* 2011; Eyerich & Novak, 2013). A wide range of allergens have been implicated in causing immune activation via a Th2 dominant mechanism in AD such as toxins produced by skin staphylococci and house-dust mites (Cardona *et al.,* 2006; Leung and Bieber, 2003). This increases the frequency of IgE receptor bearing antigen-presenting cells (APCs) in eczematous lesions that play a key role in allergen presentation to T-cells residing in atopic skin. IgE bearing Langerhans cells can also migrate to lymph nodes where naïve T cells are stimulated contributing to Th2 cell pool expansion (Eyerich and Novak, 2013). Moreover, IL-4 and IL-13 can reduce expression of some important stratum corneum proteins such as filaggrin further aggravating the barrier function (Hanel *et al.,* 2013). Persistent inflammation observed in chronic lesions may be mediated by IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) that prolongs the survival of eosinophils, and other factors that contribute to inflammations such as Langerhans cells and macrophages (Pastore *et al.,* 1997; Van Reijsen *et al.,* 1992). Acute eczematous lesions contain mostly Th1 type cytokine activity with increased levels of interleukins 4, 5 and 13, while mainly Th2 type cells such as IL-12 and IFN-γ are observed in chronic lesions. IFN-γ is a key cytokine involved in the induction of T cell mediated apoptosis in eczematous lesions contributing to the observed spongiotic process in acute lesions (Allam *et al.,* 2005). The switch from Th2 to Th1 type response is thought to be mediated by production of IL-12 from either infiltrating eosinophils or dendritic cells in the epidermis or both (Leung *et al.,* 2004).

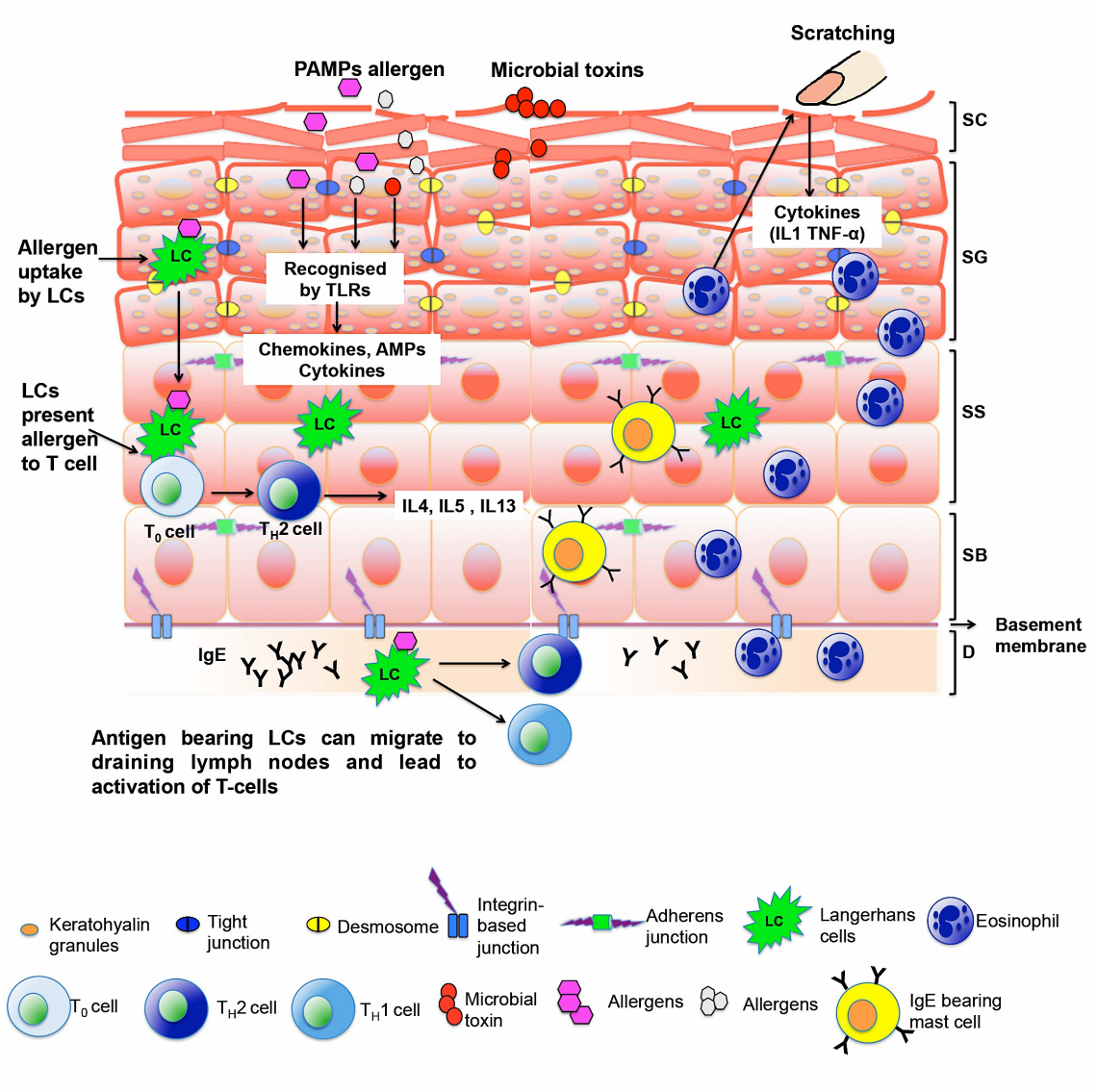

Figure 1.5 Immunological changes in AD skin.

Defective barrier can allow penetration of allergens and pathogens to trigger release of proinflammatory factors that can elicit immune responses. Scratching can damage cells and amplify inflammatory reaction in the skin. Pathogen associated molecular patterns (PAMPs) which include flagellin, peptidoglycan and nucleic acids are recognized by TLRs expressed on keratinocytes and other skin immune cells triggering the release of chemokines, antimicrobial peptides (AMPs) and cytokines which in turn can perpetuate inflammatory responses. Langerhans cells, which are key immunological sentinels in the epidermis, can take up and present the allergen to skin resident T cells activating Th2 responses. SC – Stratum corneum, SG – Stratum granulosum, SS – Stratum spinosum, SB – Stratum basale, D - Dermis

1.2.6 Barrier defect in AD

Even though abnormalities in the immune system have been strongly attributed to development of AD, the skin barrier defect is a key component in this disease and is in fact thought to be the primary cause in initiating and driving the pathogenesis of AD in the majority of AD cases therefore suggesting the observed inflammation is secondary to this inherent barrier defect (Callard and Harper, 2007; Jakasa *et al.,* 2006). Even clinically unaffected AD skin that is devoid of inflammation is dry with increased sensitization to allergens hence supporting the theory of an inherent barrier defect in AD that is susceptible for cutaneous inflammation (Suarez *et al.,* 2011; Leung *et al.,* 2013). The epidermis primarily functions as a physical barrier to the external environment, and a skin barrier defect will therefore promote water loss and permit pathogens, toxins, and other environmental allergens to penetrate the skin layers and elicit an immune response (Fig 1.6) (reviewed in Candi *et al.,* 2005). Several factors such as altered expression of filaggrin, reduced lipid component (ceramide and sphingosine) and disrupted cell-to-cell junctions have been identified as being altered in AD skin and linked to contributing to barrier defect (Bieber, 2008).

The strongest evidence for an impaired epidermal barrier being the primary cause for AD comes from the strong link between loss of function mutations in the gene encoding the key epidermal protein filaggrin (*FLG*) and AD (Baurecht *et al.,* 2007). Filaggrin is an integral component of the epidermis and has pivotal roles in barrier function by contributing to the structural integrity and hydration of the stratum corneum (Presland *et al.,* 2001). Those homozygous for *FLG* mutations lack a functional copy of *FLG*, and display dry skin and increased predisposition for AD (Fallon *et al.,* 2009; Bisgaard *et al.,* 2008). The functional roles of filaggrin will be covered in more depth in section 1.3. There is evidence however that barrier dysfunction can occur independently from the *FLG* genotype suggesting the existence of other factors impairing epidermal barrier function (Thawer-Esmail *et al.,* 2014). Amongst these is increased epidermal serine protease activity that has been identified in AD leading to stratum corneum abnormalities (Walley *et al.,* 2001). Epidermal proteases and their inhibitors are important contributors to barrier function with roles in processing of key epidermal proteins, desquamation and epidermal permeability homeostasis (Ovaere *et al.,* 2009). Serine protease (SP) hyperactivity is observed in AD with increased activity attributed due to both genetics and changes in skin pH. Serine proteases function optimally in neutral to alkaline range and AD skin has a generally higher pH level, which is

associated with high transepidermal water loss (TEWL) (Ovaere *et al.,* 2009; Walley *et al.,* 2001). Increase in SP activity can have detrimental effects on the barrier function as it can prematurely degrade corneodesmosomes and increase corneocyte desquamation (Hachem *et al.,* 2006). Excessive SP activity can also lead to disruption of lipid synthesis in epidermis leading to a decrease in stratum corneum lipid content, which is essential for epidermal permeability homeostasis (Hachem *et al.,* 2006). Finally, serine protease hyperactivity can lead to an increase in Th2 inflammation and research has shown introduction of serine protease inhibitor during sensitization has a dampening effect on allergic inflammation (reviewed in Levin *et al.,* 2013).

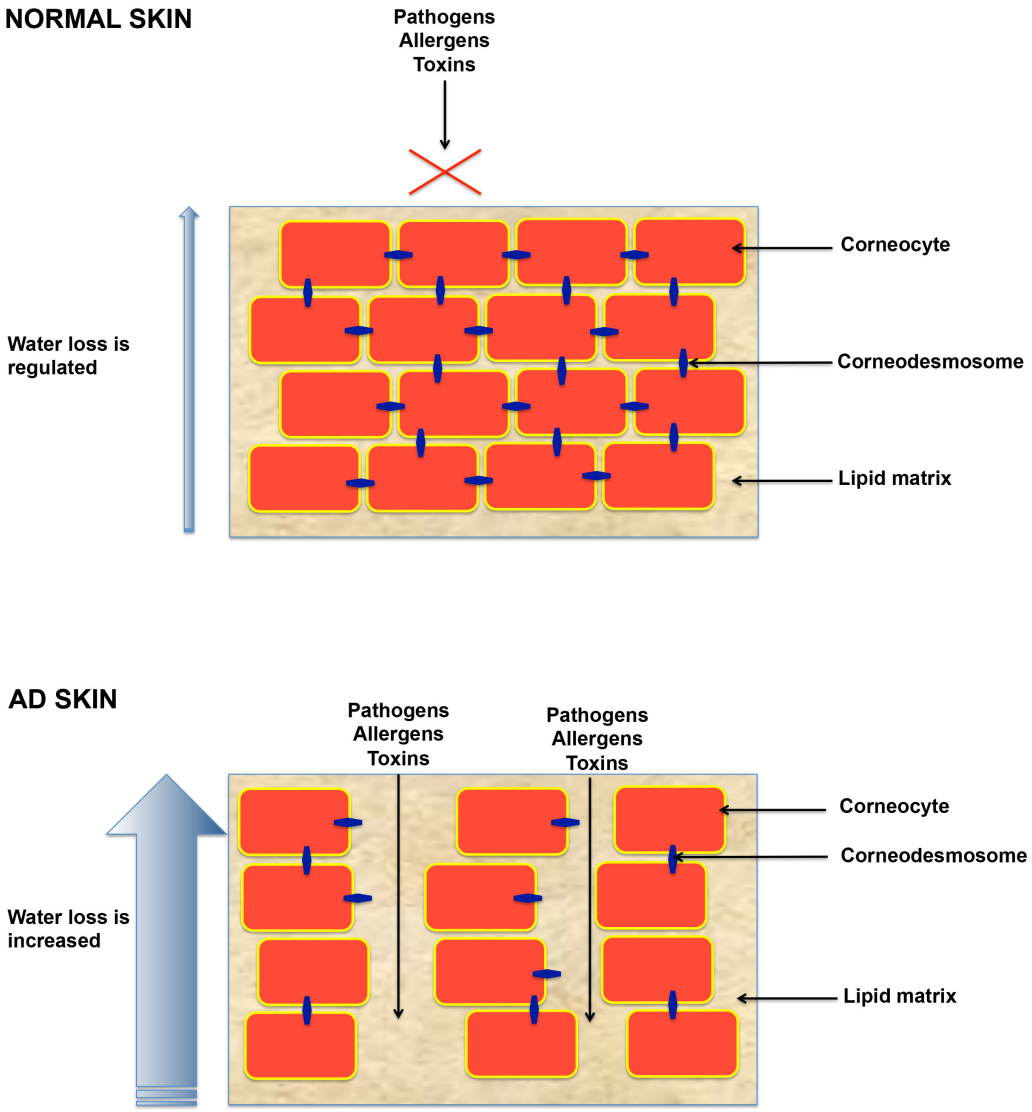

Figure 1.6 Schematic demonstrating the brick and mortar structure of the cornified layer.

Barrier defects effectively loosen this structure allowing increased water loss, entry of environmental pathogens and allergens.

1.2.7 Genetics of AD

Epidemiological research has revealed a strong genetic component to AD (Schultz, 1993; Cork *et al.,* 2006). Twin studies of AD have shown higher concordance rates in monozygotic twins (0.72 – 0.86) than dizygotic twin pairs (0.21 – 0.23) indicating there is a strong genetic influence in the development of AD (Larsen *et al.,* 1986; Bowcock and Cookson, 2004; Schultz Larsen, 1993). Additionally incidence rate of AD in children is doubled if one parent has AD and tripled if AD is present in both parents (Bieber, 2010). Various Genome wide association studies (GWAS) have revealed genes associated with AD with linkage detected on chromosomes 1, 3 4, 5, 11, 13, 15, 17, 18, 19 and 20, with *FLG* (filaggrin) at 1q21 being the most highly replicated risk locus among these studies (Barnes, 2010). Genetic linkage to the loci 1q21 was interesting as this region contains the Epidermal Differentiation Complex (EDC) comprising of genes encoding filaggrin and other structural proteins involved in keratinocyte terminal differentiation (Cookson, 2001). Null mutations in *FLG* are associated with the inherited eczematous disease and ichthyosis vulgaris (Presland *et al.,* 2000; Palmer *et al.,* 2006). In fact, presence of *FLG* mutations leads to more severe and early onset AD (Bieber, 2010). A recent GWAS study identified two new risk loci in chromosome 11 upstream of transcription factor OVO homologue-like 1 (OVOL1) and in chromosome 19 near actin like protein 9 (ACTL9), both genes implicated in keratinocyte proliferation and differentiation hence emphasizing the importance of skin barrier function abnormalities in AD development (Paternoster *et al.,* 2012). Immune responsive genes including IL-5, IL-13, IL18, CD14, toll like receptor 2 (TLR2) and those involved in cell signaling such as CSF2 (colony-stimulating factor 2) are also associated with AD (Barnes, 2010). An association was also seen between AD and *SPINK5* encoding the protein lymphoepithelial Kazal type related inhibitor (LEKTI), a serine protease inhibitor which in skin is expressed within the granular layer (Hachem *et al*., 2006; Barnes, 2010). Mutations in *SPINK5* lead to uncontrolled protease activity and underlie the disease Netherton Syndrome (NS), which is a compelling case for excessive serine protease activity leading to skin barrier dysfunction. NS patients display increased filaggrin processing and increased desquamation and frequently develop atopic manifestations including AD (Hachem *et al.,* 2006).

1.2.8 Current treatments of AD

Currently there is no cure for AD probably because it is a heterogeneous disease. The existing treatments are targeted to suppress and relieve symptoms of the disease. The patients are advised to apply emollients to keep the skin hydrated and smooth (Loden, 2003). For mild to moderate AD, first-line treatments are usually topical steroids of different strengths as they have been effective at reducing inflammation (Novak and Simon, 2011). Short courses of oral steroids are sometimes given in cases of severe flares. However, as steroid treatments can have side effects, the patient is generally kept under review by a dermatologist (Hata *et al.,* 2008). Since skin infections are quite common in AD skin, both oral and topical antibiotics are frequently prescribed to treat these secondary infections (Novak and Simon, 2011). In situations where the patients are non-responsive to steroid treatment, immunomodulators such as tacrolimus and pimecrolimus, which are calcineurin inhibitors, can be used (Buchau *et al.,* 2008). Calcineurin is an enzyme that dephosphorylates and activates nuclear-factor for activated T-cells (NF-AT), which regulates the transcription and proliferations of both Th1 and Th2 type cells. The immunomodulators can block transcription and proliferation of T cells and also downregulate T cell mediated apoptosis in AD. Tacrolimus can also reduce T cell proliferation caused by superantigens released by *S. aureus* (reviewed in Novak *et al.,* 2005). Phototherapy or ultra violet (UV) treatment can also be used but is usually reserved for severe forms of AD as it is expensive and can have side effects (Novak and Simon, 2011).

Since filaggrin is a key component of epidermal barrier function, and its expression is commonly found down-regulated in AD skin (reviewed in Irvine & McLean, 2006), filaggrin replacement therapies offer wide potential in treatment of barrier compromised disorders such as AD. Recently, Stout *et al* demonstrated that by introducing a monomeric filaggrin subunit linked to a cell-penetrating peptide into *FLG*- deficient flaky tail mouse, the phenotype can be rescued (Stout *et al.,* 2014). Although the results are promising, the introduced filaggrin peptide was detected in all layers of the epidermis including the basal layer, therefore more work is required to evaluate the consequence of filaggrin expression in these areas (Stout *et al.,* 2014). The chronic relapsing nature of AD can make the disease costly, not just financially but also in terms of the psychological and emotional impact as it can greatly affect relationships,

social activities and quality of life in general (Carroll *et al.,* 2005). Hence there is a need for better therapeutic approaches to treat this disease.

1.3 Filaggrin

1.3.1 Profilaggrin structure and function

Filaggrin is expressed as the precursor protein profilaggrin, in the terminally differentiating keratinocytes within the granular layer and is the main constituent of the keratohyalin granules (Presland *et al.,* 1992). The human profilaggrin is encoded by the *FLG* gene, which belongs to the S100 'fused' type family of gene cluster located within the epidermal differentiation complex (EDC) at 1q21 that also includes other keratinocyte differentiation related genes such as trichohyalin, hornerin and repetin. Also located in the EDC is filaggrin-2 that has strong homology to both hornerin and filaggrin (Fig 1.7A) (Presland et el., 1992; Markova *et al.,* 1993). The *FLG* gene consists of three exons and two introns, where exon 1 is non coding and protein translation is initiated with exon 2, with exon 3 encoding almost the whole profilaggrin protein (Fig 1.7B) (Presland et al., 1992). Profilaggrin is a ~400 kDa histidine-rich highly phosphorylated insoluble precursor which consists of 10 to 12 filaggrin repeats separated by small linker peptides flanked on each side with partial filaggrin repeats, a S100-type calcium binding domain, a unique C-terminal domain and an N-terminal domain containing the highly conserved A domain and slightly less conserved B domain (Presland *et al.,* 1992; Gan *et al.,* 1990). The B domain contains a bipartite nuclear localization sequence that is conserved between human, mouse and rat. In humans the filaggrin subunits are identical in size (324 aa) with some sequence similarity, whereas in mice and rats the repeats show greater than 90% amino acid sequence identity (Resing *et al.,* 1993).

1.3.2 Profilaggrin-filaggrin processing pathway

Filaggrin is a highly cationic protein whose particular charge distribution is vital for its primary function, which is to aggregate and collapse the keratin filament network of the cell, leading to nuclear degradation and formation of the cornified layer (Presland *et al.,* 1992). Profilaggrin mRNA is initially detected in the granular layer, and once transcribed, the profilaggrin gets highly phosphorylated, a process that is thought to

inhibit keratin association prematurely, as only dephosphorylated filaggrin monomers can aggregate keratins (Lonsdale-Eccles *et al.,* 1982). It is predicted that conformational changes induced by phosphorylation might block access to proteolytic activity (Resing *et al.,* 1993). Hence both transcription and processing of filaggrin molecules is presumably a tightly controlled process to prevent improper keratinocyte differentiation. The importance of correct filaggrin expression and processing is demonstrated by pathological conditions such as Ichthyosis Vulgaris (Fleckman and Brumbaugh, 2002) and Netherton Syndrome (Hewet *et al.,* 2005) where lack of functional filaggrin or excessive filaggrin processing respectively leads to skin barrier defects.

Profilaggrin processing occurs in the upper granular layer in keratinocytes, where it undergoes numerous dephosphorylation and proteolytic events with several proteases implicated in the process (Fig 1.8) (Pearton *et al.,* 2002). Dephosphorylation is thought to require more than one phosphatase with studies showing involvement of acid phosphatases and a PP2A-type enzyme (Kam *et al.,* 1993). Since filaggrin intermediates lack detectable phosphorylated residues it is thought most dephosphorylation occurs prior to processing of profilaggrin (Harding and Scott, 1983). Proprotein convertase (PC) cleavage sites are located at the N termini of profilaggrin, where PC family members' furin and PACE 4 (a subtilisin-like endoprotease) have been shown *in vitro* to process and release the N-terminus of profilaggrin (Pearton *et al.,* 2001). The cleaved N-terminus containing the sub domains A and B, relocates to the nucleus facilitated by a bipartite nuclear localization sequence where it is thought to play a role in nuclear degradation as the N terminal domain was detected in the nuclei of transitioning cells where DNA has started to fragment, a feature characteristic of cells undergoing apoptosis (Pearton *et al.,* 2002; Ischida-Yamamoto *et al.,* 1998). The precise function of the C terminal is unclear but it is somehow involved in profilaggrin processing as individuals carrying a mutations resulting in truncated filaggrin repeats without the C terminal domain are unable to process profilaggrin to filaggrin monomers (Presland *et al.,* 2000; Sandilands *et al.,* 2007). Calpain 1 and the serine protease termed profilaggrin endopeptidase 1 (PEP1) have been implicated in processing of small peptide linker regions between the filaggrin monomer units to subsequently release the functional filaggrin monomer subunit (Yamazaki *et al.,* 1997; Resting *et al.,* 1995). In mice, the filaggrin monomers are joined by two different types of linker peptides differing by either the addition or omission of a FYPV motif. The linker containing the FYPV motif is processed first leading to a transient accumulation of filaggrin

intermediate species. A second processing step yields the single 37 kDa filaggrin monomer units (Resting *et al.,* 1989). In humans the linker peptide sequences are identical (Gan *et al.,* 1990).

Several other proteases have been identified via mouse knockout experiments as being involved in profilaggrin processing. *In vivo* studies have demonstrated mice deficient for Matriptase/MT-SP1, a type II transmembrane serine protease, have impaired profilaggrin to filaggrin processing and barrier dysfunction (List *et al.,* 2003). Knockout mice of the serine protease prostasin CAP1/Prss8 also display defects in filaggrin processing and barrier defects (Leyvraz *et al.,* 2005). The *Spink5*-deficient homozygous mouse displays contrasting effects with accelerated profilaggrin processing, hence implying involvement of the inhibitor LEKTI in regulating the proteases that process profilaggrin (Hewet *et al.,* 2005). Altered profilaggrin processing is also observed in 12R-lipoxygenase (12R-Lox) deficient mice. 12R-lox, transcribed by the gene *Alox12B*, is an epidermal deoxygenase of fatty acid substrates where deficiency in the enzyme alters profilaggrin processing leading to accumulation of filaggrin intermediates and total absence of filaggrin monomers (Epp *et al.,* 2007). Although the exact mechanism is not clear, it is possible 12R lox deficiency may interfere with membrane bound or sequestering of proteases involved in end stages of profilaggrin processing. During final stages of terminal differentiation the filaggrin monomers are further processed into amino acids and their derivatives. Similar to other epidermal proteins such as keratin 1 and trichohyalin, filaggrin undergoes deamination where arginine is converted to citrulline residues. Deamination neutralizes the charge on filaggrin which is found to correlate with disruption of filaggrin-keratin complex (Tarcsa *et al.,* 1996; Ishida-Yamamoto *et al.,* 2002). Filaggrin is further broken to smaller sized fragments (approx. 12-15 kDa) by the enzymes caspase 14, calpain 1, and further degraded by bleomycin hydrolase to generate free amino acids and derivatives such as pyrrolidone carboxylic acid (PCA) (Denecker *et al.,* 2007; Kamata *et al.,* 2009). PCA is a major contributor to production of natural moisturing factors (NMFs), which are essential for keeping the stratum corneum hydrated. Another derivative of filaggrin break down products is *trans*- urocanic acid (UCA), which is converted to *cis*-UCA by UVB radiation and mediate UVB- induced immunosuppression (Fig 1.8) (Walterscheid *et al.,* 2006). In fact Caspase-14 knockout mice display reduced skin hydration, increased transepidermal water loss and sensitivity to UVB radiation (Denecker *et al.,* 2007). Hence this indicates that filaggrin is an integral part of skin barrier function by

maintaining stratum corneum integrity and hydration. In addition to hydration, NMFs also maintain optimum acidic pH and buffering capacity in the stratum corneum, and decrease bacterial colonization in the skin (Levin *et al*., 2013; Miajlovic *et al*., 2010).

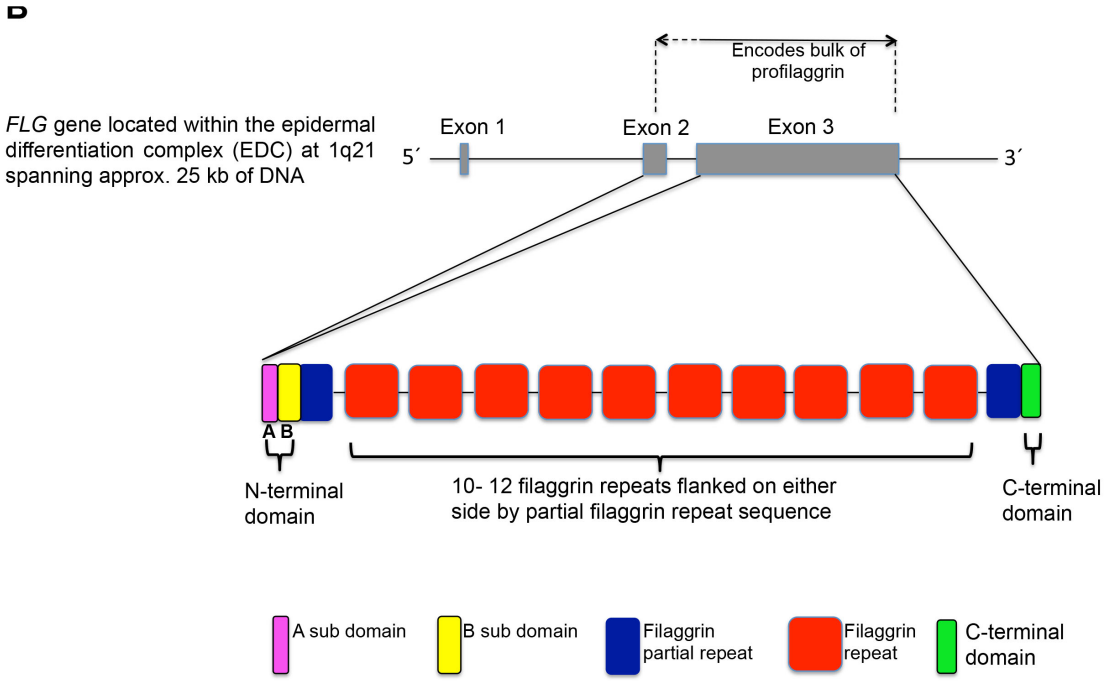

Figure 1.7 Profilaggrin gene structure.

(A) Schematic of the expanded EDC complex at 1q21 shows location of filaggrin at this loci (adapted from Strong et al., 2010). (B) The Profilaggrin protein is mostly encoded by exon 3 and has 10-12 filaggrin repeats flanked on each side by partial filaggrin repeat sequence, a C terminal domain and an N terminal domain comprised of two sub domains A and B.

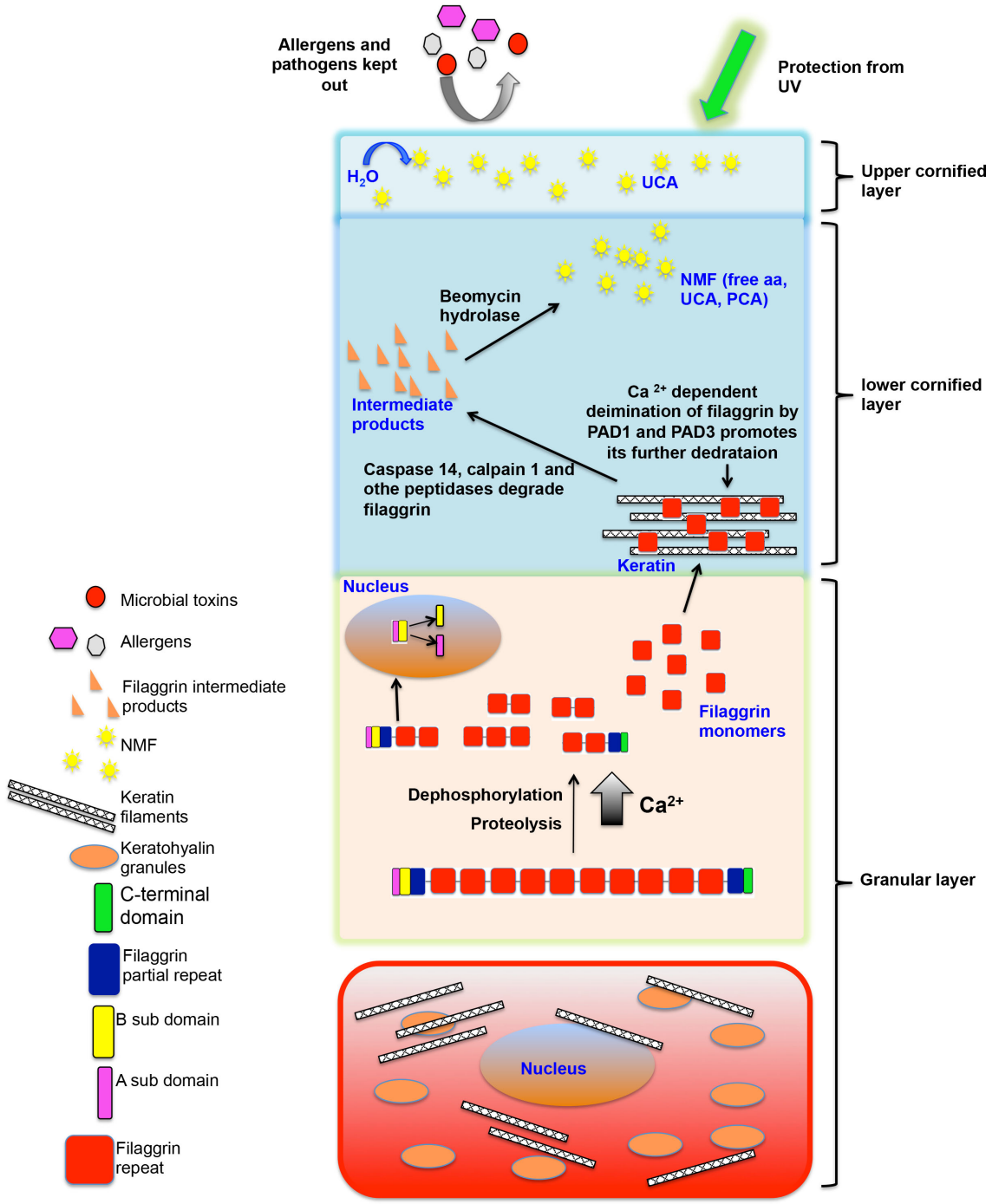

Figure 1.8 Profilaggrin to filaggrin processing pathway.

Profilaggrin is located in the keratohyalin granules in the granular layer, where increase in Ca^{2+} concentration promotes dephosphorylation and proteolysis by several proteases to release the monomeric filaggrin units. N terminal domains A and B relocate to the nucleus. Filaggrin monomers bind to and collapse the keratin filaments during the cornified envelope formation. Caspase 14, calpain and bleomycin hydrolase degrade filaggrin into free amino acids and the amino acid derivatives UCA and PCA leading to hydration of the cornified layer and protection from UV radiation.

1.3.3 Filaggrin and AD

Filaggrin deficiency has been attributed to both nonsense and frameshift *FLG* mutations resulting in mostly truncated profilaggrin or non-functional filaggrin monomers (Sandilands *et al.,* 2009). More than 20 different mutations in different AD populations have been identified, however with a combined allele frequency of 18 % to 48 % for mutations R501X and 2282del14, these two null mutations represent the strongest risk factors for AD (Fig 1.9) (Sandilands *et al.,* 2009). It is estimated that in AD patients with moderate to severe forms of the disease, 50% can be attributed to filaggrin changes (McAleer *et al.,* 2013). Additionally AD patients with filaggrin involvement have higher incidence of skin infections (McAleer *et al.,* 2013). It is clear AD can occur independently of *FLG* mutation as not all individuals with filaggrin null mutations develop AD, and there are AD patients with reduced filaggrin in absence of *FLG* mutations (O'Regan *et al.,* 2008; Thawer-Esmail *et al.,* 2014). In fact in a recent study conducted in African AD population, a significant reduction of 20 % in filaggrin degradation products PCA and UCA were observed with *FLG* genotype not making a significant contribution, hence implying a defect in the filaggrin processing (Thawer-Esmail *et al.,* 2014). Since PCA and UCA are reduced by 50 % in those carrying filaggrin mutations, it is likely a profilaggrin processing defect gives rise to a milder phenotype (Kezic *et al.,* 2011). It is therefore possible that abnormalities in pathways involved in the processing of profilaggrin can give rise to reduced filaggrin and subsequent barrier defects.

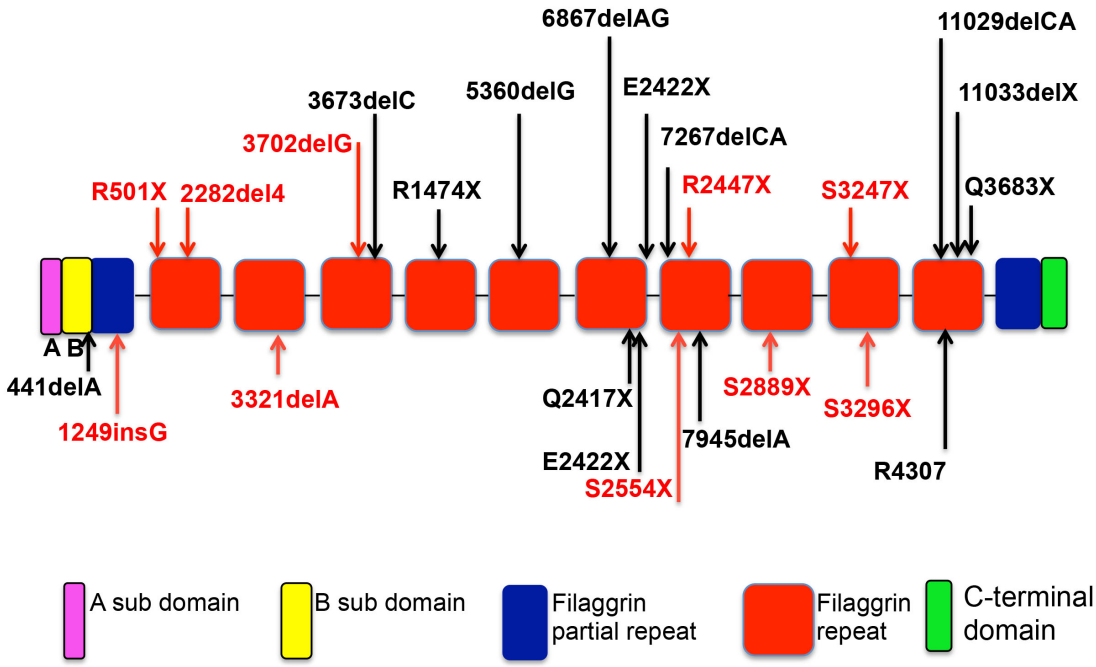

Figure 1.9 Structure of the profilaggrin showing both rare (black) and common (red) loss of function filaggrin mutations identified.

Mutations listed here include those identified in European, European American, Chinese, Singaporean Chinese and Japanese populations. Adapted from Sandilands *et al*., 2009 and Brown *et al*., 2009.

1.4 PI3 Kinase/Akt signaling pathways

1.4.1 Akt/PKB structure and activation mechanism

Akt, also known as protein kinase B (PKB), is a serine threonine kinase that is involved in a diverse array of cellular functions such as cell cycle regulation, cellular survival, glucose metabolism, cell differentiation, cell growth and proliferation (reviewed in Manning and Cantley, 2007). Originally discovered as the human homologue of the viral oncogene v-Akt (Staal, 1987; Bellacosa *et al.,* 1991), Akt has now emerged as a central signaling node in higher eukaryotes and a crucial protein kinase in human physiology and diseases including cancer, metabolic disorders and cardiovascular diseases (Bellacosa *et al.,* 2005). Akt has a highly conserved general structure across a wide spectrum of species and has three isoforms (Akt1, Akt2 and Akt3) encoded by three separate genes, which once activated can phosphorylate numerous target proteins involved in regulation of processes such as cell growth, differentiation, proliferation, survival (Bellacosa *et al.,* 1998). Akt1 is the most ubiquitously expressed isoform with Akt2 expressed at a lower level in comparison, while Akt3 is the least expressed in adult tissues with expression mostly confined to testes and brain (Hanada *et al.,* 2004). Akt structure is composed of an N-terminal Pleckstrin Homology domain (PH) that mediates membrane recruitment, a catalytic domain and a hydrophobic domain that contains a T activation loop with a conserved threonine residue (T308) and serine residue (S473) (Hanada *et al.,* 2004; Manning & Cantley, 2007).

Akt is a key downstream effector of phosphoinositide-3-kinases (PI3K). Studies have shown that membrane recruitment via the PH domain and phosphorylation (Kohn *et al.,* 1996) at the residues T308 and S473 is required for full Akt activity (Bellacosa *et al.,* 2005). Akt is activated by several extracellular signals such as growth factors, cytokines and hormones binding to receptor tyrosine kinases (RTK) in the membrane that activate PI3 kinase. Once activated PI3 kinase phosphorylates phosphatidylinositol-4,5-biphosphates (PIP2) to generate phosphatidylinositol-3,4,5-triphosphates (PIP3) which then binds to Akt via the PH domain and recruiting it to the cell membrane (Franke *et al.,* 1997). PIP3 also recruits 3-phosphoinositide-dependent protein kinase 1 (PDK1) to the membrane, which phosphorylates Akt at the site threonine 308 (T308) leading to partial activation of Akt. Full activation of Akt is only achieved when the serine 473 residue at the hydrophobic motif is phosphorylated by rictor-mTORC2

(mammalian target of rapamycin complex 2) (Sarbassov *et al.,* 2005) leading to additional substrate specific phosphorylation events (Fig 1.10A) (Guertin *et al.,* 2006). Following activation Akt may translocate to either the cytoplasm or nucleus where it exerts numerous substrate specific phosphorylation events such as inhibitory phosphorylation of pro-apoptotic FOXO proteins and activation mTORC1 pathway regulating protein synthesis and cell growth (Fig 1.10B) (Manning & Cantley, 2007).

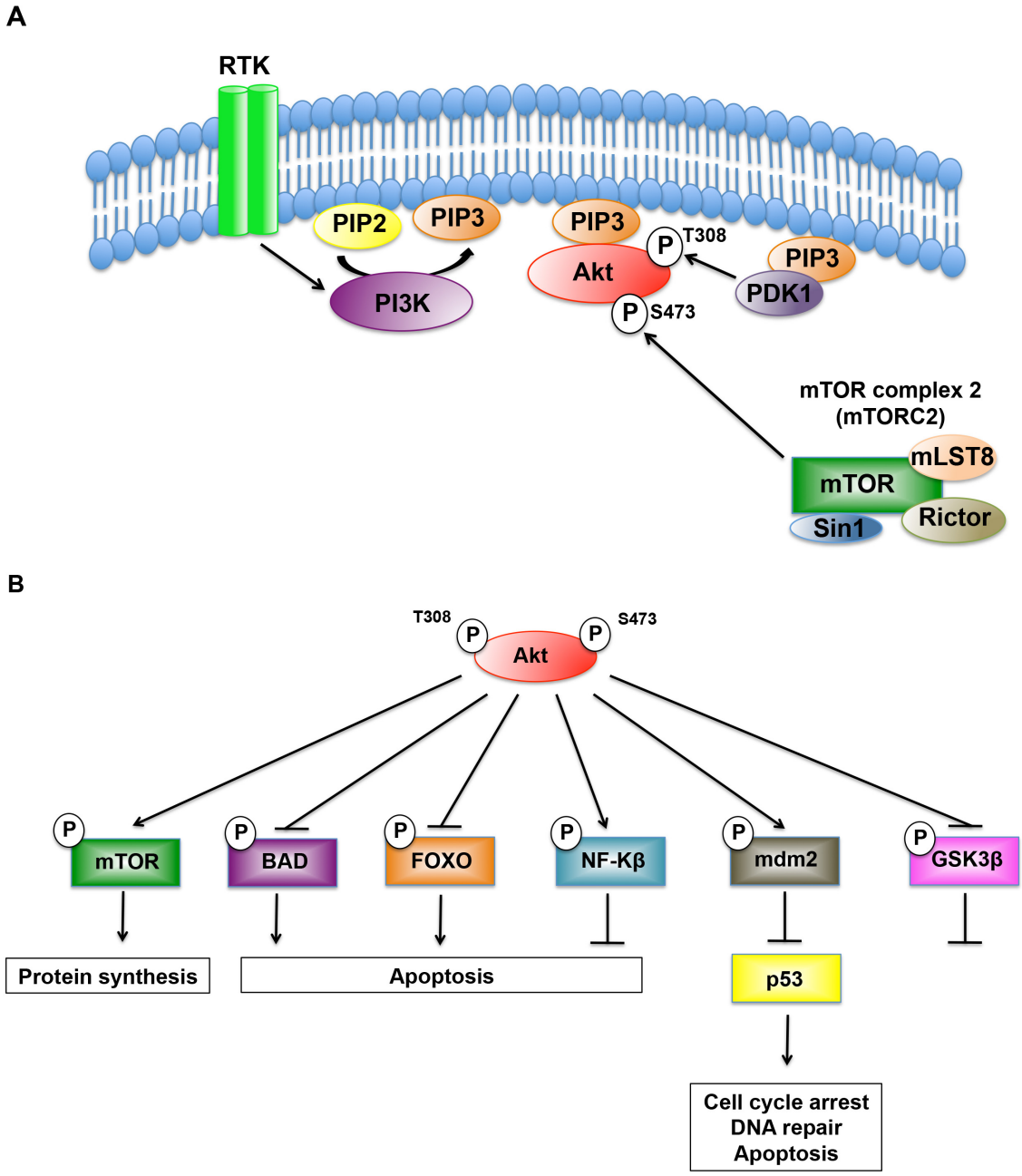

Figure 1.10 PI3 Kinase/Akt pathway.

(A) RTK receptor activates PI3 Kinase leading to generation of PIP3 which binds Akt and facilitates phosphorylation of Akt at T308 and S473 by PDK1 and mTORC2 respectively. (B) Activated Akt phosphorylates various downstream substrates resulting in increase of cell growth and cell survival pathways and decrease of apoptotic pathways.

1.4.2 Akt in cell differentiation

While Akt is most well-known for promoting cell survival and growth, it has also been implicated in controlling cell differentiation with roles in aspects of differentiation in different types of cells such as myocytes, osteocytes, neurons and adipocytes (Gardner *et al.,* 2012; Mukherkee *et al.,* 2010). Even though the different isoforms of Akt share their substrate repertoire, studies have shown that they sometimes display unique functions, which may be attributed partly to different cellular localization determining selective substrate accessibility to different isoforms (Grabinski *et al*. 2011). Mice deficient for Akt1 and Akt2 display a muscle deficiency phenotype (Peng *et al.,* 2003). Akt1 is required for initial stages of muscle differentiation and Akt2 is not needed for differentiation but rather the maturation of myofibres (Gardner *et al.,* 2012). Akt1 is essential for adipocyte differentiation (Xu *et al.,* 2004) while Akt2 is required for initiation of osteoblast differentiation (Mukherjee *et al.,* 2010). Sometimes Akt isoforms can compensate each other, like in the case of either Akt1 or Akt2 restoring myofiber development in myoblasts in the absence of Akt2 (Gardner *et al.,* 2012). However this is not always the case, as Akt1 cannot make up for lack of Akt2 in osteoblast differentiation (Mukherjee *et al.,* 2010).

1.4.3 PI3 Kinase/Akt pathway in skin development

Akt signaling is considered important for proper skin development in human with reports associating Akt with keratinocyte survival and differentiation (Calutti *et al.,* 2005; Thrash *et al.,* 2006; O'Shaughnessy *et al.,* 2007). Increased expression of Akt in keratinocytes promotes growth arrest and differentiation while inhibition of Akt activity results in selected death of terminally differentiating keratinocytes suggesting that Akt protects the cells from possible apoptotic like events induced during terminal differentiation (Calautti *et al.,* 2005). Both Akt1 and Akt2 activity has been detected in human keratinocytes, with Akt3 activity reported at significantly low levels (Calautti *et al.,* 2005; O'Shaughnessy *et al.,* 2007). Akt1 and Akt2 deficient mice display impaired skin development with reduced filaggrin expression while those doubly null for both isoforms lack stratum corneum and die shortly after birth (O'Shaughnessy *et al.,* 2007; Peng *et al.,* 2003). However Akt1 is the predominant Akt activity detected in the upper epidermis during the barrier acquisition stage of keratinocyte differentiation and targeted knock down of Akt1 leads to impaired epidermal barrier (O'Shaughnessy *et al.,* 2007; Thrash *et al.,* 2006). Furthermore sonication experiments on isolated cornified

envelopes demonstrated that Akt1 null mouse cornified envelopes displayed significantly more fragility than the wild type (O'Shaughnessy *et al.,* 2007). Taken together these findings suggest that Akt1 plays a key role in keratinocyte differentiation and barrier formation, possibly by mediating profilaggrin-to-filaggrin processing.

1.4.4 The role of RAPTOR-mTORC1 in modulating Akt activity

Akt activity can be regulated by dephosphorylation at T308 and S473 by phosphatases such as Pp2A (protein phosphatase 2A) (Andjekovic *et al.,* 1996). The tumour suppressor PTEN (phosphatase and tensin homolog) can also inhibit Akt activity by converting PIP3 to PIP2 (Stambolic *et al.,* 1998). Epidermal specific PTEN knockout mice have hyperactive epidermal Akt, display hyperplasia and hyperkeratosis (Suzuki *et al.,* 2003). This indicates that careful regulation of Akt activity is important for normal skin development as reduced or hyper active Akt can lead to impaired epidermal differentiation, structure and barrier function. Perhaps to ensure proper regulation, several negative feedback loops are thought to exist, with one negative regulation loop existing between mTORC1 and Akt. S6K1/2, a component of mTORC1 can directly phosphorylate receptor accessory protein insulin receptor substrate 1 (IRS-1) at several inhibitory residues, resulting in the degradation of the adapter molecule, which prevents the docking of PI3K and downregulate the PI3K/Akt signaling pathway (Harrington et al., 2004; Shah *et al*., 2004; Sully *et al*., 2013). Research has shown knock down of Raptor (regulatory associated protein of mTOR) increases Akt activity (Sarbassov *et al.,* 2005). Raptor forms complexes with the regulatory kinase mTORC1. A recent publication by Sully *et al*. (2013) has demonstrated that treatment of keratinocytes with rapamycin, a drug that specifically inhibits the Raptor-mTORC1, increases Akt activity, by inhibiting the negative feedback loop that exists between mTORC1 and the IRS-1 (Sully *et al.,* 2013). This indicates that this negative feedback loop is present in keratinocytes and can hence modulate Akt activity in the skin (Fig 1.11). Given the importance of Akt for epidermal barrier formation, identifying its downstream effectors involved in this process will further our understanding of keratinocyte differentiation process and aetiology of skin diseases such as AD.

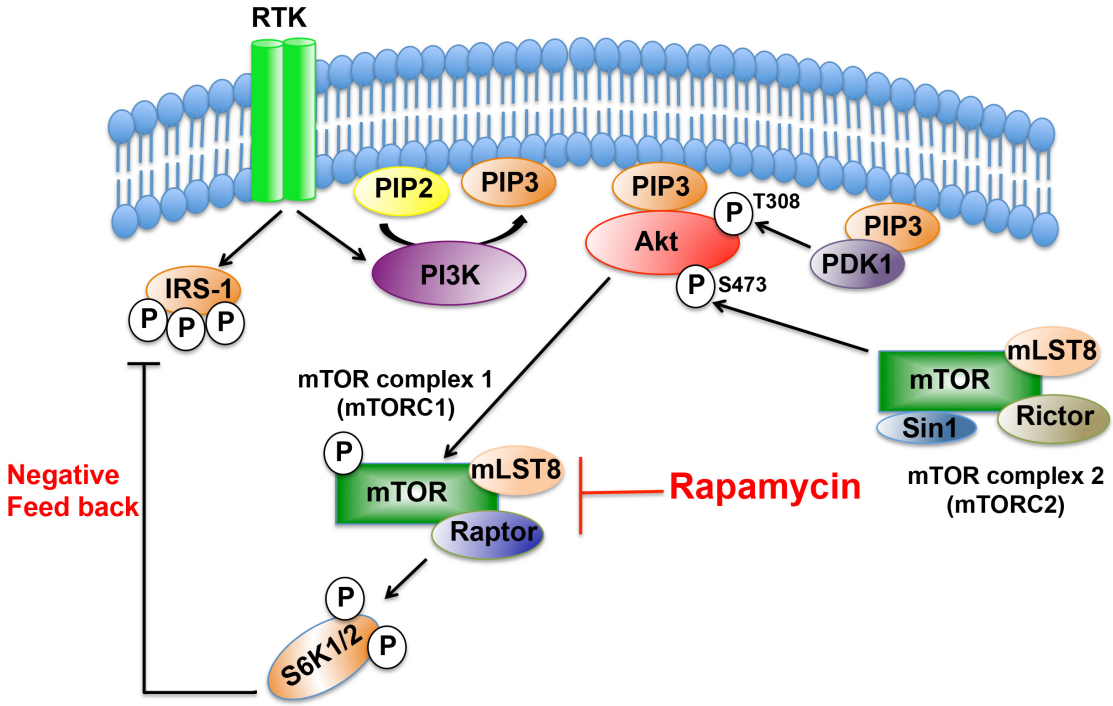

Figure 1.11 Modulation of Akt activity by mTORC1.

mTORC1 (mTOR) mediates phosphorylation of specific residues on IRS-1 which activates a negative feedback loop that inhibits PI3 Kinase activation and Akt activation.

1.5 Heat shock protein 27 and actin

Heat shock protein 27 (Hsp27) also known as Heat shock protein beta-1 (HspB1), belongs to the large family of Heat Shock Protein (Hsps). Common functions of Hsps include chaperone activity, inhibition of apoptosis, thermotolerance and stabilization of the cytoskeleton (Merck *et al.,* 1993; Garrido *et al.,* 2001). Like all members of the Hsps, HspB1 can form large oligomers and its function is often modulated by its phosphorylation status. Phosphorylated HspB1 has been reported to have a regulatory role in actin filament dynamics. Actin is one of the most abundant eukaryotic proteins and is critical for various cellular functions such as cell motility, maintenance of cell shape and regulation of transcription. Actin can exist in its monomeric form (G actin) or polymerize to form filamentous actin (F acin) (Reviewed in Dominguez and Holmes, 2011). A well characterised function of monomeric HspB1 is its ability to bind to actin microfilaments and stabilize F-actin polymerization (Benndorf *et al.,* 1994; Guay *et al.,* 1997). Actin dynamics has an important role in keratinocyte stratification with studies showing disruption of the actin cytoskeleton leading to abnormal keratinocyte differentiation (Koegel *et al.,* 2009; Luxenburg *et al.,* 2011). HspB1 is a substrate of Akt (Konishi *et al.,* 1997) and studies have shown Hsp25 phosphorylation (the murine homologue of HspB1) has a similar expression pattern to Akt1 phosphorylation with expression being more prominent in the upper epidermis during keratinocyte differentiation (O'Shaughnessy *et al.,* 2007). In addition HspB1 has been found to co-localize with cornified envelope proteins. (O'Shaughnessy *et al.,* 2007 & Jonak *et al.,* 2002). Interestingly, recent findings have established that both phosphorylation of HspB1 in the upper epidermis and its transient co-localisation with filaggrin in the keratohyalin granules in the cytoplasm occurs in an Akt1-dependent manner (O'Shaughnessy *et al.,* 2007). Furthermore targeted knock down of HspB1 in keratinocytes leads to reduced filaggrin expression and hyperkeratosis (O'Shaughnessy *et al.,* 2007).

1.6 Lamin A/C

Lamins are type V intermediate filaments that are part of the nuclear lamina, which is a matrix of proteins lining the nucleoplasmic surface of the inner nuclear membrane (Prokocimer *et al.,* 2009). Lamins are classified into two groups, A-type lamins and B-type lamins, both components of the nuclear lamina. B type lamins are expressed in most cells while A type lamins tend to be more differentially expressed

with suggestions of involvement in more specialized functions such as differentiation (Prokocimer *et al.,* 2009). In humans homozygous loss of A-type lamins result in lethality at birth (Muchir *et al.,* 2003). A-type lamins comprise of Lamin A and C, two alternately spliced products of a single transcript transcribed by the *LMNA* gene (Stewart and Burke, 1987). Lamin C is produced as a mature protein while other transcript, Prelamin A, is further processed with modifications leading to isoprenylation, carboxymethylation and endoproteolysis at the C-terminal CaaX motif before forming mature lamin A (Sinensky *et al.,* 1994). Alterations in prelamin A processing are at the centre of lamin A/C related disease called laminopathies (Maraldi *et al.,* 2011). Apart from providing structural scaffolding for the nucleus, lamin proteins can interact with various proteins including chromatin associated proteins, transcription factors and with inner nuclear membrane proteins (INM) such as SUN and nesprin that form the LINC (linker of Nucleoskeleton and cytoskeleton) complex at the nuclear envelope (Dechat *et al.,* 2008). This involves lamins in the regulation of gene expression, DNA replication and also mediate interactions between lamin and actin network.

Lamin A/C are reported to interact with specific transcription factors such as c-fos and Oct-1 suggesting that mutations in lamin A/C can lead to distinct tissue specific diseases as these lamins have tissue specific roles during differentiation (Gonzalez *et al.,* 2008). This is the case in laminopathies such as muscular dystrophy and Emery-Dreifuss muscular dystrophy (EDMD), which affects striated muscles, peripheral nerves and adipose tissue (Maraldi *et al.,* 2011). However in the case of premature ageing syndromes such as Hutchinson-Guilford progeria Syndrome (HGPS) a more widespread ageing of most tissues are observed with skin changes considered an early clue to a diagnosis of HGPS with patients displaying scleroderma (Mounkes *et al.,* 2003). HGPS in vivo mouse model displayed skin abnormalities with hyperkeratosis and parakeratosis (Sagelius *et al.,* 2008). EDMD patients also display nuclear abnormalities (Maraldi *et al.,* 2011). Research has shown that during apoptosis, lamin A/C is degraded, a process initiated by phosphorylation (Peter *et al.,* 1990). It has also been reported that uncleavable mutant lamins cause delays in chromatin condensation and nuclear degradation during apoptosis (Rao *et al.,* 1996). Interestingly, studies have shown lamin A is a downstream nuclear target of Akt and is phosphorylated by Akt in the evolutionary conserved Akt motif at the amino acid residue Ser404 (Cenni *et al.,* 2008). In view of the fact that nuclear retention in the cornified layer is a phenotype

observed in AD, it will be interesting to elucidate whether Akt may mediate nuclear degradation via lamin A during keratinocyte differentiation.

1.7 Cathepsin H

Cathepsin H (Ctsh) belongs to a group of papain-like lysosomal cysteine proteases consisting of 11 members mainly located in the endolysosomal compartments and within the exocytosis pathways of some secretory cells (Tedelind *et al.,* 2010; Reiser *et al.,* 2010). Similar to other proteases, Ctsh is synthesized as a procathepsin which consists of disulphide-linked heavy and a light chains (Baudys *et al.,* 1991). Ctsh also has a glycosylated octapeptide called mini-chain covalently linked to the main protein, the presence of which makes Ctsh predominantly an exopeptidase with limited endopeptidase activities (Guncar *et al.,* 1998). Procathepsin undergoes glycosylation and proteolysis to form the single chain 28 kDa mature Ctsh. Ctsh also can exist as its processed forms Ctsh heavy chain (22 kDa) and Ctsh light chain (6 kDa) (Popovic *et al.,* 1988). Ctsh is ubiquitously expressed in all tissues with a well characterized role in lung surfactant SP-B processing in type II pneumocytes (Brasch *et al.,* 2002; Buhling *et al.,* 2011). Ctsh activity is increased in breast, colorectal and prostate carcinoma while it is decreased in squamous cell carcinoma of the head and neck (Reiser *et al.,* 2010).

1.8 Aims and objectives

1.8.1 Rationale for the study

A skin barrier defect is the major driver of pathogenicity in AD. A defective barrier allows entry of allergens and pathogens resulting in inflammatory responses (Callard & Harper, 2007; Leung *et al.,* 2014). Skin barrier defects in AD have been shown to result from reduced expression of the skin barrier protein filaggrin, which can result from mutations in the *FLG* gene (Presland *et al.,* 2000; Palmer *et al.,* 2006). However reduced filaggrin independent of *FLG* mutations, has also been observed in AD, hence implying alternative mechanisms leading to defects in profilaggrin processing (Thawer-Esmail *et al.,* 2014). The processing of profilaggrin to filaggrin is poorly understood with only a few proteases identified (List *et al*., 2003). As filaggrin is involved in several aspects of barrier function, further knowledge about the profilaggrin-processing pathway will broaden the knowledge of later stages of keratinocyte differentiation leading to barrier formation. The Akt signaling pathway is

involved in skin barrier formation (Thrash *et al.,* 2006; O'Shaughnessy *et al.,* 2007), however the epidermal downstream targets of the Akt signaling pathway are not well characterized. In particular, Akt activity is important for filaggrin processing as reduced Akt activity leads to reduced filaggrin protein levels and hyperkeratosis (O'Shaughnessy *et al.,* 2007). A substrate of Akt, HspB1, was found to be involved in filaggrin processing in an Akt mediated manner however the exact mechanism was not determined (Fig 1.12) (O'Shaughnessy *et al.,* 2007 & Jonak *et al.,* 2002). Even though Akt activity in AD skin has not been evaluated, it is possible that Akt activity in the skin is reduced in AD as experimental evidence shows a reduction in granular Akt phosphorylation can give rise to mild barrier defects similar to AD (Thrash *et al.,* 2005; O'Shaughnessy *et al.,* 2007).

This study will therefore focus on evaluating Akt activity in uninvolved AD skin and identifying downstream targets of Akt signaling pathway that are involved in filaggrin processing and other aspects of keratinocyte differentiation leading to barrier formation. As lesional AD skin has elevated immune responses, which can further exacerbate barrier defects, uninvolved skin sections are chosen for this study so any abnormalities observed should be due to skin barrier defects only. The study will also evaluate RAPTOR as a modulator of Akt activity in skin. A better understanding of the molecular components involved in AD-associated epidermal barrier and differentiation defects can hopefully lead to novel targeted therapies.

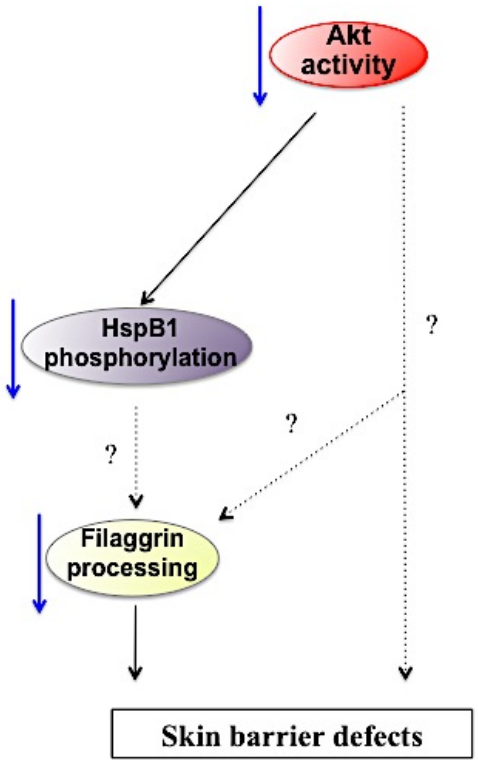

Figure 1.12 Model describing established and possible downstream effects of Akt signaling pathway leading to barrier defects.

Dotted arrows indicate relationships with mechanisms unknown.

1.8.2 Hypothesis

In AD, reduction in epidermal granular Akt phosphorylation leads to misprocessing of profilaggrin and barrier defects, potentially due to up-regulation of epidermal RAPTOR expression

1.8.3 Objectives:

1. Generate an Akt1 shRNA knockdown rat epidermal keratinocyte culture model in both submerged monolayer culture and organotypic culture to examine the effects on profilaggrin processing and other aspects of barrier formation.

2. Profile differentially expressed genes in this Akt1 knocked down (kd) culture model.

3. Obtain mechanistic insights of how Akt-mediated HspB1 phosphorylation is involved in filaggrin processing.

4. Extend the analysis of relevant differentially expressed proteins, RAPTOR and pSerAkt in the granular layer of unaffected skin from AD patients.

5. Clarify the role of RAPTOR in modulating Akt activity and profilaggrin processing.

6. Stimulate and inhibit function of components differentially expressed in our Akt1 knockdown 'model' to determine their function in epidermal differentiation and barrier disruption.

Chapter 2 : Materials and methods

2.1 Reagents and Solutions

Table 2-1 Reagents list

Item	Supplier	Catalogue Number
0.25 % Trypsin	Sigma Aldrich Co.	T3924
1 Kb DNA ladder	NEB	N0467S
100 bp DNA ladder	NEB	N0468S
100x Antimycotic (Antibiotic Antimycotic)	Life Technologies	15240-062
100x Penicillin/Streptomycin with L-Glutamine	Life Technologies	15140-122
2-Mercaptoethanol	Sigma Aldrich Co.	M6250
2x Laemmli Sample Buffer	Bio-Rad	161-0737
Agarose	Sigma Aldrich Co.	A9539
AGN193109	Santa Cruz Biotechnologies	Sc210768
All Trans Retinoic Acid (ATRA)	Fisher Scientific	10552611
Ammonium persulphate	VWR	443073
BCA protein assay kit	Fisher	23227
Bovine Serum Albumin	Sigma Aldrich Co.	A9418
Bouin's Fixative	Polysciences, Inc.	16045-1
Chloroform solution	Sigma Aldrich Co.	25666
Cholera Enterotoxin stock	Merk Biosciences	227036
Concentrated HCl (Conc. HCl) (37%)	Science Company	NC-5844
Coomassie Blue G250 colloidal pre-mixed	Fisher Scientific	12778039
OCT embedding cryoembedding Matrix	Thermo Scientific	0370024
DEPEX	Fisher Scientific	50-980-371
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich Co.	D2650
Dulbecco's Modified Eagle's Medium (DMEM)	Life Technologies	11960-044
Eosin	Sigma Aldrich Co.	E4009
Epidermal Growth Factor (EGF)	Peprotech EC Ltd	100 15
Fish skin gelatin	Sigma Aldrich Co.	1001444578
Haematoxilin Solution	Merck	105174
Ham's F12	Invitrogen	41966-029
Hydrocortisone (HC)	Merk Biosciences	386698

Item	Supplier	Catalogue Number
Insulin stock	Sigma Aldrich Co.	15500
Lipofectamine plus Reagent	Life Technologies	15338030
Mitomycin C	Sigma Aldrich Co.	M0503
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma Aldrich Co.	T9281
Novex® Tris-Glycine SDS Running buffer (10x)	Invitrogen	LC2675
Novex® Sharp Pre-stained Protein Standard	Life Terchnologies	LC5800
Phenol Solution	Sigma Aldrich Co.	P4557
Phenylmethanesulfonyl Fluoride solution (PMSF)	Sigma Aldrich Co.	93482
Phosphate Buffer Solution (PBS)	Life Technologies	14190-094
Phosphate Buffer Solution (PBS) without Ca^{2+} and Mg^{2+} (for cell culture)	Life Technologies	14190-169
Pierce® Western Blot Transfer Buffer 10x Methanol-free	Fisher Scientific	10565604
Ponceau S Staining Solution	Sigma Aldrich Co.	09276
QIAfilter Plasmid Midi kit	Qiagen	12243
Quantitect SYBR® Green PCR Kit	Qiagen	204141
Rapamycin	Cell Signalling	9904
RNase OUT	Life Technologies	10777019
Sodium dodecyl sulphate (SDS)	Sigma Aldrich Co.	71727
SuperScript II RT	Life Technologies	18064014
SYBR®Green JumpStart™ Taq ReadyMix™	Sigma Aldrich Co.	S4438
Taq DNA Polymerase	New England Biotechnologies (NEB)	M0480L
One Shot® Top10 Competent Cells	Invitrogen	C4040-03
Tri Sodium Citrate	Sigma Aldrich Co.	S1804
Triton X-100	Sigma Aldrich Co.	T8787
Trypsin Neutralizing Factor (TNF)	Lonza	CC-5002
Tween® 20	Sigma Aldrich Co.	P5927
Wortmannin	Sigma Aldrich Co.	W1628

Table 2-2 Media and stock solutions

DMEM+

Component	Amount (ml)	Final concentration
DMEM	450	-
FBS	40	10%
100x AM	5	1x
100x AB	5	1x

FAD + 10% FBS

Component	Amount (ml)	Final concentration
DMEM	300	-
Ham's F12	100	-
FBS	45	10%

100x HCE

Component	Amount (ml)	Final concentration
Hydrocortisone (HC)	1	10%
Cholera Enterotoxin	0.1	1%
Epidermal Growth Factor	1	1%
FAD + 10% FBS	7.9	-

FAD HCE

Component	Amount (ml)	Final concentration
FAD + 10 % FBS	44.5	-
100x HCE	5	1x
Insulin	0.4	1%

Versene

Component	Amount	Final concentration
10x PBS	50 ml	1x
0.5 M EDTA stock	54 ml	2%
Distilled water	adjust to 500ml	

Total Lysis Buffer

Component	Amount (ml)	Final Concentration
2-Mercaptoethanol	1	2%
SDS	1	2%
1M Tris HCl pH 7.5	0.5	10mM
Distilled H_20	47.5	-

RIPA Lysis Buffer

Component	Amount (ml)	Final Concentration
1M Tris-Hcl pH7.4	2.5	50mM
5M NaCl	1.5	150ml
0.5M PMSF	0.1	1mM
0.5M EDTA	0.2	1mM
Triton x100	0.5	1%
Roche Inhibitor Tablet	1 per 50 ml	-
Distilled water	adjust to 50 ml	-

10mM NaCitrate pH 6.0

Component	Amount	Final Concentration
Tri Na Citrate	2.94 g	10mM
Tween 20	0.5 ml	0.05%
Conc. HCl	few drops to adjust pH to 6.0	-
Distilled water	adjust to 1L	-

10x PBS

Component	Amount	Final concentration
PBS tablets	100	10x
Distilled water	adjust to 1 L	-

1x PBS

Component	Amount	Final concentration
10x PBS	100 ml	1x
Distilled water	adjust to 1 L	-

PBST

Component	Amount	Final concentration
PBS	1 L	1X
TWEEN-20	1 ml	0.1%

TBS

Component	Amount	Final concentration
Tris	6.05 g	50 mM
NaCl	8.76 g	150 mM
HCl	few drops to adjust to pH 7.5	-
Distilled water	adjust to 1 L	-

TBST

Component	Amount	Final concentration
TBS	1 L	-
Tween-20	1ml	0.1%

Table 2-3 Primary and secondary antibodies

Primary Antibodies	Type	Company	Catalogue Number	IHC/IF dilution	WB dilution
Agarose-conjugated Hsp27	Rabbit polyclonal	Santa Cruz	Sc1048 AC	-	1:50
Akt phophorylated (pSer473)	Rabbit polyclonal	Cell Signalling	9271	1:10	1:100
Akt-1	Mouse monoclonal	Cell Signalling	2967	1:10	1:1000
Flag	Mouse monoclonal	Sigma	8146	1:10	1:100
β-Actin	Mouse monoclonal	Millipore	MAB1501 R	-	1:1000
c-myc	Mouse monoclonal	Sigma	9E10	-	1:500
Cathepsin H	Rabbit polyclonal	Santa Scruz	Sc6496R	1:50	1:200
Filaggrin	Rabbit polyclonal	Santa Cruz	Sc30230	1:50	1:500
GAPDH	Mouse monoclonal	Millipore	MAB374	-	1:5000
Hsp27	Rabbit polyclonal	abcam	ab12351	1:100	1:500
Hsp27 phospho-Ser82	Rabbit polyclonal	abcam	ab17938	1:100	1:500
Involucrin	Mouse monoclonal	Genetex	GTX7241 5	1:10	1:50
Keratin 1	Rabbit polyclonal	Covance	PRB-165P	1:100	1:1000
Keratin 5	Rabbit polyclonal	Covance	PRB-160P	-	1:1000
Keratin 10	Rabbit polyclonal	Covance	PRB-159P	1:10	1:1000
Lamin A/C	Mouse monoclonal	Santa Cruz	Sc-7293	1:200	1:500
Loricrin	Rabbit polyclonal	Covance	PRB-145P-100	1:100	1:1000
Raptor	Rabbit polyclonal	Cell Signalling	2280	1:10	1:500
Rictor	Rabbit polyclonal	Cell Signalling	2114	1:10	1:500
Total Akt	Rabbit polyclonal	Cell Signalling	9272	-	1:500

Secondary Antibodies	Company	Catalogue Number	IHC/IF dilution	WB dilution
HRP conj. Swine anti rabbit	DakoCytomation	P0447	-	1:2000
HRP conj. Goat anti mouse	DakoCytomation	P0399	-	1:2000
Alexa Flour 488 Goat anti mouse IgG	Invitrogen	A11001	1:500	-
Alexa Flour 594 Goat anti rabbit IgG	Invitrogen	A11012	1:500	-
Alexa Flour 488 Goat anti rabbit IgG	Invitrogen	A11008	1:500	-
Biotinylated Goat anti Rabbit	Vector	BA-1000	1:200	-
Biotinylated Goat anti mouse	Vector	BA-9200	1:200	-
Donkey anti-goat	Santa Cruz	Sc-2020	-	1:2500

2.2 General cell culture techniques

All tissue culture work was performed in class II safety cabinets using sterile techniques. Cell cultures including organotypic cultures were carried out in humidified incubators at 37°C and 5% CO_2. Mouse fibroblast cell cultures were carried out in humidified chambers at 37°C and 10% CO_2. Tissue culture flasks and plates were standard tissue culture treated plates purchased either from CORNING® or Nunc (Thermo Fisher Scientific).

2.2.1 General trypsinisation protocol

The culture medium was removed and the cells gently rinsed twice with sterile versene. Fresh 5-10 ml of sterile versene was added to the flask again and incubated at room temperature for 5 min. The versene was removed and replaced with 4 ml of 0.25 % Trypsin and incubated for 5 min at 37 °C in the incubator. Excess DMEM+ medium neutralized the trypsin and the suspension was then centrifuged at 1600 x *g* for 20 minutes. The cell pellet was resuspended in fresh DMEM+ medium. For continuous culture, cells were split 1 in 10 into a new T75 flask. For experiments, cell count was done and appropriate number of cells plated in culture plates or flasks.

2.2.2 Freezing and thawing of cells

For long term storage of viable cells they were prepared as follows- Cells were counted and pelleted after centrifugation for 20 min at 1600 x *g*. Supernatant was discarded and the pellet resuspended in 10% DMSO (v/v) (Sigma Aldrich) in FBS at a concentration of 1 million cells/ml. 1 ml of the suspension was aliquoted to labeled cryovials (Nunc, Thermo Fisher Scientific). To freeze the cells slowly, the vials were placed in an isopropanol container (Nalgene) and placed in -80° for 24 hours before transferring to liquid nitrogen for long-term storage. Frozen cells were recovered by thawing an aliquot quickly in a 37°C water bath before transferring to 14 ml of prewarmed DMEM+ to dilute the cell suspension. Cells were plated in a T75 flask and media was replaced 24 hours later to remove traces of DMSO.

2.2.3 Growth assays

Growth assays were performed by plating 100,000 REK cells per well (6 well plate) containing DMEM+ media with increasing concentrations of G418 (0.1 ng/ml, 1ng/ml, 10ng/ml, 30ng/ml and 100ng/ml). Cells were cultured for a week with media changes with the indicated G418 concentrations every other day.

2.2.4 Rat Epidermal Keratinocyte (REK) and Swiss mouse 3T3 fibroblast (NIH 3T3) culture

Aliquots of single cell suspensions were transferred to culture plates or flasks with modified DMEM (DMEM+) and placed in the incubator. Media was replaced with fresh DMEM+ every two or three days and cells were subcultured before they reach 90 % confluency. For transfected cell lines, the cells were selected using DMEM+ supplemented with G418 at a concentration of 10 ng/ml as this is the concentration that prevented REK cell growth in assays (section 2.2.3). Cells were collected after washing the flasks or plates 3 times with PBS, followed by trypsinisation with 0.25 % Trypsin for 5 minutes, and pelleted after centrifugation at 1,600 x g for 20 minutes at room temperature. Note: The NIH 3T3 cells (ATCC) were a gift from Dr. Wei Li Di.

2.2.5 Human keratinocyte cell culture

Human keratinocyte culture was established by plating aliquots of single cell suspension in a flask or plate coated with mitocycin C (Sigma Aldrich) treated Swiss mouse 3T3 fibroblasts. *Preparation of mitomycin treated NIH 3T3 plates***:** Mitomycin C was added to 3T3 fibroblasts suspended in a 50 ml falcon tube (Corning) with DMEM+ medium at a concentration of 40 ng/ml. This was then plated in a T25 flask and placed in an incubator for 3 hours after which the medium was aspirated, plate gently washed three times with PBS to remove traces of mitomycin c treatment. 3T3 cells were then trypsinized for 5 minutes, cells pelleted and resuspended in FAD HCE media and plated at a density of 1.0-1.5x10^5/cm^2. Aliquots of single cell suspension Normal Human epidermal keratinocytes (NHEKs) were resuspended in FAD HCE and transferred to mitomycin C treated 3T3 coated plates or flasks. For collection of cultured NHEKs, cells were trypsinised with 0.25 % Trypsin for 5 min. Trypsin was neutralized using Trypsin Neutralising Solution (TNS) (Trypsin: TNS = 1:2). Cells were spun down 300 x *g* and resuspended in FAD HCE.

2.2.6 General transfection protocol

All transfections were done using Lipofectamine™ 2000 reagent (Invitrogen) according to manufacturer's protocol. Briefly, 24 hours before transfection, 8 x10^5 cells in 2 ml of DMEM with 10% v/v FBS (without antibiotics) were plated in each well of a 6 well plate so the cells will reach 90 – 95% confluency at the time of transfection. The transfection complexes were prepared as follows: Complexes were prepared using the plasmid DNA (μg) to Lipofectamine™ 2000 (μl) of 1:2. For each transfection sample 1g of plasmid DNA was mixed with 250 μl of DMEM and mixed gently. 2 μl of lipofectamine was mixed with 250 μl of DMEM and mixed gently. These were incubated for 5 minutes at room temperature before combining the diluted plasmid DNA and lipofectamine solution that was allowed to stand at room temperature for 20 minutes. 500 μl of the complex was transferred to each well and mixed gently. Plates were then incubated at 37°C in an incubator with a media change to fresh DMEM+ after 6 hours of transfection. For stable transfection, cells were split 1 in 10 24 hours after transfection and media replaced with 0.1 mM G418 selection media. For transient transfection, lysates were collected 24 hours after trasnsfection.

2.2.7 shRNA plasmids, RAPTOR over expression

Four SureSilencing shRNA plasmids (Qiagen, Paisley, UK) were used to knockdown Akt1 expression in REKs; shRNA1-GCA CCG CTT CTT TGC CAA CAT, shRNA2-AAG GCA CAG GTC GCT ACT AT, shRNA3-GAG GCC CAA CAC CTT CAT CAT, shRNA3-GCT GTT CGA GCT CAT CCT AAT, and of these 1 and 3 were used for further experiments. Ctsh knockdown was successfully achieved by transfecting NHEKs and REKs with two shRNA plasmids (shRNA1-CAA GAA TGG TCA GTG CAA ATT; shRNA2-CTA GAG TCA GCT GTG GCT ATT) using lipofectamine 2000 according to manufacturers' protocol briefly described above (Qiagen, Paisley, UK). One scrambled control was used (GGAATCTCATTCGATGCATAC). Lysates were collected after selection in 0.1mM G418 for 2 weeks. A Myc-tagged RAPTOR expressing plasmid (OriGene, Rockville, Maryland, USA) was transiently transfected into REKs using lipofectamine 2000 at increasing plasmid concentrations 0.1 μg/ml, 0.5 μg/ml and 1 μg/ml.

2.2.8 Lamin contructs

Flag tagged wild-type and 3 mutant lamin A contructs (Cenni *et al*., 2008) were transfected into REKs and NHEKs using lipofectamine 2000 according to manufacturers' protocol briefly described in section 3.2.5. Stable transfection was achieved after selection under 0.1 mM G418 for two weeks. Lamin A mutant constructs comprise of single mutants S301A, S404A and double mutants S301A/S404A where serine resides have been mutated to alanine. **Acknowledgements:** I would like to thank Professor Sandra Marmiroli (University of Modena and Reggio Emilia) for providing the wild type and mutant lamin constructs.

2.2.9 Post Confluent cultures

Cells were defrosted, put in culture and allowed to recover for a week. Before reaching 90 % confluency, cells were then trypsinised, counted, pelleted and resuspended in DMEM+ for untransfected cells and DMEM+ with 0.1 mM G418 for transfected cells. For all post confluent cultures, 100,000 cells were plated in a 6 cm plate and media changed every other day. With day of plating designated day 0, lysates were collected on days 2,3,4,5 and 6.

2.2.10 Organotypic culture

Organotypic culture was performed using keratinocytes seeded onto de-epidermalised dermis (DEDs) (Euro Skin Bank) with a fibroblast feeder layer on the lower side of DED. To prepare the feeder layer, 200,000 NIH 3T3 cells were transferred into a culture chamber ring placed in centre of the DED. After 24-48 hours, the DEDs are turned over and 200,000 sample cells (REKs transfected with Akt1 shRNA or scrambled shRNA) are transferred into a culture chamber ring in centre of DED. After 24 hours, the REK organotypic culture was raised to liquid/air interface and grown under selection in DMEM+ with 0.1 mM G418 for 14 days. Media was changed every two days. On the 14th day, the DEDs were cut in two with one half embedded in cryo freezing medium and stored in -80°C until required. The other half was fixed in Bouin's solution for 2 hours on a shaker at room temperature, replaced with 70% ethanol for 5 min (repeated with fresh 70 % ethanol a few times until colour of fixative fades). The fixed DEDs are then embedded in paraffin or frozen in OCT and stored at -80° C as appropriate.

2.2.11 Cell treatments

Cells were treated with the inhibitor Rapamycin at 10 nM for four hours prior to lysate collection. Wortmannin (Sigma), a PI3 kinase inhibitor, was introduced into REK cells at 2 μM for 24 hours (O'Shaughnessy *et al*., 2007a) prior to lysate collection. Human keratinocytes were treated with 5 μM all-trans retinoic acid (ATRA) and 0.1 μM Retinoic acid receptor antagonist (AGN193109) for 24 hours prior to lysate collection. DMSO was used as a control at a concentration not exceeding 0.1 %.

2.3 Protein expression analysis by western blotting

2.3.1 Sample preparation

Cells were lysed using radio immunoprecipitation assay (RIPA) buffer for cytoplasmic protein extraction. After rinsing the cells with ice cold PBS, 150 μl of RIPA buffer was added to each well of a 6 well plate on ice and placed on ice for 10 minutes. Cells were then scraped off into labeled eppendorfs and sheared with a 2 ml 31 G needle approximately 8 times. The samples were centrifuged at 10,000 RPM at 4°C for 10 minutes. The supernatants were then aliquoted into labeled eppendorfs and stored

at -20°C until required. Total cellular protein was extracted from cells using total lysis buffer as follows. Cells in culture plates were rinsed with ice cold PBS approximately 4 or 5 times. 150 μl of lysis buffer was added to each well of a 6 well plate on ice and placed on a shaker for 10 minutes. The cells were then scraped off the wells using a cell scaper, transferred to labeled eppendorfs and placed on a 95°C heat block for 10 minutes. Cells were incubated on ice for 1 min and cell suspension was sheared at least 8 times using a 2 ml syringe with a 25 G needle. The samples were centrifuged for 5 minutes at 10,000 RPM, and supernatant aliquoted into labeled 1.5 ml tubes and stored at -20°C until required.

2.3.2 Protein quantification

Protein was quantified using Bicinchoninic Acid Protein Assay Kit (BCA) as follows. Protein standards were prepared using Bovine serum albumin at concentrations: 10, 200, 400, 800 and 1000 μg/ml. BCA working solution was prepared according to instructions: 100 μl of $CuSO_4$ to 5 ml of BCA solution. Master mixes of total volume 100 μl were prepared of 2 dilutions from each lysate: 1: 2 and 1: 10 dilutions. 25 μl of each dilution was added to a 96 well plate with each dilution loaded in triplicate. 25 μl of each standard in triplicate was also loaded into the plate and one well with 25 μl RIPA solution as a blank. Each well including the blank was then topped with 200 μl of the BCA working solution. Plate was covered with foil and allowed to incubate for 30 minutes. A Plate Reader (Bio-Tek Instruments, Ascent Software Version 2.6) was used to measure the absorbance at 620 nm. Absorbance value of the blank was deducted from the rest of the readings. The protein concentrations were calculated using the line equation generated from a standard curve.

2.3.3 SDS-PAGE gel electrophoresis

The glass plates (Biorad) were assembled and gel chamber between the plates filled up to two thirds of the way with 8 % resolving buffer made up according to Table 2.4. Gel was overlaid with a layer of water and gel was left to set at room temperature for 30 minutes. Once the gel has set the stacking gel was made up (Table 2.4) and poured into the gel chamber and a 1.5 mm comb (Biorad) placed between the plates. Stacking gel was allowed to set for 30 minutes at room temperature. After the gel has

set, the comb was carefully removed and plates were set up in a Mini protean 3 gel tank system (Biorad). The gel tank was filled with 1x Running Buffer with final concentration of 50 mM Tris-base, 384 mM Glycine and 0.4 % SDS. 1 x Running Buffer was obtained by diluting 10x Running Buffer stock (Invitrogen). Approximately 15 µg of each protein sample combined with 2x loading buffer was loaded with a pre-stained protein ladder (Life technologies). The gel was run at constant voltage of 130 V for 1 hour.

Table 2-4 Recipe for 8% SDS-PAGE gel and loading buffer

Resolving gel	**Company**	**Quantity**	**4x Resolving bufffer**	**Company**	**Qunatity**
Protogel	Sigma	4 ml	Trizma Base	Sigma	36 g
4x Resolving buffer	-	3.75 ml	HCl	Science Company	5.5 ml
MilliQ	-	7.25 ml	10 % w/v SDS	-	8 ml
10 % APS	-	50 µl	Milli Q	-	up to 200 ml
Temed	Sigma	10 µl			

Stacking gel	**Company**	**Quantity**	**4x Stacking buffer**	**Company**	**Qunatity**
Protogel	Sigma	0.65 ml	Trizma Base	Sigma	12.1 g
4x Stacking buffer	-	1.25 ml	HCl	Science Company	7.5 ml
MilliQ	-	3 ml	10 % w/v SDS	-	8 ml
10 % APS	-	25 µl	Milli Q	-	up to 200 ml
TEMED	Sigma	5 µl			

10 % APS	**Company**	**Quantity**	**10 % SDS**	**Company**	**Quantity**
APS	VWR	1 g	SDS	Sigma	10 g
Milli Q	-	10 ml	Milli Q	-	adjust to 100 ml

2x Loading buffer	**Company**	**Quantity**
2-Mercaptoethanol	Sigma	50 µl
2x Laemmli Buffer	Bio-Rad	950 µl

2.3.4 Transfer

The gel was carefully placed inside the gel cassette with one side in contact with a wet blotting paper and other side in contact with presoaked Hybond™- C Nitrocellulose membrane (Amersham, Biosciences). Presoaked sponge pads and more blotting paper were placed on either side of this assembly and the assembled gel cassette was placed inside the transfer apparatus in the gel tank. The tank was filled with 1x transfer buffer (diluted from 10x transfer buffer stock, Thermo Scientific

Pierce). Transfer was done at constant current of 0.1 A (approximately 30-35 V) for 1.5 hours.

2.3.5 Ponceau staining and blocking

After transfer, the nitrocellulose membrane was washed in a clean tray with Milli Q water for 2 minutes on a shaker. Water was removed from the tray and replaced with Ponceau S solution (made up by dissolving 70 ml of Ponceau S stock in Milli Q water topped up to 250 ml) to determine even loading of the gel. The tray was placed on the shaker for 5 minutes after which the Ponceau S solution was removed and membrane rinsed with Milli Q.

For blocking, membrane was placed in 5% w/v skimmed milk proteins (Marvel) made up in TBST buffer (Table 2.5) and placed on a shaker at room temperature for 30 minutes. After which the membrane was washed for 3 times 5 minutes each with TBST buffer prior to primary antibody incubation.

Table 2-5 Recipe for blocking solutions used in western blot analysis

5 % w/v milk protein blocking solution	**Company**	**Quantity**	**5 % w/v BSA blocking solution**	**Company**	**Qunatity**
Skimmed milk powder	Marvel	2.5 g	Bovine serum albumin	Sigma	1 g
TBST	-	50 ml	TBST	-	20 ml

2.3.6 Protein detection

Primary antibodies were diluted in either 5% bovine serum albumin (Sigma) or 5% skimmed milk proteins dissolved in TBST (Table 2.5) and incubated overnight at 4ºC. After primary antibody incubation membranes were washed 3 times for 5 minutes each in TBST. Secondary antibody incubation was for 1 hour at room temperature at concentrations; goat anti-mouse horseradish peroxidase 1:3000, swine anti-rabbit horseradish peroxidase 1:3000 (both DakoCytomation). Following 3 5-minute washes in TBST, the protein was visualized using an ECL™ Prime Western Blotting Detection Reagent (Amersham, GE Healthcare) according to manufacturer's instructions. Briefly, the detection agents A and B were mixed in the ratio 1:1 and the mixture spread over the entire membrane and incubated at room temperature for 4 minutes. In a dark room

the Hyperfilm ECL (Amersham, GE Healthcare) was placed on top of the membrane for the required exposure time and developed in a autoradiography developer by Xograph imaging systems.

2.4 Co-immunoprecipitation assay

Cell extracts for co-immunoprecipitation assay were obtained using ice cold RIPA buffer as described in section 3.3.1. 500 μl of RIPA lysates from each sample were combined with 20 μl of agarose-conjugated HspB1 antibody (Santa Cruz) in labeled eppendorfs to form immunocomplexes. Mixture was vortexed and incubated on ice on a shaker for four hours. After incubation, the tubes were centrifuged at 5000 RPM for 4 min and supernatant discarded retaining the viscous 'pellet'. The 'pellet' was washed 4 times with 300 μl of ice cold RIPA buffer and centrifuged at 5000 RPM for 5 min and the supernatant discarded each time. For final wash, fresh RIPA buffer was added to the pellet, centrifuged at 10,000 RPM for 5 minutes and the supernatant was discarded. The 'pellet' was dissolved in 80 μl of 2x loading buffer (table 2.4) and either loaded to a gel (20 μl per well) and proceeded with western blot analysis as described above or stored at -20° for later use. In this protocol the agarose beads are not recycled. Just prior to leading for western blot analysis, the samples which are resuspended in 2x loading buffer, are boiled for 5 min at 95°C which elutes the antibodies from the beads.

2.5 AD uninvolved skin sections and normal skin sections.

AD uninvolved skin sections were a gift from Dr. Wei Li Di. They were archived punch biopsy samples from non-affected areas from the arm of AD children (age 3 to 5 years). Samples were formalin-fixed paraffin embedded.

Normal skin sections were formalin-fixed and paraffin embedded tissue array sections obtained from Biomax (US, Biomax Inc).

2.6 Immunoflourescent Analysis

Prior to primary antibody addition, the samples were prepared as follows. Frozen sections were cut at a thickness of 7 μm using a 5030 Microtome Cryostat (Bright Instrument Co. Ltd,. Huntington, UK). These sections were fixed for 5 min at

room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.2 % Triton X-100 (Sigma) (Table 2.6). Cultured cells on coverslips were also fixed as above. Paraffin embedded sections and formalin embedded sections were cut at 4 μm using a microtome (Microm International GmbH) and incubated at 37°C overnight. These sections were then dewaxed with xylene and hydrated through alcohol gradients. Following this, formalin-fixed paraffin embedded sections were subjected to antigen recovery where slides were heated to over 65°C for 4 minutes in 10mM Na-Citrate buffer.

All sections and cells were then blocked for 30 min in 0.4% fish skin gelatin dissolved in TBS and 0.2 % Triton X-100 (Table 2.6). For sections primary antibody incubation was overnight at 4 °C and secondary antibody incubation was 45 minutes at room temperature with Alexa fluoro-conjugated antibodies (Molecular Probes). Tissue sections were mounted in Prolong Gold anti-fade reagent (Invitrogen) with 4',6-diamidino-2-phenylindole (DAPI), which was used as a counter stain. For cultured cells on coverslips, both primary and secondary antibody incubation was for 45 minutes each at room temperature. Images were taken with a Nikon Eclipse E600 microscope with x20 (NA 0.4) objectives, using CoolSnap digital camera (QImaging Inc., Canada) using QCapturePro software. Image J software suite was used to perform image analysis. Confocal images were obtained using a Zeiss LSM 710 inverted confocal microscope (Zeiss).

Table 2-6 Recipes for fixative and blocking solutions used in immunofuorescent analysis

0.4% Fish skin gelatin blocking solution	**Company**	**Quantity**
Fish skin gelatin	Sigma	80 µl
PBS	Life Technologies	20 ml
Triton X-100	Sigma	40µl

Fixing solution	**Company**	**Quantity**
4 % Paraformaldehyde in PBS	-	10 ml
Triton X-100	Sigma	20 µl

4 % Paraformaldehyde in PBS	**Company**	**Quantity**
Paraformadlehyde	Sigma	4 g
1M NaOH	-	few drops to adjust pH to 7.4
PBS	-	100 ml

2.6.1 Confocal microscopy and Nuclear Volume calculations

Confocal images were obtained using Zeiss LSM 510 (Zeiss) laser confocal microscope with a Plan Apochromat 63x NA 1.40 oil immersion lens. Using the Z-stack function of ZEN 2009 software (Zeiss), a quick scan along XY axis was done to determining the end and start point of each stack. Once these were chosen, confocal Z-stacks of lamin constructs transfected REKs immunostained for anti-flag and loricrin were acquired with Z-stacks, each consisting of 65 optical slices. Once images were acquired threshold settings and background elimination was done using Fiji Image J software suite. The files were then converted to 8-bit images in greyscale and were uploaded to Volocity®3D Image Analysis Software. Nuclear volumes were thus determined setting the parameters to find objects based on intensity and exclude objects smaller than 3000 μm^3.

2.6.2 Immunofluoresent analysis of Ctsh -/- and Ctsh +/- mouse epidermis, and cornified envelope analysis

Both wild type and Ctsh deficient mice skin were fixed in Bouin's solution and embedded in paraffin, sectioned and stained as described in section 2.5. Cornified envelopes were extracted from whole neonatal skin by boiling for 10 min in Buffer IB (2% SDS in 0.1M Tris and 2mM EDTA). Large pieces of skin were discarded and remaining solution containing the cornified envelopes were pelleted by centrifugation at 10,000 RPM for 5 minutes and washed in PBS, pelleted again and resuspended in PBS for counting under microscope. Sonication was done for 5 seconds on 50 µl of cornified envelope suspension using Vibra-Cell™ Ultrasonic Processor (Sonics®).
Acknowledgement: I would like to thank Professor Thomas Reinheckel (Institute of Molecular Medicine and Cell Research, University of Freiburg) for providing wild type, Ctsh +/- and -/- mouse skin samples.

2.7 Haematoxylin and Eosin (H&E) staining

Sample sections were cut and dewaxed as described above (section 2.6), they were stained in haematoxylin solution Gill III (Merck) for 3 minutes. Slides were then placed under running tap water for 2 min before briefly dipping acid alcohol solution (0.1 M HCl in 70 % ethanol). After the acid differentiation step the sections were placed in running tap water for 2 min and stained with eosin for 30 sec. Sections were washed again in running tap water for 2 min, dehydrated through 95 % ethanol (5min), 2 changes of 100% ethanol (5 min each) and cleared in 2 changes of xylene (5 min each), and mounted in DEPEX.

2.8 Gene expression analysis

2.8.1 RNA extractions

RNA was extracted from REKs was obtained using a standard Trizol:Chloroform extraction method followed by using RNeasy Plus mini Kit (Qiagen). Briefly, Cells were homogenized in chilled Trizol. Chloroform was added to separate the mixture into a phenol-chloroform phase, an interphase and an aqueous phase which contained the RNA. RNA was precipitated from the aqueous layer using isopropanol, pelleted and washed in 70 % ethanol, centrifuged at 7,500 x g for 4 min at 4°C, and the pellet redissolved in RNase free water and proceeded with RNeasy Plus

mini Kit as per manufacturers protocol. Briefly, homogenizing buffer RLT was added to the sample and the solution was transferred to a genomic DNA eliminator spin column placed in a collection tube and centrifuged for 30s at 8000x g. Spin column was discarded and ethanol was added to the flow through and transferred to an RNeasy spin column and centrifuged for 15 sec at 8000x g and the flow through was discarded. The spin column membrane was washed couple of times with buffer RPE by centrifuging for 15 sec at 8000x g to remove traces of ethanol. The RNA was finally eluted by adding RNase-free water directly to the spin column placed in a new collection tube and centrifuging at 8000x g for 1 min. The concentration of RNA was determined by a Nanodrop Spectrophotometer (Agilent).

2.8.2 cDNA synthesis and PCR

First strand cDNA was synthesized from RNA using random hexamers with 400 ng/μl random hexamers (Life Technologies) per 1 μg of RNA. Master mix (Qiagen) was made up according to Table 2.7 and these were added to each RNA:hexamer mix and placed in a covered heated block for 42° C for 60 min and 70° C for 10 min. Both normal PCR and Real Time PCR was performed on cDNA from RNA extracted from normal REKs, transfected and overexpressed cells using the master mix recipe in Table 2.7 and program in Table 2.8. Gene expression was normalized using the housekeeping gene *ACTB* (β-Actin). Quantitect primers were purchased from Qiagen and are listed in Table 2.9. For Real Time PCR, fold differences were calculated using the ΔΔCt method (Bustin and Mueller, 2005). Briefly, the cycle threshold (CT) was recorded for the target genes of interest and the housekeeping gene *ACTB* (β-Actin). Mean values for triplicate wells for each gene was calculated and using the following equation: ΔΔ CT = ΔCT (gene of interest) - ΔCT (*ACTB*), where $2^{(-_{\Delta\Delta} CT)}$ calculated fold change in target gene normalized to the endogenous house keeping gene.

Table 2-7 Master mix solutions for cDNA synthesis and PCR

Master mix for cDNA conversion

Reagent	Per reaction (µl)
5x buffer (Qiagen)	4
10mM dNTP (Qiagen)	2
AMV RT (Qiagen)	1
RNase out (Life Technologies)	1
RNase free H20	up to 20

Normal PCR Master mix

Reagent	Per 20 µl reaction (µl)
10 x PCR mix (Qiagen)	2
Primer (F + R) (Qiagen)	2
50 mM MgCl2 (Qiagen)	0.6
10 mM dntp (Qiagen)	1
Taq polymerase (NEB)	0.05
H20	11.35
cDNA template	3

Real Time PCR Master mix

Reagent	Per 20 µl reaction (µl)
Sybr green MM	10
Primers	2
H20	5
cDNA	3

Table 2-8 PCR programs

Program for both Normal PCR and Real Time PCR

Initial heat activation	95°C	15 min	
	94°	15 sec	x 35 cycles
	55°C	30 sec	
	72°	30 sec	
	4°	∞	

Table 2-9 QuantiTect Primer Assays

Product	Company	Catalogue number
Rat Filaggrin	Qiagen	QT00425691
Rat β-Actin	Qiagen	QT00193473
Rat Serpinb9	Qiagen	QT00413840
Rat Serpin b3	Qiagen	QT00457800
Rat F2rl2	Qiagen	QT00454818
Rat Ctsh	Qiagen	QT00182105

2.9 Microarray analysis and IPA analysis

0.1 mg RNA was extracted from 2 control (scrambled) REK lines and 2 biological replicates of each Akt1 shRNA knockdown cell line. After second strand synthesis using Superscript II kit (Invitrogen), biotinylated cRNA was made from this cDNA using BioArray HighYield RNA Transcript Labeling Kit (Enzo Diagnostics). cRNA was then purified, fragmented and hybridised to the exon array rat genome chip (Affymetrix, Santa Clara, California) according to manufacturers' specifications. Scrambled controls were used to provide baseline values in all analysis. Genes that were tagged as present and differentially expressed with ≥ 1.5 fold with a p-value ≤ 0.05 by Mann-Whitney analysis and a p-value less than 0.05 after Benjamini-Hochberg False Discover Rate correction were considered for analysis. For background correction and normalisation data was imported to GeneSpring (Agilent) for correction using the RMA 16 (Robust Multi-array Analysis) summarizing method (Institute of Child Health Microarray and High Throughput Sequencing Facility, UCL Genomics). Differentially regulated genes were further analysed using Ingenuity Pathway Analysis (IPA) Software. Data sets including the gene identifiers and expression differences were uploaded into the application; these genes were overlaid onto a global molecular network produced by information available in the Ingenuity Pathways Knowledge Base (www.ingenuity.com).

2.10 Mass spectrometry

Total protein lysate of scrambled controls and Akt1 kd cells were separated on an SDS-PAGE gel, stained with colloidal Coomassie Blue for 30 minutes on a shaker at room temperature. Differentially expressed bands were excised, and de-stained overnight in distilled water. The gel pieces were then dehydrated and proteolysis performed by adding Trypsin and incubated overnight in a water bath at 37°C. Peptides were analysed by Kevin Mills and Wendy Heywood of the UCL proteomic facility using a combination of Nanoflow HPLC unit with ElectroSpray Ionisation-Time-of-Flight Mass Spectrometry (ESI-QTOF MS) allowing desalting and separation of peptides prior to analysis which increases sequence coverage and number of peptides sequenced. Identified proteins were given a UniProt accession number and those with a PLGS (ProteinLynx Global SERVER) score of greater than 46 where $p < 0.05$ were considered for analysis. The formula for calculating the PLGS score is -10*log (P),

where P is the probability that the observed match between experimental and database sequence is a random event.

2.11 Statistics

Data is expressed as means (±SEM), with differences between means analysed with GraphPad Prism™ Software. The following tests were applied: Fishers exact test, Analysis Of Variance (ANOVA) and non-parametric Mann-Whitney U test with two-tailed test of significance. Differences at $P < 0.05$ were considered to be statistically significant.

Chapter 3 : Akt1 controls key processes in epidermal terminal differentiation and cornified envelope formation

3.1 Introduction

Skin is comprised of a stratified epidermis with histologically distinct four layers, basal, spinuous, granular and cornified layer, each layer characterised by specific cell morphology and unique stage of differentiation (Fuchs, 2007; Watt, 2002). The keratinocyte is the main cellular constituent found within the human stratified squamous epithelium, and by a tightly controlled process of growth arrest and differentiation, the highly cross-linked protein component is enveloped by covalently linked lipid molecules thus forming the cornified layer or stratum corneum (Fuchs & Raghavan 2002; Hardman *et al*., 1999). During the early stages of keratinocyte differentiation the early differentiation markers K1 and K10 are expressed and as the cells move into the granular layer, they begin the expression of cornified envelope precursors, also known as late differentiation markers, such as involucrin, loricrin and profilaggrin (Coulombe and Wong, 2004; Segre, 2006; Presland, 1992). The keratinocyte terminal differentiation culminates in the formation of the cornified layer, which begins when filaggrin binds to the keratin filament network in the upper granular layer of the epidermis ending with the formation of compact dead squame that forms the stratum corneum (Watt and Green, 1981; Manabe *et al*., 1991). These differentiated cells are eventually shed from the stratum corneum and continuously replaced through proliferation of stem cells in the basal layer (Proksch *et al*, 2008).

Filaggrin is an important component of the stratum corneum contributing largely to its physical integrity, hydration, antimicrobial activity and pH amongst other properties (Rawlings and Harding, 2004; Kezic *et al*., 2008). Additionally filaggrin is also suggested to play a role in nuclear degradation, hence the extensive proteolytic processing of its precursor profilaggrin is a highly controlled process both temporally and spatially, to ensure the mature filaggrin monomers are produced in a timely manner that does not compromise the integrity of the epidermal barrier (Presland *et al*, 1997). In fact aberrant filaggrin processing has been reported to lead to defects in stratum corneum formation (Hewett *et al*., 2005 & List *et al*., 2003), and mutations in filaggrin predispose individuals to skin disorders such as AD and ichthyosis vulgaris (Palmer *et*

al., 2006; Smith *et al*., 2006 ; Weidinger *et al*., 2007). Filaggrin mutant mice (flaky tail) have a defective skin barrier and increased allergic sensitization, both of which are common characteristics of AD skin (Fallon *et al*., 2009; Oyoshi *et al*., 2009). Taken together these reports highlight the necessity for proper filaggrin expression in skin barrier formation. However only about 25 % of AD patients have filaggrin mutations suggesting the importance of other mechanisms involved in the processing of this protein in stratum corneum formation (Brown *et al*., 2009; Smith *et al*., 2006; Enomoto *et al*., 2008).

Multiple signalling pathways act both sequentially and in parallel to regulate keratinocyte differentiation (Dotto, 1999). Signalling cues from both Notch and Wnt gene families have been reported to regulate commitment to differentiation (Fuchs and Raghavan , 2002; Dotto, 1999). Specific protein kinase C family members have been shown to regulate expression of late differentiation markers required for cornified envelope formation (Dotto, 1999). The p63 pathway is reported to be important for key events in the development and differentiation of the epidermis, including barrier formation (Koster *et al*., 2002). However, the complex events leading to skin barrier competence are still poorly understood and with the increase in prevalence of skin disorders (Aberg *et al*., 1995; Simpson *et al*., 2009; Danielsen *et al*., 2013), a wider knowledge is required for better therapeutic options. Akt, or protein kinase B, a major downstream effector of insulin and growth factor signalling pathway, has been implicated in regulating differentiation of many tissues such as bone, muscle and skin (Peng *et al*., 2002). In fact, Akt1 activity has been found to be required for filaggrin processing and skin barrier competence (O'Shaughnessy *et al*., 2007; Thrash *et al*., 2006), and previous findings have established that both phosphorylation of heat shock protein 27 (Hsp27), a known substrate of Akt, in the upper granular layer and its transient co localisation with filaggrin in the keratohyalin granules in the cytoplasm occurs in an Akt1 dependent manner (O'Shaughnessy *et al*., 2007; Jonak *et al*., 2007).

In this study, we generated Akt1 knockdown rat epidermal keratinocyte (REK) cell lines to more fully understand the role of Akt signalling in the keratinocyte differentiation program, with a specific aim to evaluate further the role of Hsp27 in filaggrin processing. We hypothesised that in the absence of or in reduced Akt1 phosphorylation, Hsp27 forms complexes or interacts with other molecules and

therefore is not accessible to form interactions with filaggrin, hence interfering with filaggrin processing.

3.2 Results

3.2.1 Post confluent REKs mimic terminally differentiating cells in granular and transitional layer

As stages of keratinocyte differentiation are defined by specific expression patterns of keratinocyte markers (Fuchs, 2007), REKs were grown to post confluence and expression of differentiation markers at both pre- and post-confluent cultures were determined. Compared to pre-confluent cultures, post-confluent cultures expressed higher levels of early keratinocyte marker K1 and increased expression of profilaggrin and the monomeric mature subunit filaggrin, suggesting these cells are more differentiated and are a good model for granular and transitional layer keratinocytes (Fig 3.1). pSer473Akt phosphorylation is detected only in post confluent cultures and increases as cells become more confluent at days 5 and 6 (Fig 3.1). Previous findings have reported that Akt phosphorylation peaks in the terminally differentiating granular layer during barrier acquision stage of mouse embryonic development (O'Shaughnessy *et al*., 2007), suggesting that REKs at day 5 and 6 are likely to be at this terminal differentiation stage.

Heat shock protein 27 (HspB1), co-localises with filaggrin in keratohyalin granules and the phosphorylated form (pSerHspB1) is most prominent in the granular layer during the mouse embryonic barrier acquisition (O'Shaughnessy *et al*., 2007). This report also showed the trimeric (75 kDa) phosphorylated form was increased during barrier acquisition while total HspB1 remain unchanged (O'Shaughnessy *et al*., 2007). Agreeing with this data, an increase in the pSerHspB1 75 kDa phosphorylated form was observed in post confluent cultures while no obvious change was observed in monomeric total HspB1 25 kDa form (Fig 3.1). Taken together these results indicated that as REKs are grown to post confluence they achieve a differentiated state mimicking the granular and transitional layer of the skin. Hence in all further experiments, we chose the optimum time-point where the cells are terminally differentiated; all subsequent analyses were performed on cells cultured to post-confluent day 5.

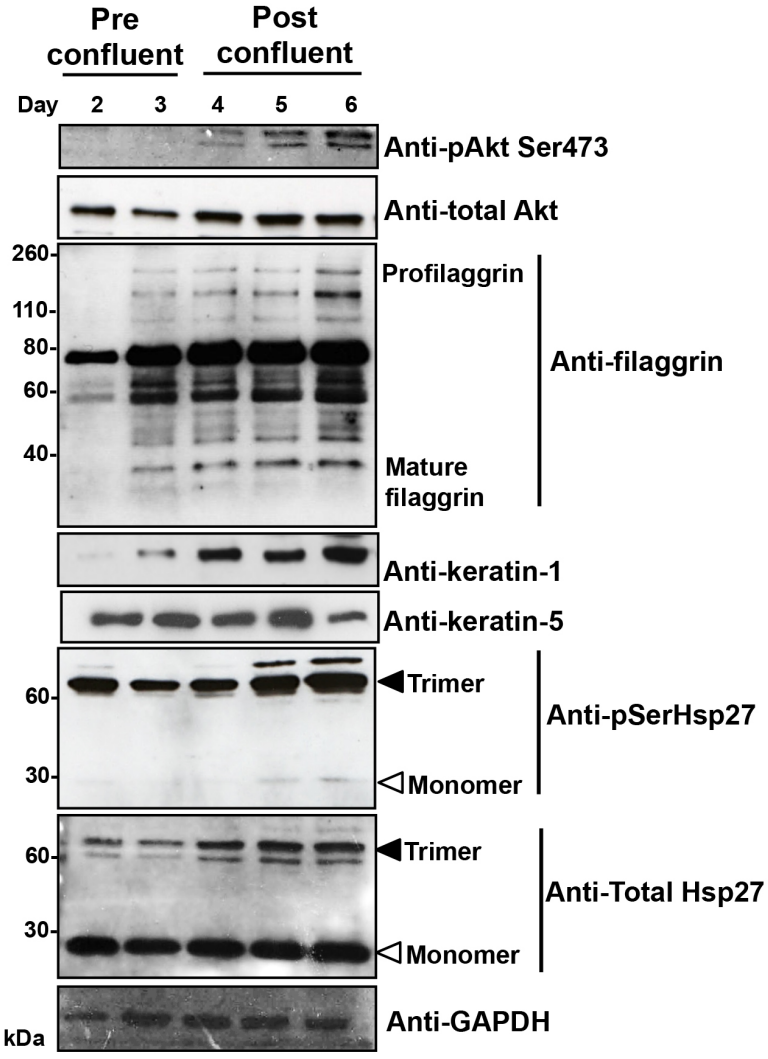

Figure 3.1 Post confluent REKs mimic terminally differentiating cells in stratum granulosum.

Western blot of epidermal differentiation markers, Akt and Hsp27 in pre and post-confluent REKs. Pre confluent cell cultures at days 2 and 3 showed low levels of the early differentiation marker K1, late differentiation marker profilaggrin and its mature subunit filaggrin in comparison with post confluent cultures at days 4, 5 and 6. Monomeric total Hsp27 remained unchanged with increase in the trimeric forms in both total and phosphorylated forms of Hsp27 in post confluent cultures. pAkt was undetectable in pre confluent cultures with increasing levels in post confluent cultures. Total Akt remained unchanged while basal keratin 5 showed a decrease in protein levels in day 6. All westerns are representative images from n=2. Protein lysates were prepared using total lysis buffer.

3.2.2 Filaggrin processing is Akt dependent and the Akt1 shRNA REK organotypic model phenocopies AD skin

Akt phosphorylation was reduced in REKs using Akt1 shRNA-mediated knockdown of Akt1 expression and by using the pharmacological PI3 kinase inhibitor wortmannin, a drug that can inhibit all downstream signaling pathways of PI3 kinase including Akt (O'Shaughnessy *et al*., 2007a; Lam et al., 1994). Real time PCR analyses of Akt1 knock down REKs showed no significant change in mRNA levels of filaggrin (Fig 3.2). In both A1 and A3 Akt shRNA REKs there was a reduction in Akt phosphorylation of more than 60 % in post confluent cultures (Fig 3.3A & B). Overall filaggrin processing, and total filaggrin levels were reduced with a significant reduction in the mature monomeric filaggrin subunit in both shRNA Akt1 knockdown REKs and Wortmannin-treated cells (Fig 3.3A & B) suggesting that Akt activity is required for filaggrin processing. No appreciable change was observed in the early differentiation markers K1 and K10 (Fig 3.3A). The late differentiation marker loricrin was reduced in the wortmannin treated cells (Fig 3.3B), and also in the Akt1 shRNA expressing cells, with line A3 showing the largest reduction. Expression levels of total Akt and keratin 5 remained unchanged. These results were confirmed in post confluent human keratinocytes where a reduction in pAkt and overall filaggrin levels processing was observed after wortmannin treatment (Fig 3.4).

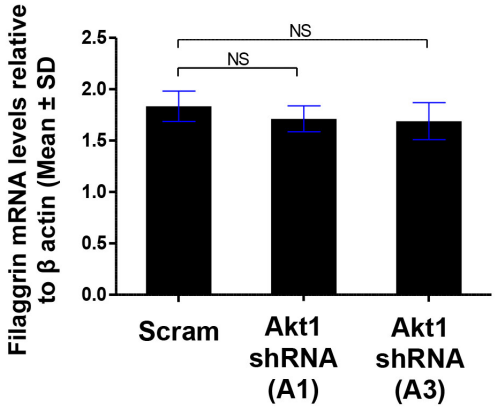

Figure 3.2. Real Time PCR results of filaggrin mRNA expression levels in scram and both Akt1 shRNA expressing cell lines.

Statistical analysis was performed using the non-parametric Mann-Whitney U-test. Significance was defined as $p<0.05$. NS = not significantly different

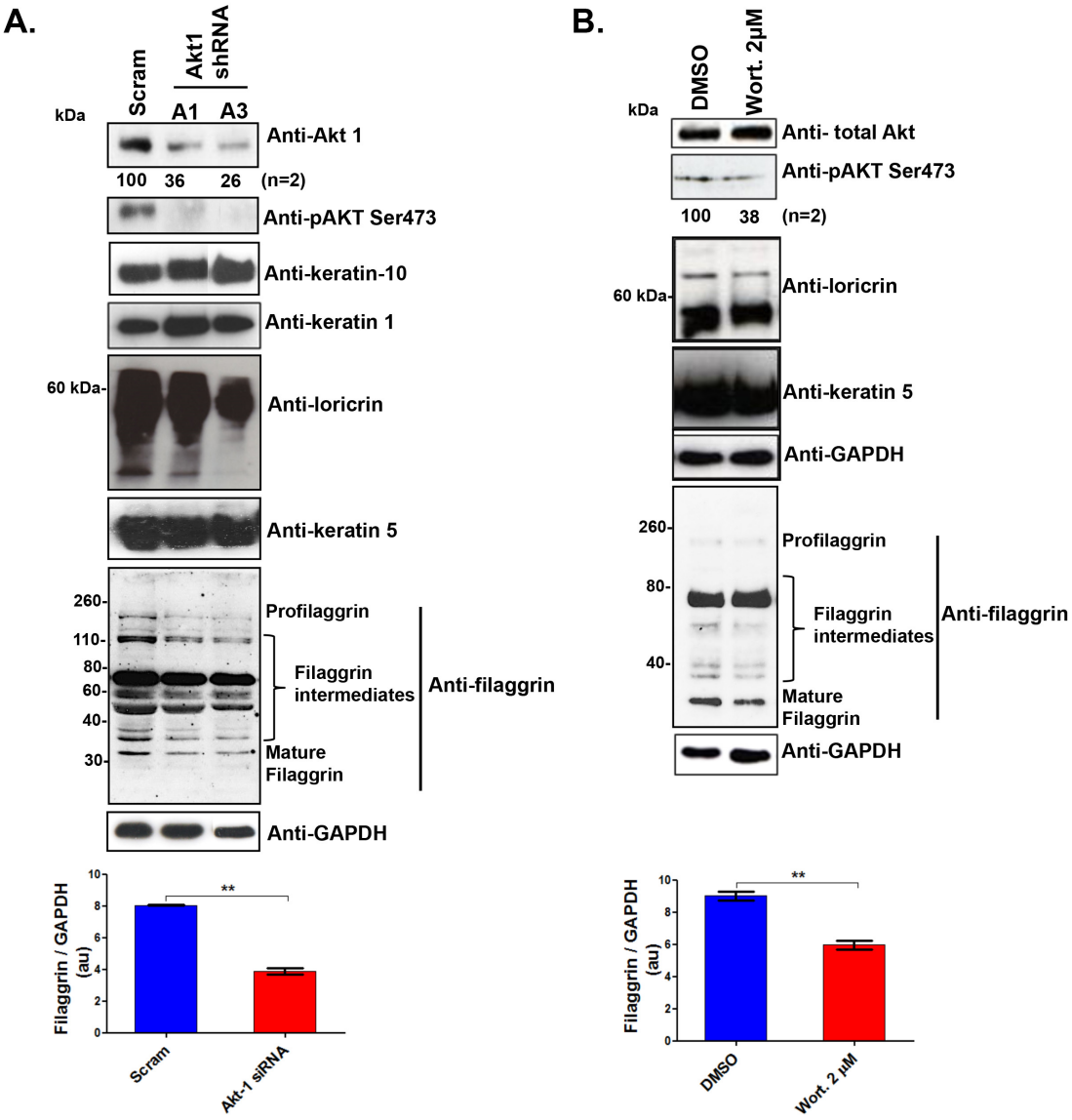

Figure 3.3. Reduced Akt phosphorylation is associated with decrease in filaggrin processing.

*(A) Immunoblot of anti Akt-1 showed a reduction of Akt1 protein levels by more than 60% in both Akt1 shRNA expressing cells. Western analysis also showed a reduction in pAkt and a reduction in late differentiation marker loricrin, greatest in A3. Overall filaggrin processing and levels were significantly reduced (n=3) in both Akt-1 shRNA expressing cells. The basal keratin 5 remained unchanged and no appreciable difference was observed in the early differentiation markers K1 and K10 in the Akt1 siRNA expressing cells. (B) Western analysis also showed a reduction of pAkt by more than 60 % in wortmannin treated REKs. Immunoblots of anti-loricrin showed a reduction in loricrin while the basal keratin 5 remain unchanged in Akt inhibited cells. Overall filaggrin processing and levels were decreased significantly (n=3) in wortmannin treated cells. ** $p \leq 0.01$(Mann-Whitney U test). Filaggrin western images are representative images from n=3. All the other western images are representative images from n=2. Protein lysates were prepared using total lysis buffer.*

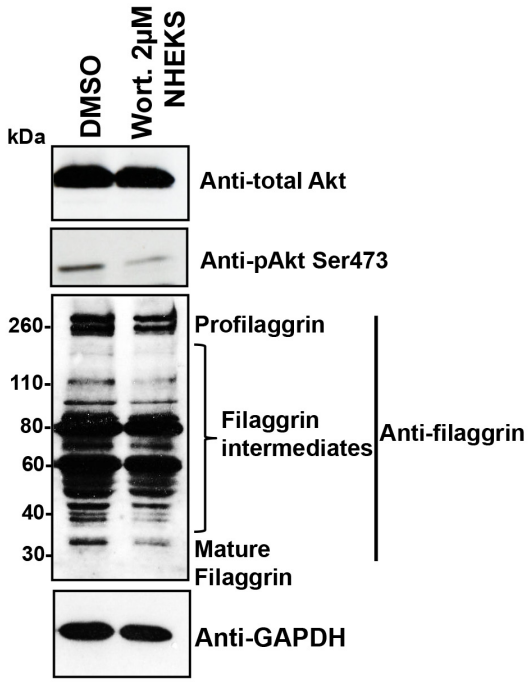

Figure 3.4. Reduced Akt activity is associated with over all reduction in filaggrin processing in human keratinocytes.

Western analysis showed a reduction in pAkt and overall filaggrin processing and levels in cells treated with the pharmacological PI3 Kinase inhibitor, wortmannin. All western images are representative images from n=2. Protein lysates were prepared using total lysis buffer.

When Akt1 knockdown REKs were grown in organotypic culture on a de-epidermalized dermal scaffold and examined, they displayed hyperkeratosis, a thickened cornified layer, and parakeratosis (nuclei retention in the stratum corneum) (Fig 3.5 H&E), two features which are characteristic of AD skin (Guttman-Yasskey *et al*., 2011; Nakamura *et al*., 2011). The expression patterns of keratinocyte differentiation markers were observed by immunofluorescent analysis. Consistent with the western data, confirmation of Akt1 knock down was observed along with a reduction in filaggrin protein expression in the granular layer of Akt1 shRNA expressing cultures. Similar to the western analysis, no obvious change was observed in the K1 or K10, while a reduction in loricrin was observed in A3 (Fig 3.5).

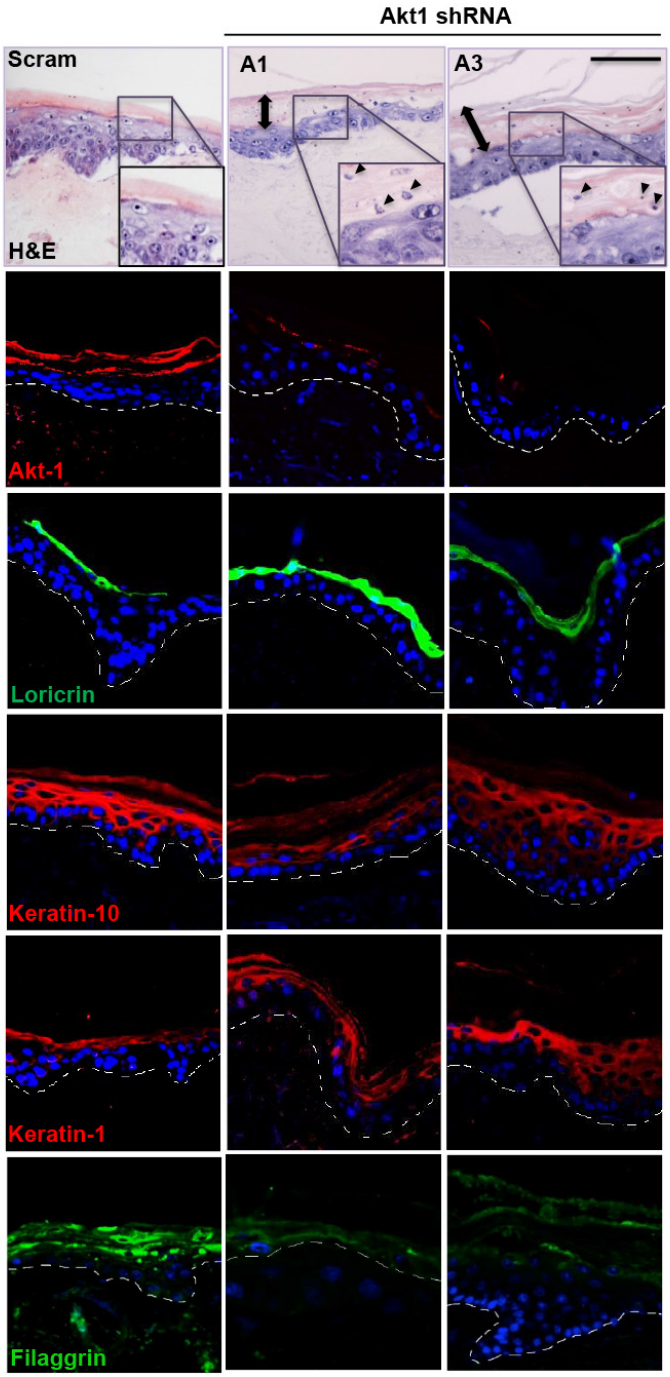

Figure 3.5. Akt1 shRNA REK model phenocopies AD skin.

H&E sections of Akt1 deficient organotypic cultures displayed thickening of the stratum corneum (hyperkeratosis) and retention of nuclei (inset; black arrow heads) in the stratum corneum (parakeratosis) in comparison with the scrambled. Double sided black arrows indicate the thickened cornified layer. Organotypic cultures of scrambled and 2 cell lines expressing shAkt1 (Akt1 shRNA) were immunostained for expression of Akt1, early keratinocyte differentiation markers Keratin 1,Keratin 10 and late differentiation markers loricrin and filaggrin. A reduction in Akt1 was observed in both shAkt1 expressing cells. While no change was observed in K1 and K10, a small reduction was observed in loricrin in A3. Filaggrin protein levels were decreased in both shAkt1 expressing cell cultures. The sections were counterstained using Dapi (blue). Scale bar 50 μM. All images are representative images from n=2.

3.2.3 Akt inhibition reduced filaggrin and pSerHspB1 co-localisation

HspB1 phosphorylation in the epidermis is Akt-dependent (O'Shaughnessy *et al*, 2007). Consistent with this, western blot analysis showed a reduction in pSerHspB1 of both the trimeric 75 kDa form and the monomeric 25 kDa form in both Akt1 knock down cell line (A3) and Akt inhibited cells while the total HspB1 levels remained unchanged (Fig 3.6A & B). It is possible reduction of pSerHspB1 is dependent on level of Akt1 knock down which may explain why no change in pSerHspB1 monomeric and trimeric forms was observed in Akt1 A1 shRNA. Unphosphorylated HspB1 exists as a monomeric form but can form large oligomers (minimum 22 mers and over), whereas phosphorylation of HspB1 breaks down these large oligomers and dramatically changes the dynamics towards formation of small oligomers such as the dimeric and trimeric forms (Hayes *et al*., 2009).

HspB1 phosphorylation and its transient interaction with filaggrin in the keratohyalin granules in the granular layer is Akt1 dependent (O'Shaughnessy *et al*., 2007). To determine whether this interaction is observed in Akt inhibited REKs, co-immunofluorescence was performed on confluent cells treated with wortmannin. Results indicated co-localisation with pSerHspB1 in the DMSO (control) treated cells, which was greatly reduced in the Akt inhibited REKs, consistent with the published data (O'Shaughnessy *et al*., 2007). In addition, in the Akt inhibited cells, pSerHspB1 expression was more prominent in the periphery of the cells (Fig 3.7A), reminiscent of the F-actin staining observed in keratinocytes (Fig 3.7B) prompting the question whether F-actin and pSerHspB1 might be interacting.

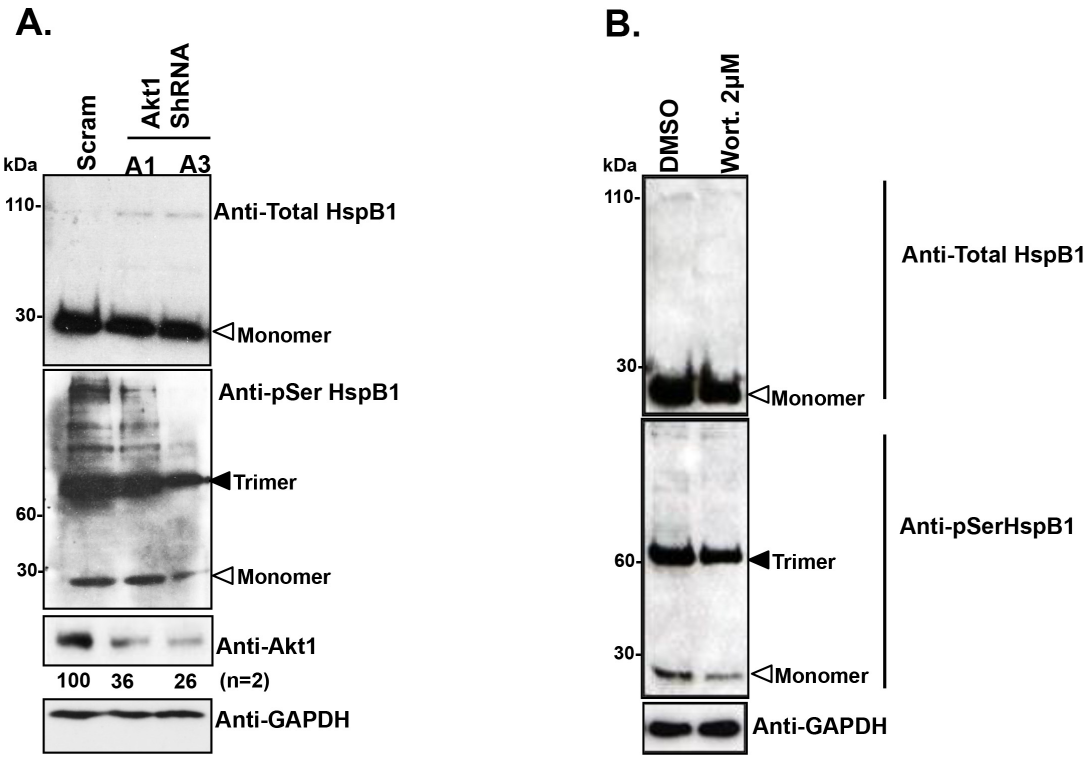

Figure 3.6. Reduced Akt activity reduced levels of pSerHspB1.

(A) Western blot analysis of Akt1 shRNA expressing cells showed a reduced protein expression levels of both the monomeric and trimeric pSerHspB1. (B) Treatment with the PI3 kinase inhibitor, wortmannin also decreased both the monomeric and trimeric forms of pSerHspB1 while total HspB1 remained unchanged. All westerns are representative images from n=2. Protein lysates were prepared using total lysis buffer.

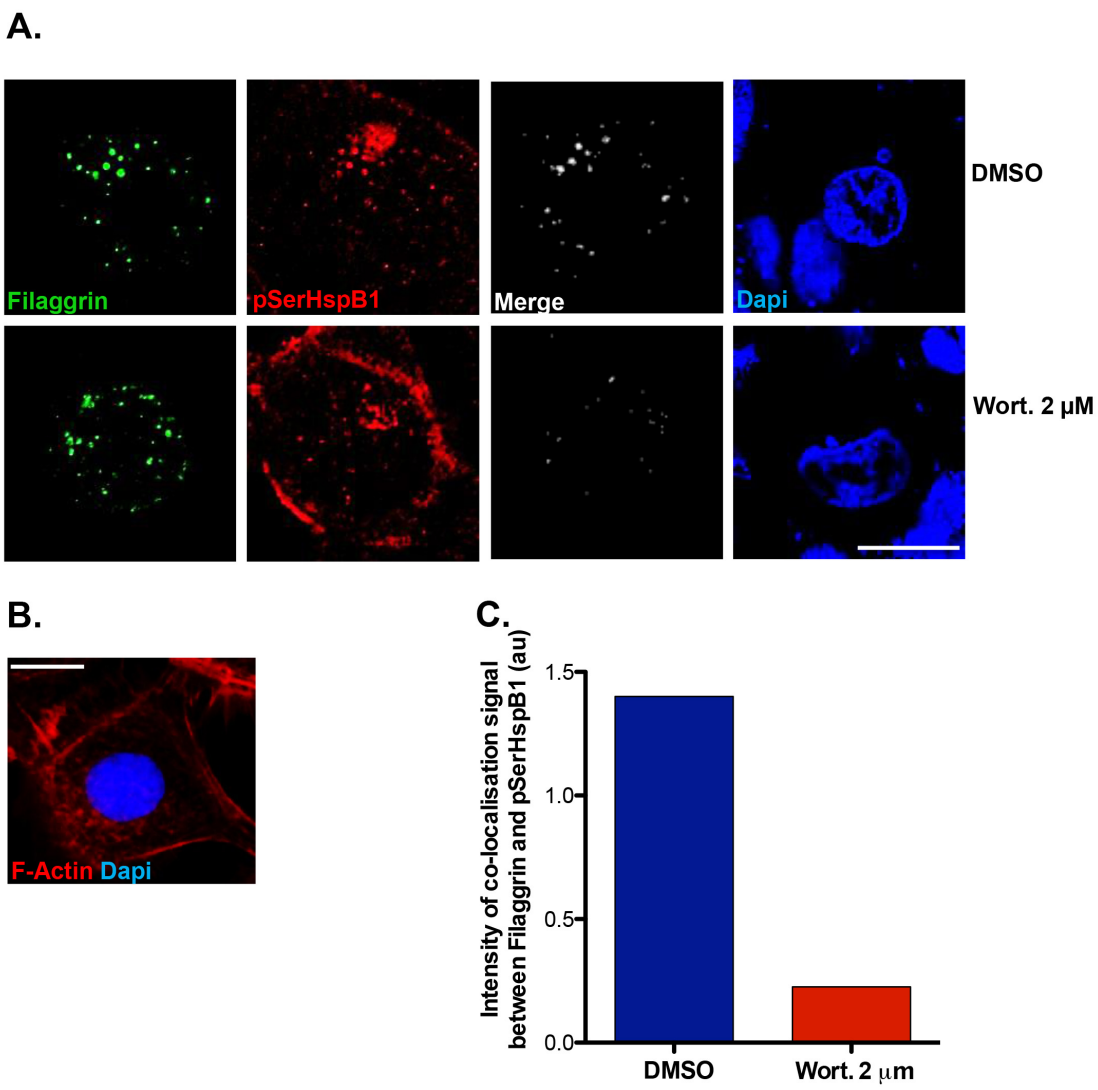

Figure 3.7. Akt inhibition reduced filaggrin and pSerHspB1 co localisation.

(A) Co immunofluorescent analysis of filaggrin (green) and pSerHspB1 (red) on post confluent REKs treated with PI3 kinase inhibitor wortmannin compared with the control (DMSO). (B) Phalloidin stained F-actin (red) at the cell periphery in post confluent REKs. DAPI (blue) was used as a counter stain. Scale bar 10 µM. (C) Densitometric quantification of co-localised signals between filaggrin and pSerHspB1 in DMSO and wortmannin. Images displayed in this figure are representatives from n=2.

3.2.4 Increased HspB1-Actin interaction in Akt1 shRNA cells

Phosphorylated HspB1 has been reported to have a regulatory role in actin filament dynamics. In fact a well characterised role of monomeric HspB1 is its ability to bind to actin microfilaments and inhibit F-actin polymerization by capping the ends or by binding to G-actin and sequestering them (Lavoie *et al*., 1993; Guay *et al*, 1997), whereas pSerHspB1 inhibits G-actin sequestering (Guay *et al*, 1997). To investigate the potential interaction between HspB1 and actin, co-immunoprecipitation (co-IP) with an agarose-conjugated anti-HspB1 antibody, was performed on both Akt inhibited (Wortmannin treated) and Akt1 shRNA expressing REKs.

IP western blot analysis showed an increased interaction between HspB1 and actin in both Akt inhibited and Akt1 knock down cells in comparison with the controls where the interaction was reduced. IP western analysis showed reduced interaction between HspB1 and filaggrin in Akt inhibited cells consistent with the reduced co-localization observed in the co immunofluorescence results in Fig 3.7A (Fig 3.8). A specific subset of filaggrin intermediates associated with HspB1 consistent with previous reported data (O'Shaughnessy *et al*., 2007). Interestingly immunostaining of F-actin in wortmannin treated REKs showed enhanced F-actin polymers at the cell periphery compared to the control (Fig 3.9).

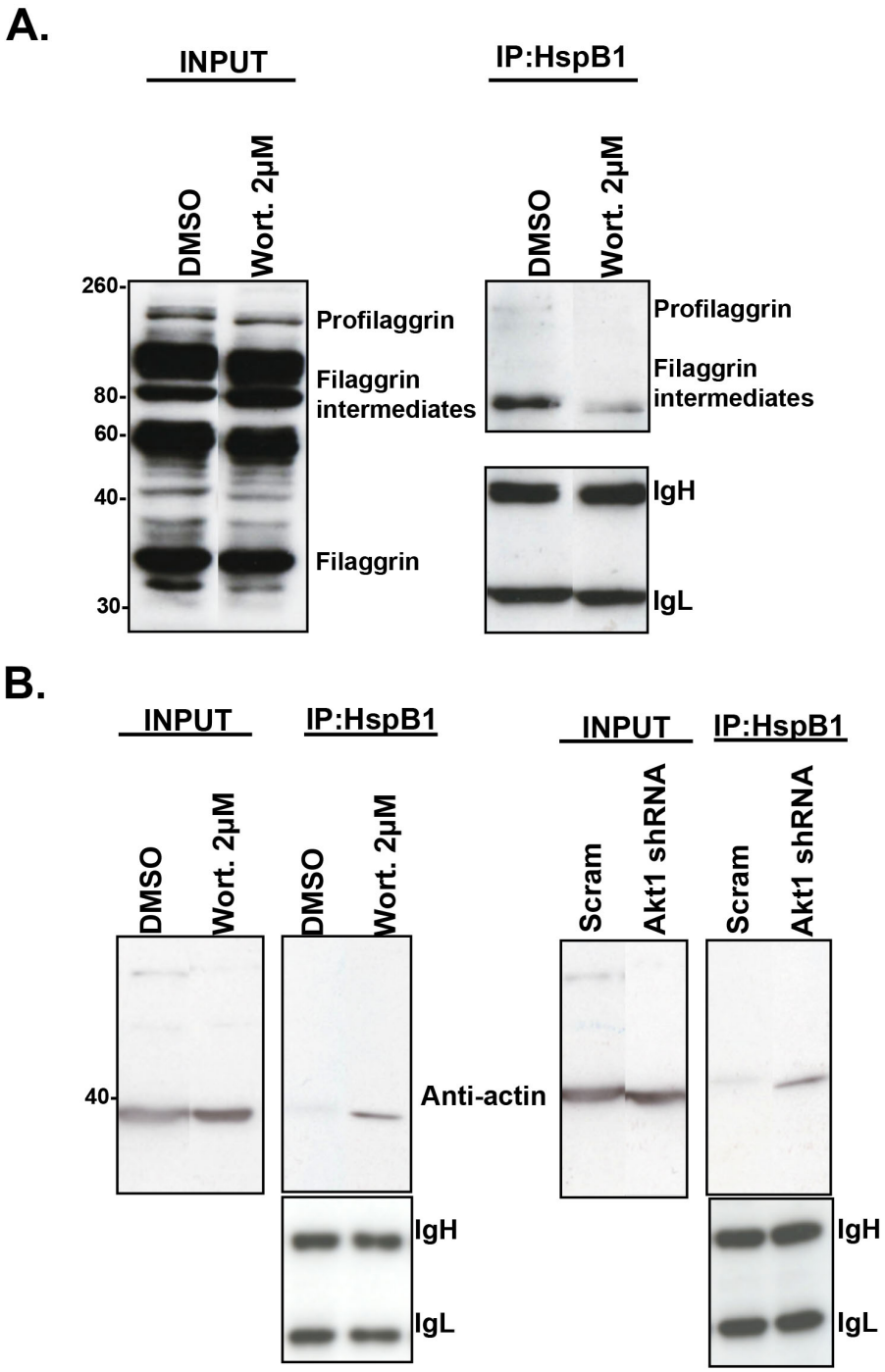

Figure 3.8. Decreased HspB1-filaggrin and increased HspB1-Actin interaction in REKs with reduced Akt phosphorylation.

(A) Co-IP western blot analysis of interaction between filaggrin and HspB1 in wortmannin treated cells showed a decreased HspB1-filaggrin association in comparison with the control (DMSO). (B) Co-IP western analysis of interaction between actin and HspB1 in both Akt1 shRNA cells and wortmannin treated cells showed an increased HspB1-actin association in both treatments in comparison with control (DMSO). Westerns are representative images form n=2. Protein lysates were prepared using RIPA lysis buffer.

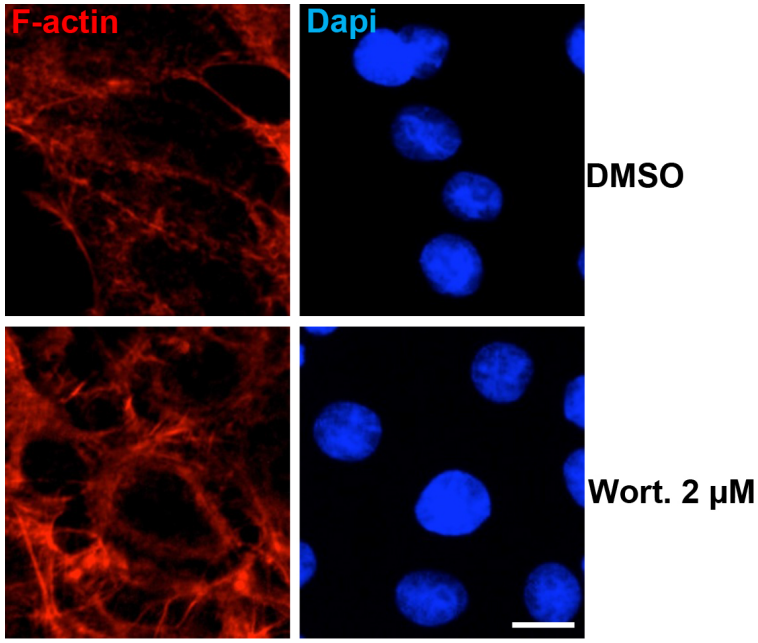

Figure 3.9. Enhanced F-actin staining at the cell periphery in cells with reduced Akt phosphorylation.

Phalloidin stained F actin (red) at the cell periphery. Dapi (blue) was used as a counter stain. Scale bar 10 µM. All images are representative images from n=2.

3.3 Discussion

Although the filaggrin gene (*FLG*) and the association of *FLG* null mutations with AD has been well characterised, the exact mechanism of the processing of profilaggrin to filaggrin is still incompletely understood. The data described here support Akt1 as an important signalling molecule in keratinocyte differentiation and identifies Akt1 as a key component in filaggrin processing, and furthermore establishes the Akt1 shRNA organotypic and cell culture model as an appropriate model to investigate the role of Akt1 in late stage terminal differentiation.

The reduction in phosphorylated HspB1 observed in Akt1 knockdown cells and wortmannin inhibited cells, is consistent with previous findings (O'Shaughnessy *et al*., 2007), and the reduction in filaggrin processing in both treatments suggests that Akt1 activation is required for both HspB1 phosphorylation and filaggrin processing. Since HspB1 shRNA knockdown organotypic models displayed hyperkertosis and reduced filaggrin expression (O'Shaughnessy *et al*, 2007), the role of HspB1 in filaggrin processing may be Akt1 mediated. The Akt1 shRNA expressing organotypics demonstrated both hyperkeratosis and parakeratosis, which are two characteristics of AD skin (Leung and Bieber, 2003) suggesting that the Akt1 shRNA model phenocopies AD.

These data show an Akt1-dependent co-localization of filaggrin with pSerHspB1 consistent with previously reported data (O'Shaughnessy *et al*., 2007), where Akt1 activation was required for filaggrin-HspB1 co-localisation. Results also indicated that reduction in Akt activity caused a change in pSerHspB1 localisation, leading to more pronounced expression in the cell periphery similar to F-actin staining observed in keratinocytes. Immunoprecipitation data showed that reduction in Akt1 activity resulted in an increased association between HspB1 and actin. These results would imply that switch from a HspB1-actin interaction to a HspB1-filaggrin interaction is Akt1 mediated. Actin dynamics have been reported to change dramatically during proliferation and differentiation of cells (Watt, 1986; Ben-Ze'ev, 1987). It has been reported that in more stratified keratinocyte cultures, actin staining at the cell periphery in cells in the suprabasal layer is less defined and more diffuse in comparison with basal cells (Kubler *et al*., 1991; Lewis *et al*., 1987). However in the differentiated

cell cultures with reduced Akt activity, actin staining was more defined and prominent at the cell periphery, where as in the control cultures the F-actin staining was less defined similar to published data (Kubler *et al*., 1991). In stratified keratinocyte cultures, the subrabasal cells have more diffuse and less defined F-actin staining at the cell periphery similar to what is observed in the DMSO treated cells in Fig 3.9 (Kubler *et al*., 1991; Lewis *et al*., 1987). Hsp27 binds F actin, an action favoured by monomeric unphosphorylated Hsp27 (Graceffa, 2011; During *et al*., 2007). Furthermore, unphosphorylated Hsp27 binding is concentrated at F-actin polymerizing sites, and once phosphorylated, Hsp27 does not localize to these regions (During *et al*., 2007). Taken together, this data suggests that the unphosphorylated monomeric Hsp27 binds to the actin stabilizing the actin cytoskeleton, which will explain the persistent actin staining in Akt1 shRNA knock down cells. Since the phosphorylated trimeric form does not bind to actin, it thus binds to filaggrin facilitating proper filaggrin processing. It is also interesting to note that phosphorylation of Hsp27 enhances its chaperone activities (Hayes *et al*., 2009).

The data presented here demonstrates that pSerHspB1-filaggrin interaction and filaggrin processing are both Akt1 dependent, and we propose that the reduced filaggrin processing observed in Akt1 shRNA knock down cells is due to maintained HspB1-actin interaction.

Chapter 4 : Cathepsin H, an Akt1 dependent epidermal protease in profilaggrin processing, is reduced in eczema

4.1 Introduction

Abnormalities in the immune system have been strongly attributed to the development of AD (Von Bubnoff *et al*., 2001; Novak *et al*., 2003). However, current evidence suggests that primary cause for disease development in the majority of AD cases is a defective skin barrier, suggesting the observed inflammation is a secondary effect of this inherent barrier defect (Callard and Harper, 2007; Jakasa *et al*., 2006). Epidemiological research has revealed a strong genetic component to AD and to skin barrier function in the disease (Schultz-Larsen, 1993; Cork *et al*., 2006). One protein of particular interest is the epidermal structural protein filaggrin, where null mutations in the *FLG* gene are associated with an inherited eczematous disease, ichthyosis vulgaris, and have been established as being a major predisposing factor for AD (Presland and Dale, 2000; Palmer *et al*., 2006). However several studies have shown that only ¼ of AD patients have filaggrin mutations (Smith *et al*., 2006; Weidinger *et al*., 2007; Brown *et al*., 2009), and not all individuals with filaggrin mutations have AD (reviewed in O'Regan *et al*., 2008), suggesting that other mechanisms might give rise to the barrier defect observed in AD patients. The importance of filaggrin for proper barrier function has been demonstrated by numerous studies using filaggrin mutant mouse models where filaggrin deficient mice display barrier dysfunction and heightened percutaneous immune responses (Man *et al*., 2008; Fallon *et al*., 2009; Oyoshi *et al*., 2009). Profilaggrin to filaggrin processing is a complex process requiring dephosphorylation and numerous proteolytic events (Presland *et al*., 2004; Sandilands *et al*., 2007). Several distinct proteinases have been identified that cleave profilaggrin at specific sites releasing the filaggrin monomers and both the N and the C termini (reviewed in De Veer *et al*., 2014). Proteases such as Elastase 2 (Bonnart *et al*., 2010), SASPase (Matsui *et al*., 2011), and serine proteases Matriptase/MT-SP1 (List *et al*., 2003) are reported to be involved in profilaggrin to filaggrin processing.

Microarray analysis was performed to profile differentially expressed genes in Akt1 kd keratnocytes to identify proteases downstream of the Akt signaling pathway and elucidate further their potential involvement in filaggrin processing and barrier function. Additionally, expression patterns of any identified protein of interest from the

array analysis were characterized in skin biopsies from non-affected skin of AD patients.

4.2 Results

4.2.1 Ctsh is down regulated in Akt1 knock down REKs (microarray data)

Affymetrix DNA microarray analysis was performed on both shRNA Akt-1 knock down REK cell lines, and analysis was limited to genes that demonstrated a fold change of ≥1.5 within the dataset. Scrambled shRNA was used as a control. A total of 86 genes were differentially expressed with 34 genes up-regulated and 52 genes down regulated (Fig 4.1A). Of these 86 genes, 17 genes had differential expression ≥ 2 –fold, with the most differentially expressed gene, Cathepsin H (*Ctsh*), being down regulated over 4-fold. Ctsh belongs to a group of lysosomal cysteine proteases that are part of the papain super family of cysteine proteases and unlike most of other members of cysteine proteases, Ctsh can function in the cytosol, nucleus and also outside the cell (Tedelind *et al*., 2010; Reiser *et al*., 2010). Few proteases were identified as being differentially regulated and out of these Ctsh, F2rl2, Serpinb9 and Serpinb3 were altered ≥ 2 –fold (Fig 4.1B). Real time PCR was performed on these 4 proteases to validate the array data and results indicated only Serpinb9 and Ctsh were significantly down regulated in both Akt1 shRNA expressing cell lines in comparison with the normal scrambled shRNA expressing control (Fig 4.1C).

Using Ingenuity Pathways Analysis (http://www.ingenuity.com), relevant canonical pathways, gene regulation networks and biological functions significant to the data set were determined. To achieve this, data set containing gene identifiers of the 86 genes and corresponding expression values were uploaded into the application. The canonical pathways with most significant changes were lipid metabolic pathways and LXR/RXR pathway (Fig 4.2). Retinoid X Receptors (RXR) are nuclear receptors that are involved in retinoic acid mediated gene activation (Chawla *et al*., 2001). RXR dimerizes with other type II nuclear receptors such as the Liver X Receptor (LXR), and the resulting heterodimer, RXR/LXR, is an important regulator of keratinocyte anti inflammatory responses and lipid synthesis in the skin (Chawla *et al*., 2001; Fowler *et al*., 2003). Furthermore RXR/LXR activation stimulates keratinocyte differentiation and

lipid synthesis leading to barrier formation (Fowler *et al*., 2003; Kömüves *et al*., 2002; Man *et al*., 2008).

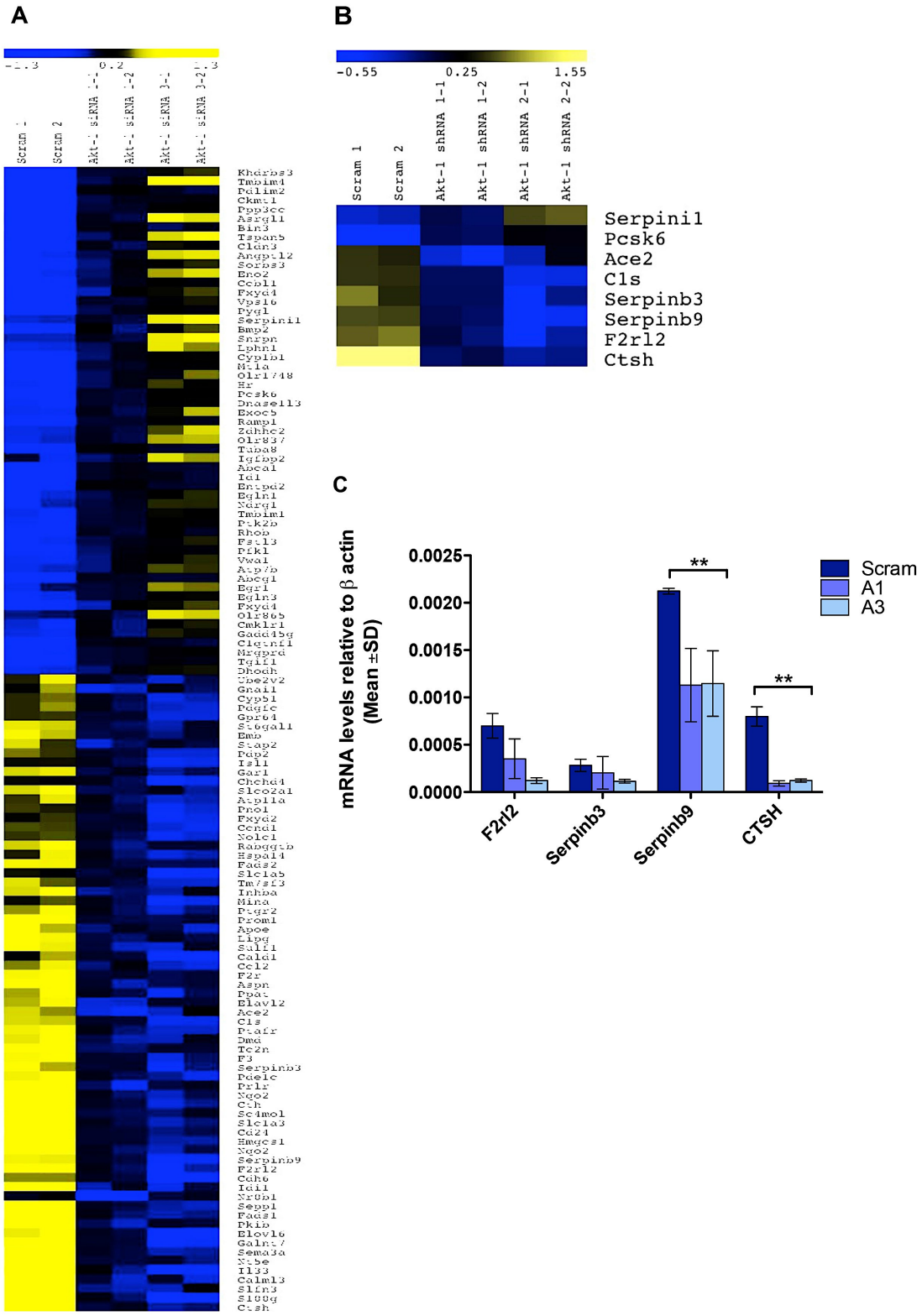

Figure 4.1 Cathepsin H is downregulated by 4 fold in Akt-1 knock down REKS.

*(A) Heat map showing differently regulated genes with a fold change ≥ 1.5. Scrambled shRNA was used as a control. Yellow corresponds to high expression, blue corresponding to low expression and black corresponding to intermediate expression. (B) Heat map of differentially regulated proteases in Akt-1 shRNA expressing cells. (C) Real Time PCR mRNA levels of F2rl2, Serpinb3 Serpinb9 and Ctsh. Statistical analysis was performed using GraphPad Prism by two-way analysis of variance (ANOVA). Significance was defined as $p<0.05$. ** $p \leq 0.01$.*

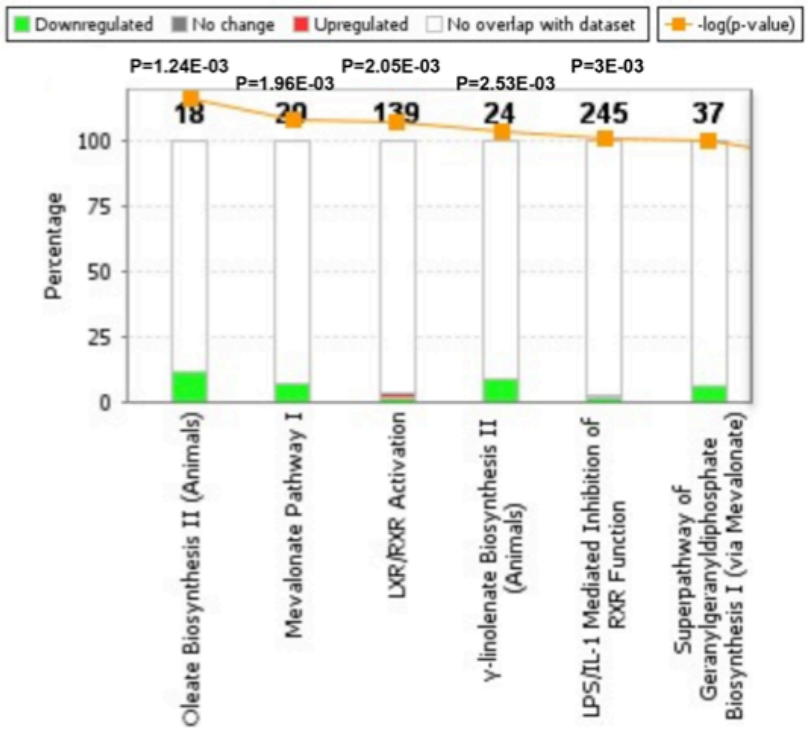

Figure 4.2 Canonical pathways and gene networks in Akt1 knock down cells.

Ingenuity Pathway Analysis (www.ingenuity.com) was used to identify the most significant canonical pathways and relevant gene networks based on the data set. A. The Most significantly overrepresented pathways altered included lipid metabolic pathways and LXR/RXR pathway. Numbers above the bars indicate the number of genes in the canonical pathway identified from the IPA knowledge base. Y-axis represents the percentage of these genes present in the microarray data set. Fisher's Exact Test was used to calculate p-value to assess significance of enrichment of the gene expression data.

Almost half of the 86 genes studies clustered into two major networks; one associated with lipid metabolism and molecular transport where majority of the genes were down regulated (Fig 4.3A). Interestingly PCSK6 (also known as PACE4) which is a protein convertase involved in profilaggrin N-terminal processing (Presland *et al*., 2009) was up regulated (Fig 4.3A). The other potential network is associated with cell morphology and cellular development (Fig 4.3B). This network was centered on Akt, which included Ctsh as one of the clustered genes. The protein metalloproteinases (MMPs), which are elevated in AD skin (Harper *et al*., 2010), and the transcription factor complex AP-1 whose activity is dysregulated in AD (Pastore *et al*., 2000) was also present in this network suggesting a potential association with Akt (Fig 4.3B). As the array analysis identified Ctsh as the most downregulated gene in the Akt1 knock down cells, this protease was further investigated to determine its role in keratinocyte differentiation.

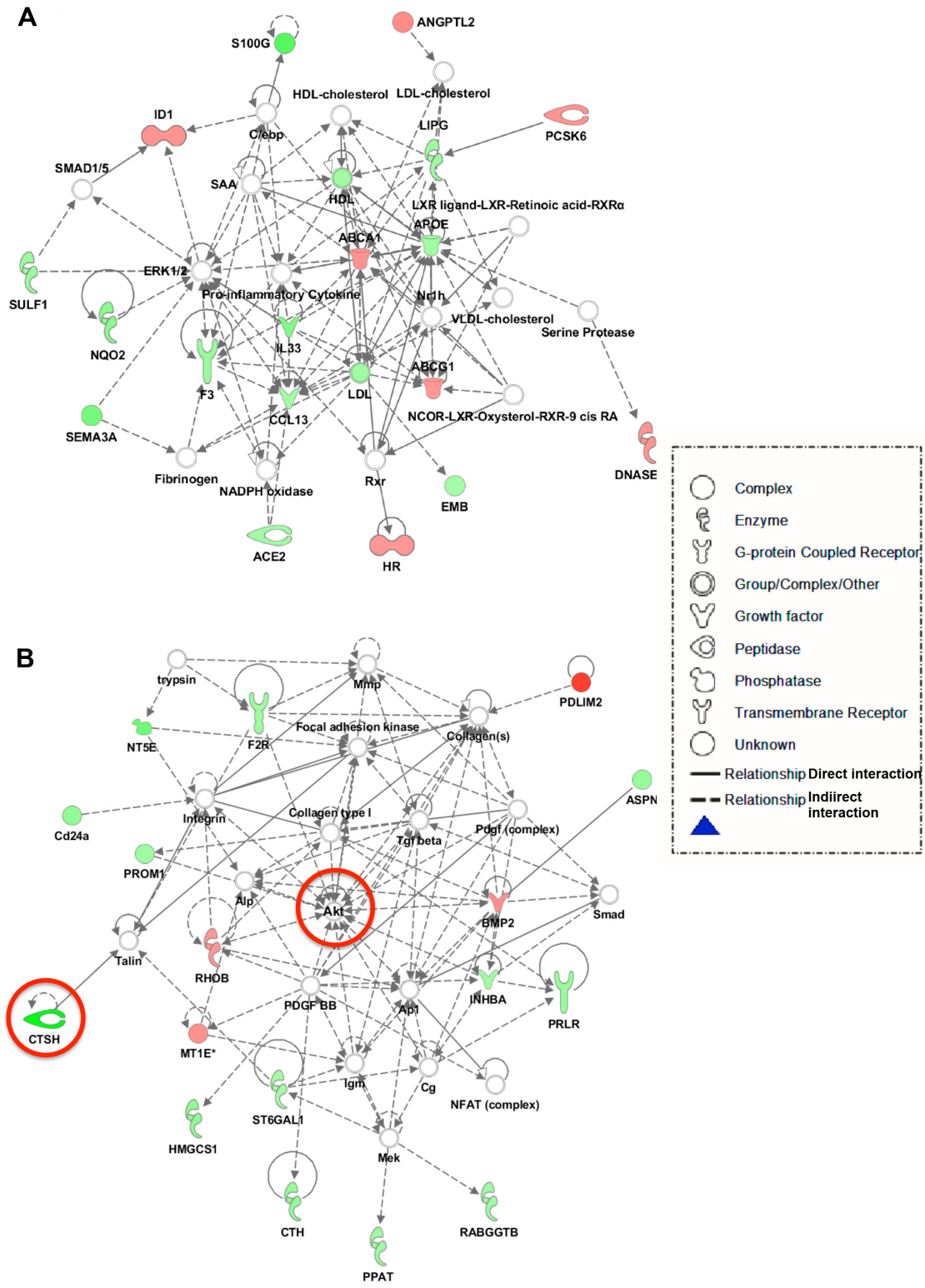

Figure 4.3 Two major networks based on 86 genes differentially regulated in Akt1 knockdown cells.

(A) & (B) Networks were generated using Ingenuity Pathways Analysis (IPA: www.ingenuity.com) in which, using information from IPA Knowledge Base, focus genes are clustered together as nodes in a network based on their interactions. Green represents down-regulated genes and red represents up-regulated genes. Colour intensity is an indication of the degree of fold change. The network is completed by filling in genes from the Knowledge Base (nodes in white).

4.2.2 Cathepsin H, a novel epidermal protease that co localizes with filaggrin

Epidermal barrier acquision in the mammalian fetus occurs during late intra-uterine development (Hardman *et al*., 1999). In mice the period of barrier acquisition takes place between embryonic days 16.5 (E16.5) and 18.5 (E18.5) with a competent barrier achieved by E18.5 (Hardman *et al*., 1998; O'Shaughnessy *et al*., 2009). Expression of granular pSer473Akt activity and Akt1 along with the cornified envelope protein filaggrin peaks in expression during this barrier acquisition stage (O'Shaughnessy *et al*., 2009). Consistent with this, Akt1 expression was at its highest at days E17.5 and 18.5 (Fig 4.4). Similarly granular layer expression of Ctsh also increased at days E17.5 and 18.5 suggesting a possible involvement in the barrier acquisition process in mice.

To evaluate whether Ctsh is involved in keratinocyte differentiation, western blot analysis was performed on both pre-confluent and post-confluent REK cultures. Ctsh protein levels were detected only in the more differentiated post confluent cultures mirroring the expression patterns of pSerAkt (Fig 4.5A and Fig 3.1) and keratin 1 (Fig 3.1), whose expression levels increase during keratinocyte differentiation (Schweitzer and Winter, 1983; O'Shaughnessy *et al*., 2007), thus implying a possible role for Ctsh in keratinocyte differentiation (Fig 4.5A). The total Akt and pSer473Akt western blots in Fig 4.5A are also displayed in Fig 3.1. The doublet Ctsh bands observed in fig 4.5A are the mature 28 kDa form and the 22 kDa form. Ctsh often migrates as the single chain 28 kDa mature form and the processed 22 kDa heavy chain (Kiahimoto & Itoi, 2013; Lu *et al*., 2007). It has been reported that the 22 kDa form can sometimes be lost in the process of sample preparation. This smaller species is suspected to be more susceptible to catalytic process involved in Ctsh turn over (Lu *et al*., 2007). The single Ctsh band detected in Fig 4.5B and C are therefore likely to be the 28 kDa form. Western blot analysis of post-confluent REKs showed a reduction in Ctsh protein levels in both Akt1 shRNA knock down REKs (Fig 4.5B) and in normal human epidermal keratinocytes (NHEKs) treated with the PI3 kinase pharmacological inhibitor wortmannin, suggesting a role for Akt1 in regulating Ctsh expression (Fig 4.5C). Judging from the size, it is likely the single Ctsh band detected here is the mature 28 kDa form.

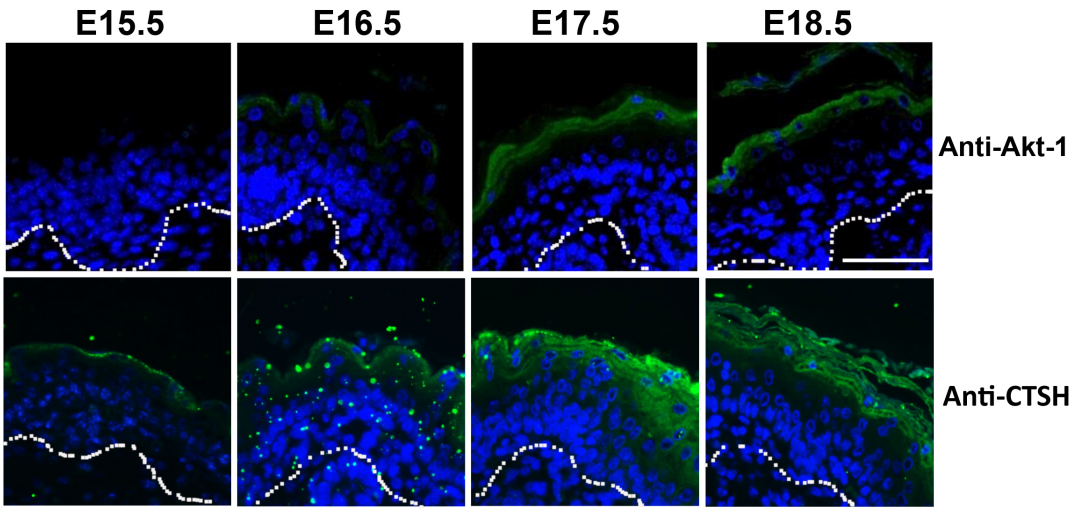

Figure 4.4 Granular Ctsh expression peaks during barrier acquision.

Immunofluorescence of mouse embryonic sections from day 15.5 (E15.5) to day 18.5 (E18.5). Both Ctsh and Akt1 staining peaks in the granular layer at E17.5 and E18.5. These are representative images from n=2.

Most cathepsins are constitutively expressed in several mammalian tissues (Brasch *et al*., 2002), while some cathepsins display cell specific expression such as cathepsin K which is expressed mainly in osteoclasts where they are involved in bone remodeling (Bromme *et al*., 1996) and cathepsin L is found to have an important role in hair follicle morphogenesis and epidermal homeostasis (Roth *et al*., 2000). Ctsh is found in high concentrations in specific tissues such as kidney, spleen, macrophages (reviewed in Guha & Padh, 2008) and in type II pneumocytes where it is critically involved in the N- terminal processing step of pro- surfactant protein C (Buhling *et al*., 2011), however there is no data about its role and expression patterns in the epidermis.

To determine Ctsh expression patterns in the skin, immunofluorescence staining for Ctsh on paraffin embedded Akt1 knock down organotypic REK sections were performed. Consistent with the western analysis, both Ctsh and filaggrin protein levels were reduced in Akt1 shRNA knock down organotypic culture models in comparison with the control (scram). Interestingly results indicated that filaggrin co-localized with Ctsh in the granular layer both in the Akt1 knockdown organotypic cultures and in normal human skin (Fig 4.6).

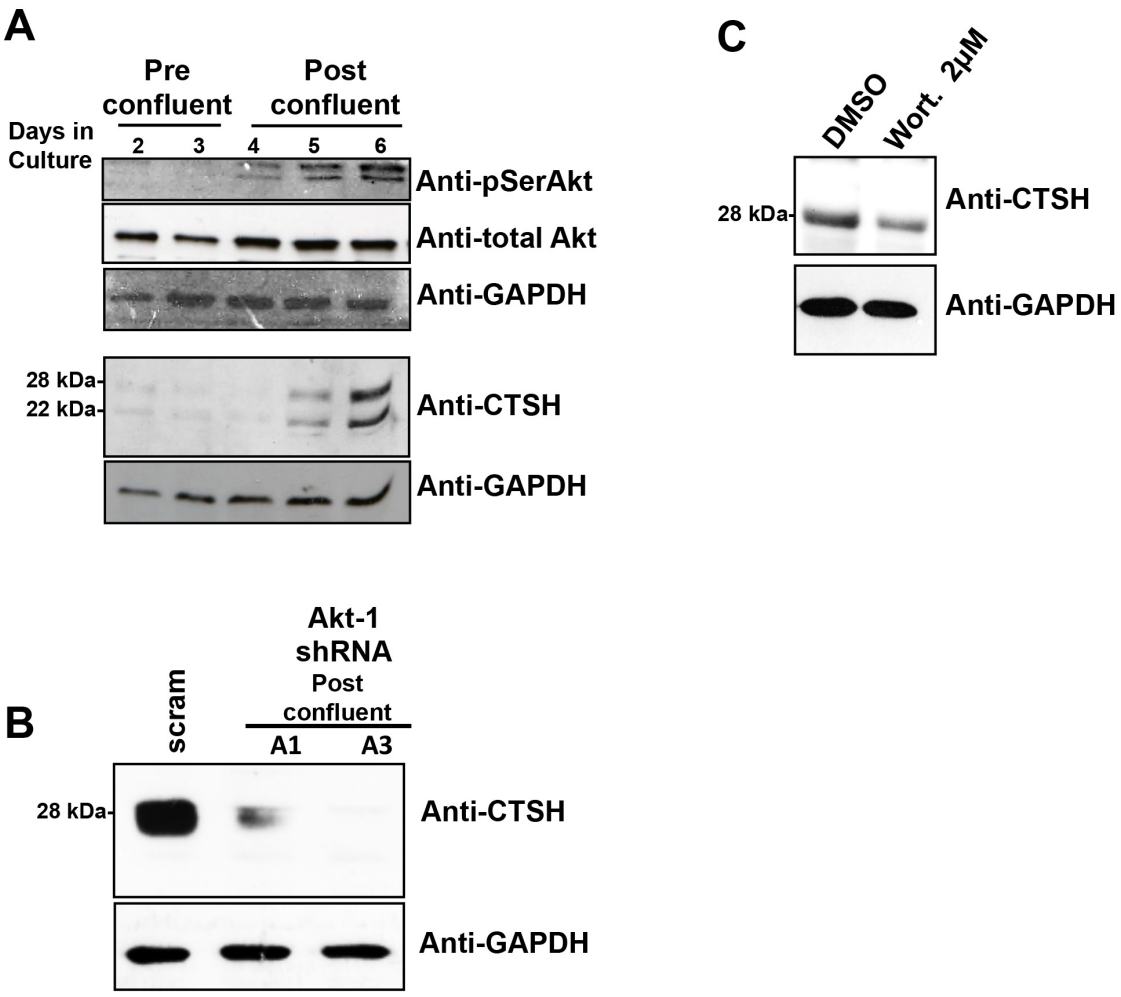

Figure 4.5 Ctsh protein is detected only in differentiated cells and is reduced in Akt-1 knockdown cells.

A. Western blot analysis of anti-pSer473Akt, anti-total Akt, anti-Ctsh and anti-GAPDH in both pre and post confluent REKs. Note now Ctsh is detected only in post-confluent day 5 and 6 lysates. The Ctsh bands detected here are the 28 kDa mature form and 22 kDa heavy chain form. B. Immunoblot showing anti-Ctsh in Akt1 shRNA expressing REKs. Only the mature 28 kDa mature Ctsh form is detected here. C. Immunoblot of anti-Ctsh and anti GAPDH in human keratinocytes (NHEKs) treated with inhibitor wortmannin at a concentration of 2μM for 24 hours. All westerns are representative images from n=2. Western blots for pSer473Akt and total Akt are also displayed in fig 3.1. Protein lysates were prepared using total lysis buffer.

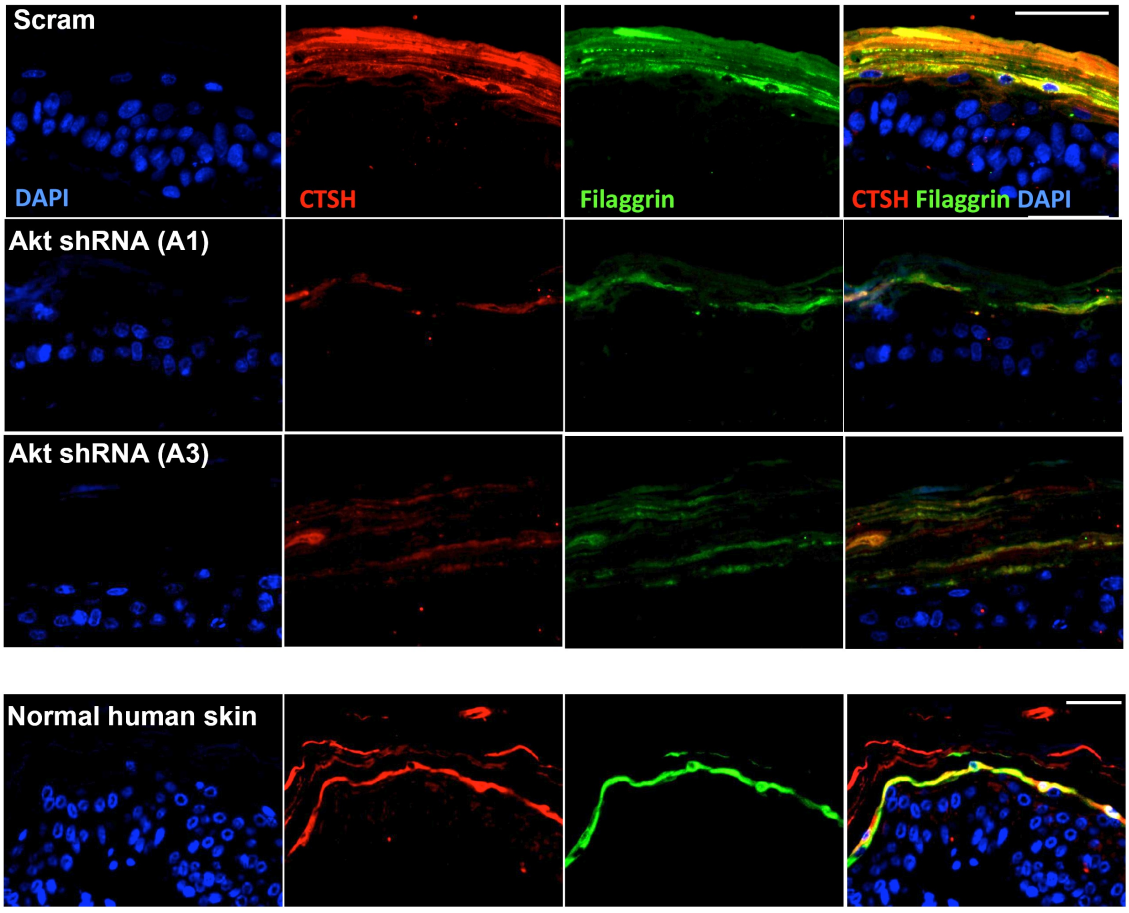

Figure 4.6 Ctsh co-localizes with filaggrin in the granular layer.

Normal human skin sections and rat epidermal keratinocyte organotypic culture expressing scrambled shRNA and 2 Akt1 shRNA expressing cell lines were co-immunostained with anti-Ctsh (red) and anti-filaggrin (green). First three rows are from REK organotypic cultures, last row represent normal human skin sections. All sections were counterstained using Dapi (blue). Scale bar, 50 μM. All images are representative images from n-3.

To further evaluate the role of Ctsh in filaggrin processing, REKs were transfected with Ctsh shRNA. Knock down of Ctsh was confirmed by western blot analysis, which showed a reduction in Ctsh protein levels in both Ctsh shRNA cell lines (Fig 4.7A). Results also showed a reduction in monomeric filaggrin and overall filaggrin levels in day 5 post confluent cells hence suggesting the protease Ctsh has a role in filaggrin processing. Interestingly, at day 6, filaggrin levels decrease is greater in Ctsh shRNA expressing cells suggesting filaggrin is getting degraded. No change in filaggrin was observed at the transcriptional level suggesting Ctsh knockdown caused changes either in the overall profilaggrin protein levels, or in proteolytic processing of profilaggrin to filaggrin (Fig 4.7B). To determine whether filaggrin levels could be rescued by increasing Ctsh levels, post confluent Akt1 shRNA knock down cells were transfected with a Ctsh expressing plasmid. Western blot analysis confirmed elevated Ctsh expression levels and an increase in overall filaggrin levels in Ctsh over-expressing cells in compared with the vector transfected cells (Fig 4.7C).

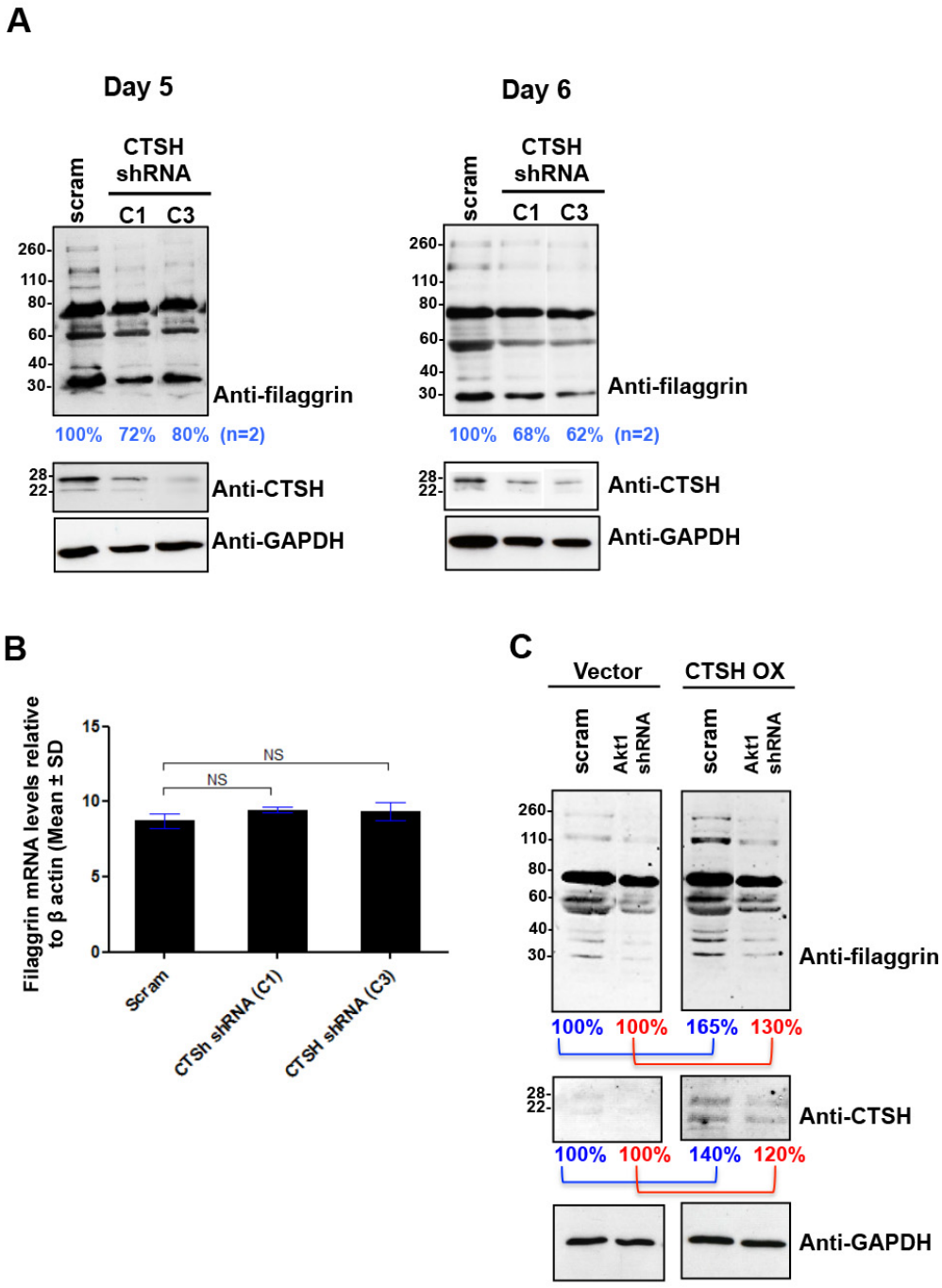

Figure 4.7 Ctsh over expression restores filaggrin protein levels in Akt-1 knock down REKs.

A.Immunnoblot of anti-filaggrin, anti-Ctsh and anti GAPDH on scram and Ctsh shRNA expressing REKs collected on days 5 and 6. B. Real Time PCR results of filaggrin mRNA expression levels in scram and both Ctsh shRNA expressing cell lines. Statistical analysis was performed using the non-parametric Mann-Whitney U-test. Significance was defined as $p<0.05$. NS = not significantly different. C. Western analysis of anti-filaggrin, anti-Ctsh and anti-GAPDH on scram and Akt1 shRNA expressing cells transfected with vector and Ctsh overexpressing plasmid. Note filaggrin increase of 65% and 30% in Ctsh overexpressed scram and Akt1 shRNA cells respectively in comparison with vector. Ctsh expression increase of 40% and 20% is also seen in scram and Akt1 shRNA expressing cells respectively in comparison with vector. All westerns are representative images from n=2. Protein lysates were prepared using total lysis buffer.

4.2.3 Aberrant profilaggrin processing and hyperkeratosis observed in both Ctsh $^{+/-}$ and Ctsh $^{-/-}$ mice.

One of the most characterized roles of Ctsh is its involvement in the processing and secretion of lung surfactant protein B (Sp-B) (Ueno *et al*., 2004; Buhling *et al*., 2011). Reinheckel and colleagues demonstrated that Ctsh knockout mice have reduced secretion of Sp-B leading to an impaired surface tension in the lungs (Buhling *et al*., 2011). Although no gross phenotype was reported in the Ctsh null mice (Buhling *et al*., 2011), closer inspection of the skin is necessary to discern whether barrier is compromised to a degree that could result in AD. Hence to understand role of Ctsh in barrier formation, isolated neonatal skin was analysed from 4 Ctsh homozygous mutant mice (–/–), 5 heterozygous mutant mice (+/–) and 2 control wild type mice (+/+). Sonication experiments on isolated cornified envelopes showed that both Ctsh$^{+/-}$ and Ctsh$^{-/-}$ cornified envelope more labile to destruction by sonication compared with wild type (Fig 4.8).

Histological analysis of paraffin embedded skin sections showed hyperkeratosis in both Ctsh$^{+/-}$ and Ctsh$^{-/-}$ in comparison with the wild type (Fig 4.9, top panels). Immunofluorescence staining with anti-Ctsh primary antibody confirmed reduction in Ctsh protein levels in the granular layer in Ctsh$^{+/-}$ and Ctsh$^{-/-}$ epidermis (Fig 4.9). Filaggrin staining in the granular layer was also reduced in both Ctsh$^{+/-}$ and Ctsh$^{-/-}$ in comparison with the wild type (Fig 4.9). Taken together these results indicated that Ctsh is important for key aspects of the physical skin barrier and that this may be mediated this by either direct or indirect involvement in filaggrin processing.

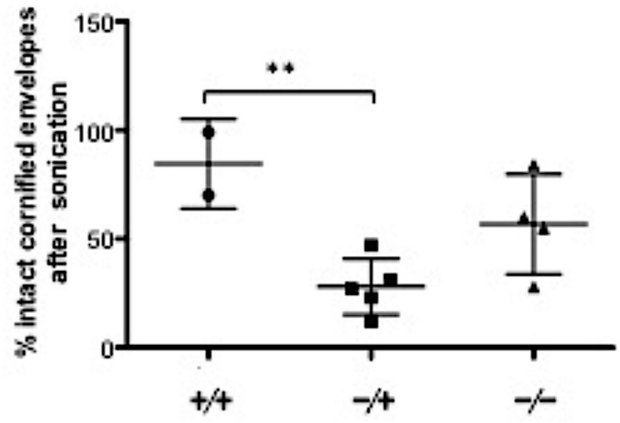

Figure 4.8 Increased cornified envelope fragility in Ctsh$^{+/-}$ mice.

*Intact cornifed cells were counted after a 5 second sonication on isolated cornified envelopes from samples Ctsh$^{+/+}$ (n=2), Ctsh$^{+/-}$ (n=5) and Ctsh$^{-/-}$ (n=4). Data is expressed as mean ± SEM. Statistical analysis was performed using the non-parametric Mann-Whitney U-test. ** $p \leq 0.01$.*

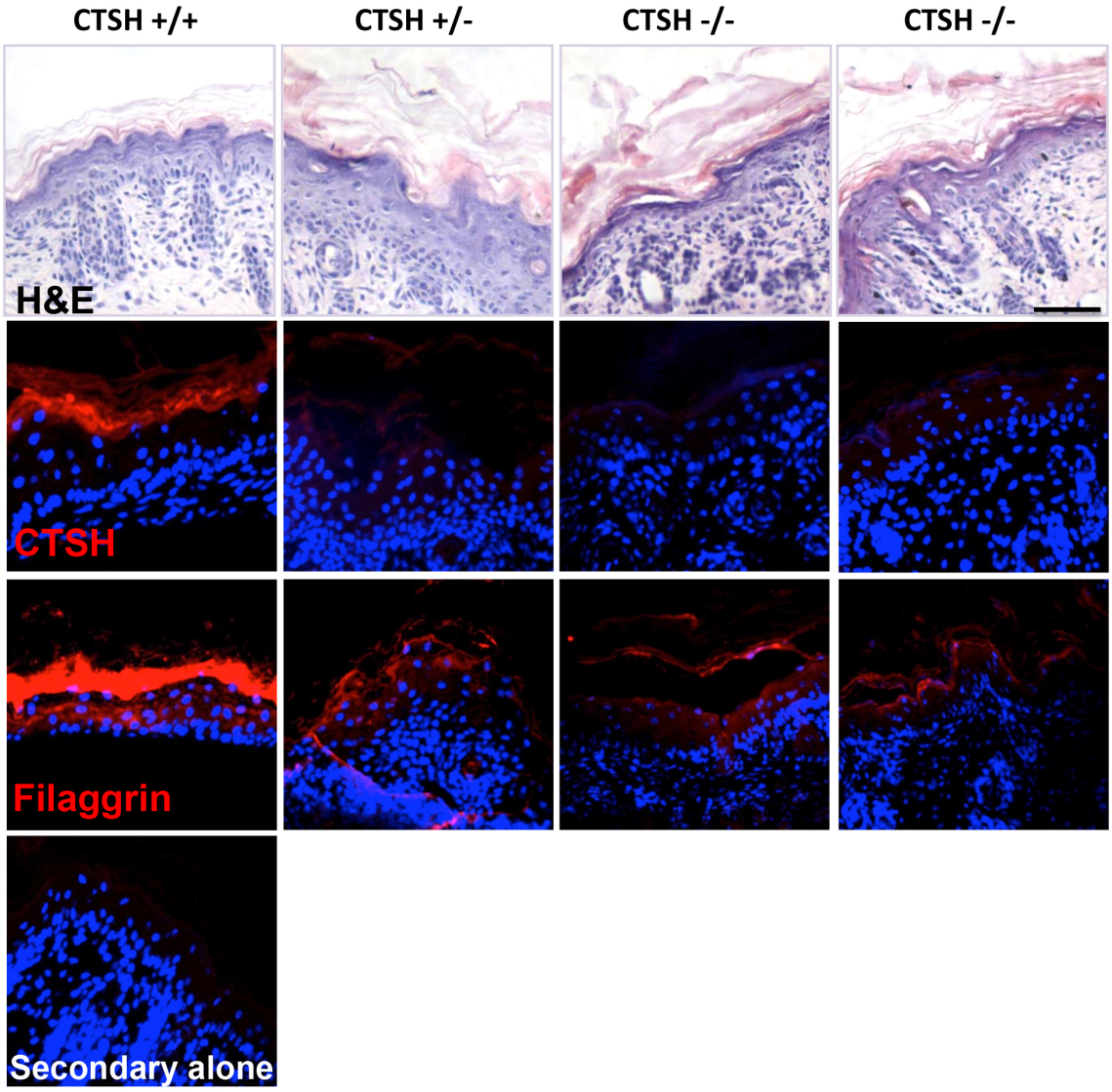

Figure 4.9 Hyperkeratosis observed in both Ctsh$^{+/-}$ and Ctsh$^{-/-}$ skin.

H&E staining (top row panels) and immunostaining of anti-Ctsh (red, 2nd row of panels), anti-filaggrin (red, 3rd row of panels) of Ctsh$^{+/+}$, Ctsh$^{+/-}$ and Ctsh$^{-/-}$. These are representative images of Ctsh$^{+/+}$ (n=2), Ctsh$^{+/-}$ (n=3) and Ctsh$^{-/-}$ (n=3). DAPI (blue) was used as a counter stain. Scale bar 50 μM.

4.2.4 Granular layer Akt phosphorylation and Ctsh reduced in AD non-affected skin

Genetic abnormalities leading to altered serine protease / serine protease inhibitor or filaggrin protein levels have led to the development of AD to be linked to a defective barrier (Walley *et al*., 2001; Smith *et al*., 2006; Fallon *et al*., 2009). One interesting example is where mutations in a serine protease inhibitor, SPINK 5 (SP inhibitor lymphoepithelial Kazal-type trypsin inhibitor), lead to Netherton syndrome (NS) where infants with NS develop severe AD (Walley *et al*., 2001; Hachem *et al*., 2006). Both *in vitro* and *in vivo* studies have identified other proteases such as Elastase 2, aspartic protease SASpase and Matriptase as being required for profilaggrin processing pathway, where a deficiency in any of these proteases lead to barrier anomalies (reviewed in De Veer *et al*., 2014).

Results reported here have so far established Ctsh as a downstream target of the Akt signaling pathway and an important epidermal protease for barrier formation. Akt knockout mouse studies and *in vitro* studies presented both here and in the literature have demonstrated the requirement of Akt in keratinocyte differentiation and barrier acquisition (Thrash *et al*., 2006; O'Shaughnessy *et al*., 2007). Furthermore reduced Akt activity resulted in hyperkeratosis and parakeratosis, 2 features commonly observed in AD (section 3.2.2), however pSerAkt expression levels have not previously been characterized in non-affected skin of AD patients. Immunofluorescence staining of non-affected skin sections (with entire skin intact) of 5 AD patients showed a reduction in granular pSerAkt activity in comparison with the normal skin (Fig 4.10A). Additionally granular layer filaggrin staining was reduced in all 5 AD patients (Fig 4.10A). Densitometric quantification showed both pSerAkt activity and filaggrin protein levels in AD non-affected skin sections were significantly reduced over all in comparison with normal skin (Fig 4.10B & C). Consistent with the Akt1 knockdown cells, granular layer CTSH expression was also decreased in all AD patients with a significant overall reduction in comparison with normal skin (Fig 4.11).

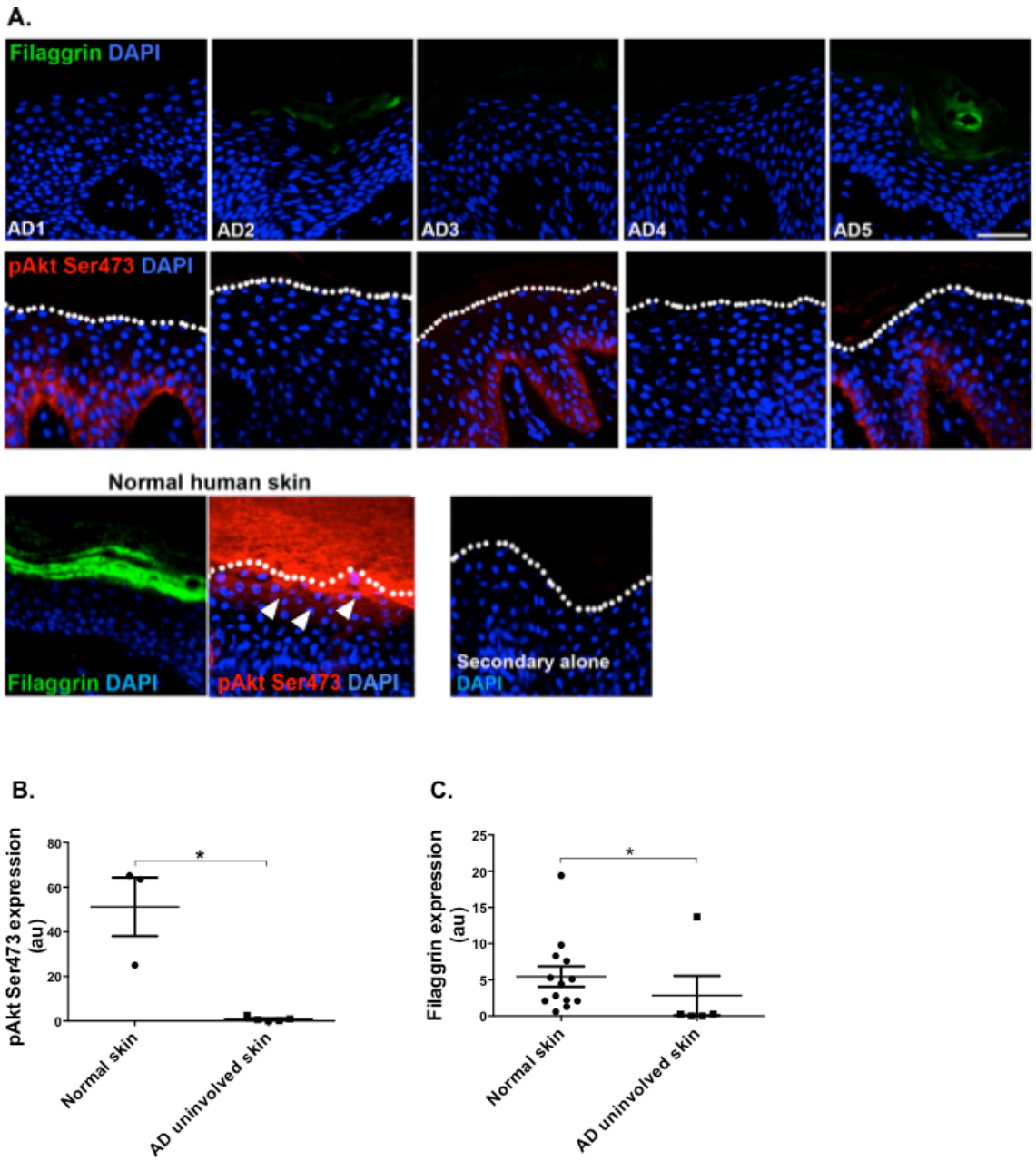

Figure 4.10 Significant reduction in both granular pSerAkt and filaggrin in AD non-affected skin.

*(A) Immunofluorescence staining for anti-filaggrin (green) and anti-pAktSer473 (red) in AD non-affected skin sections (n=5) and normal human skin (n=3). DAPI (blue) was used as a counter stain. White arrowheads denote positive granular pSerAkt staining in normal human skin. Dotted line indicates the boundary between granular layer and stratum corneum. (B & C) Densitometric quantification of immunofluorescence of pSerAkt expression and filaggrin in both normal and AD non-affected skin. Normal skin used in B are frozen sections. Normal skin used in C are paraffin embedded tissue sections. Densitometric quatification of immunofluorescence signals of both filaggrin and pAktSer473 were determined using ImageJ software. Data expressed as mean ± SEM. Statistical analysis was performed using the non-parametric Mann-Whitney U-test. Significance was defined as $p<0.05$. * $p \leq 0.05$, ** $p \leq 0.01$. au (arbitrary units). Scale bar 50 μM.*

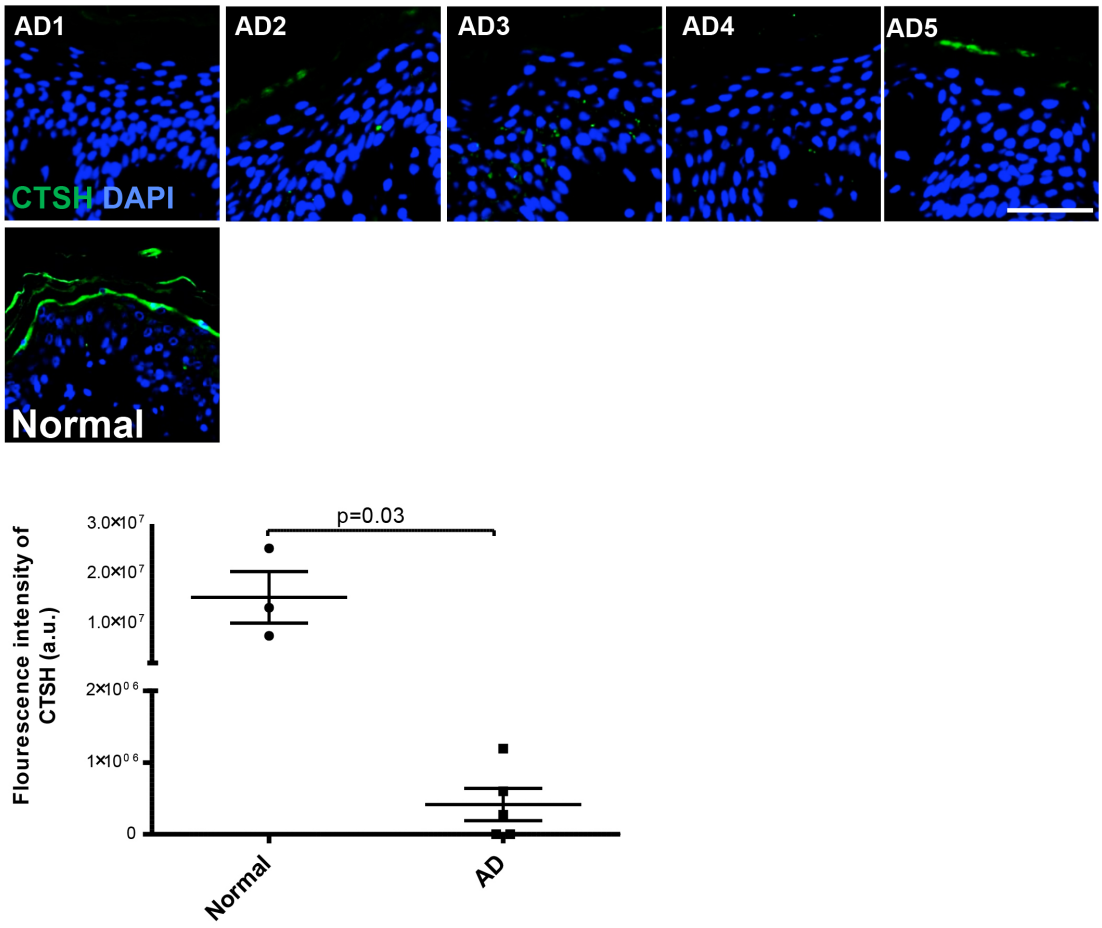

Figure 4.11 CTSH expression reduced in AD non-affected skin.

Immunofluorescence staining for anti-CTSH (green) in uninvolved AD skin (n=5) and normal skin. DAPI (blue) was used as a counter stain. Densitometry quantification of CTSH immunofluorescence signals analysis in AD skin (n = 5) in comparison with normal skin (n = 3). Densitometric quantification of immunofluorescence signals were determined using ImageJ software. Data expressed as mean ± SEM. Statistical analysis was performed using the non-parametric Mann-Whitney U-test. Significance was defined as $p<0.05$. au (arbitrary units). Scale bar 50 μM.

4.2.5 Increased RAPTOR leads to decreased filaggrin expression

The previous section showed that Akt activity is reduced in non-affected skin of a subset of AD patients. Research has shown that knock down of RAPTOR (regulatory associated protein of mTOR) increases Akt activity (Sarbassov *et al*., 2005). RAPTOR forms complexes with the regulatory kinase mTOR, which is one of the main effectors of Akt signalling (Wullschleger *et al*., 2006; O'Reilly *et al*., 2006). Sully *et al*. (2013) have demonstrated that treatment of keratinocytes with rapamycin, a drug that specifically inhibits the RAPTOR/mTORC1 complex, increases Akt activity by inhibiting the negative feedback loop that exists between mTORC1 and the receptor accessory protein insulin receptor substrate 1 (Sully *et al*., 2013).

To investigate whether reduced granular pSerAkt expression observed in AD non-affected skin could be the result of an increase in RAPTOR, RAPTOR was overexpressed in REKs using increasing doses of myc-tagged RAPTOR plasmid and the effect on Akt phosphorylation and filaggrin processing determined. Results indicated successful transfection showing increasing protein levels of both myc-tagged protein and RAPTOR. Western analysis also showed a dose dependent decrease in both Akt phosphorylation and filaggrin protein levels and potentially proteolytic processing in RAPTOR over-expressing cells (Fig 4.12).

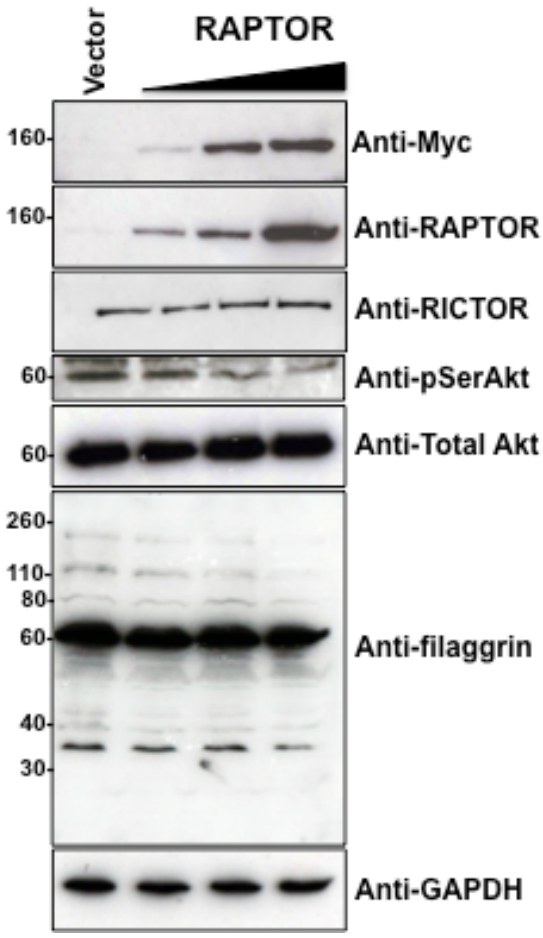

Figure 4.12 Raptor increase causes dose dependent decrease in pSerAkt and filaggrin.

Immunoblot of anti-myc, anti-RAPTOR, anti-RICTOR, anti-pSer473Akt, anti-total Akt, anti-filaggrin and anti-GAPDH on post confluent REKs transfected transiently with both vector and increasing doses of myc-tagged RAPTOR clone. All westerns are representative images from n=2. Protein lysates were prepared using total lysis buffer.

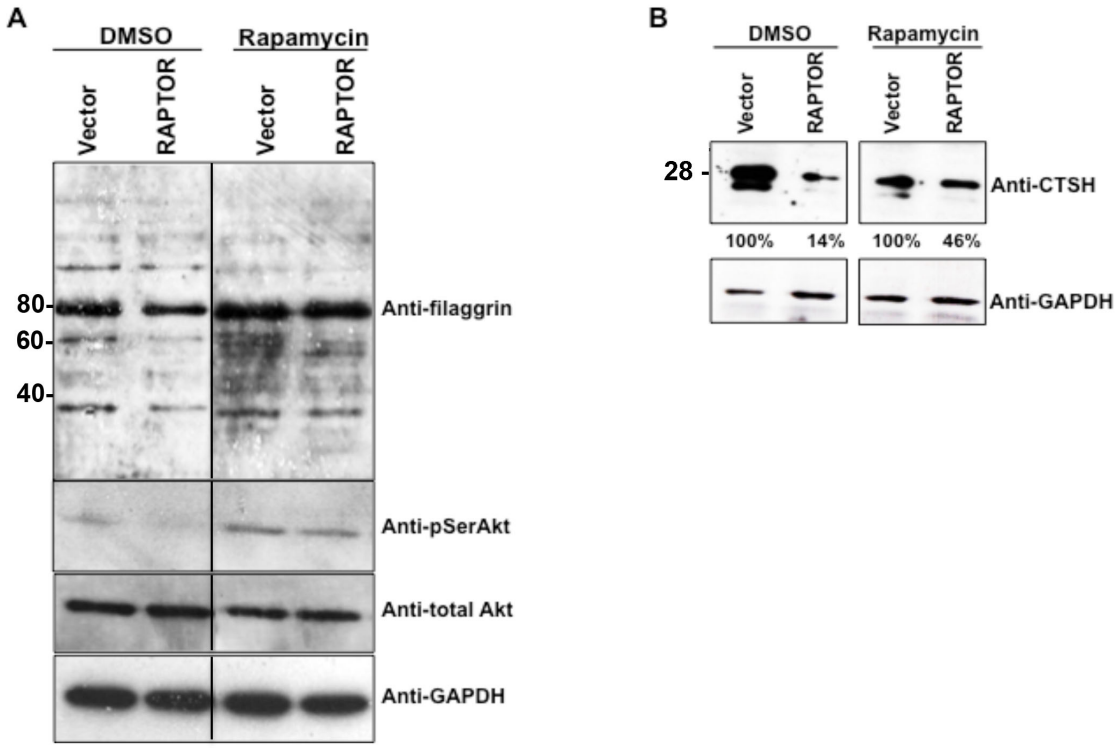

Figure 4.13 Rapamycin restores pSerAkt, Ctsh and filaggrin protein levels and processing in Raptor overexpressing cells.

Western blot analysis of post-confluent REKs transiently transfected with RAPTOR clone after treatment with 10nM Rapamycin for 2 hours. DMSO was used as a control. (A) Western analysis of anti-filaggrin, anti-pSer473Akt, anti-total Akt and anti-GAPDH. (B) Immunoblot of anti-Ctsh and anti-GAPDH. Note: Numbers indicate fold change (as a percentage) of Ctsh levels in RAPTOR overexpressed cells in comparison with vector in each treatment group (DMSO and rapamycin). All westerns are representative images from n=2. Protein lysates were prepared using total lysis buffer.

Rapamycin treatment in RAPTOR over-expressing cells restored both pSerAkt levels and filaggrin processing (Fig 4.13A). Similarly, Ctsh protein levels that were reduced in RAPTOR over expressing cells, was also increased after rapamycin treatment (Fig 4.13B). Taken together these results suggest that an increase in RAPTOR causes a decrease in Ctsh expression, filaggrin protein levels and processing. Immunofluorescence staining was performed on non-affected skin sections from AD patients and compared with normal skin sections to determine how RAPTOR expression varied with filaggrin expression. Results showed positive granular RAPTOR expression in majority of AD patients and in a subset of normal skin sections as shown in representative images in fig 4.14A, where RAPTOR positive expression is indicated by a clearly detectable signal in the suprabasal epidermis. Results also indicated that positive RAPTOR expression in AD was greater than that in normal skin. Consistent with the western analysis, positive granular RAPTOR expression was accompanied with reduced granular filaggrin expression in AD and significantly reduced in normal skin (Fig 4.14A & B). These results suggest that abnormal granular positive of RAPTOR leads to a decrease in filaggrin, and in AD RAPTOR expression is increased in comparison with normal sections.

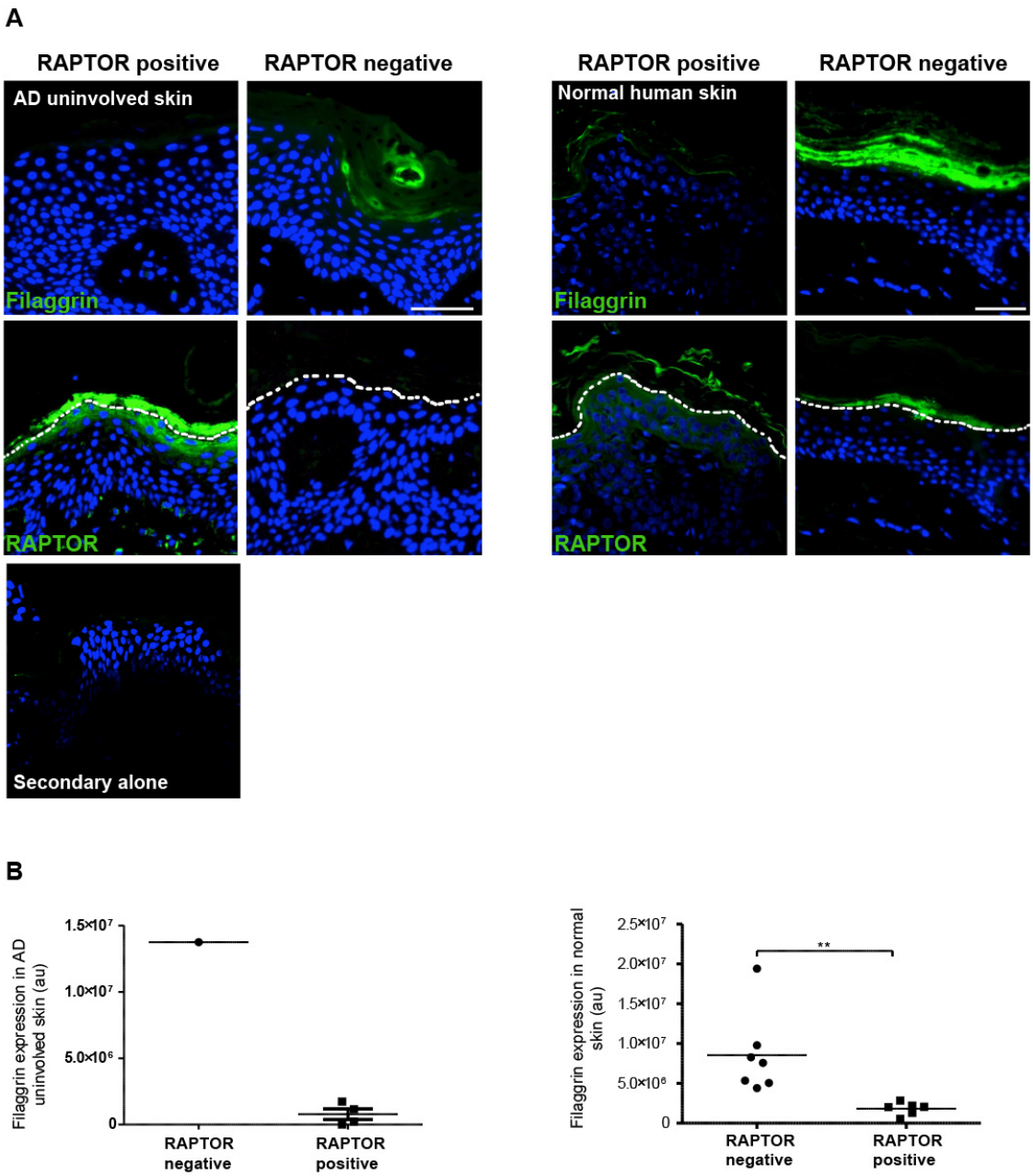

Figure 4.14 Increase in granular RAPTOR expression correlates with decrease in filaggrin.

*(A) Representative images showing immunofluorescence staining of filaggrin and RAPTOR in both AD non-affected skin (n = 5) and normal skin (n = 13). Dotted line indicates the boundary between granular layer and stratum corneum. RAPTOR positive indicate a clearly detectable signal in the suprabasal epidermis (below the dotted white line). Note how positive RAPTOR expression in suprabasal layer in both AD and normal skin are accompanied with reduced filaggrin expression. (B) Densitometric analysis of the immunofluorescence staining shows RAPTOR increase is accompanied with a significant reduction filaggrin levels in normal skin. Densitometric quatification of immunofluorescence signals were determined using ImageJ software. Data expressed as mean ± SEM. Statistical analysis was performed using the non-parametric Mann-Whitney U-test. Significance was defined as $p<0.05$. ** $p \leq 0.01$. au (arbitrary units). Scale bar 50 μM. The images of AD uninvolved skin sections with filaggrin staining are also displayed in Fig 4.10*

The Retinoid signaling pathway, which is involved in skin homeostasis, is dysregulated in skin disorders including atopic dermatitis (Gericke *et al*., 2013; Mihaly *et al*., 2011). Retinoid based therapy has been beneficial in treating skin conditions (Orfanos and Zouboulis, 1998) and has shown successful results in chronic hand eczema patients (Bissonnette *et al*., 2010). To evaluate RAPTOR expression in response to retinoic acids, REKs were treated with the retinoic acid receptor (RAR) agonist all trans retinoic acid (ATRA) and RAR antagonist AGN193109. Western analysis showed RAPTOR expression decreases with ATRA treatment, while the antagonist, AGN, increased RAPTOR protein levels (Fig 4.15). Interestingly retinoic acids have been reported to increase Ctsh expression (Flentke *et al*., 2004). Based on these results and the reported improvement in barrier following retinoid treatment (Bissonnette *et al*., 2010), it is possible the retinoids in skin are working via a mechanism where decrease in RAPTOR leads to an increase in Ctsh and filaggrin (Fig 4.16).

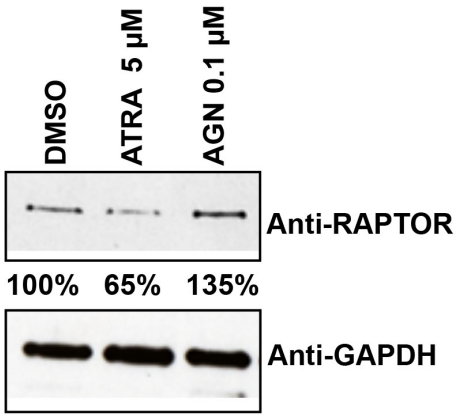

Figure 4.15 Retinoic acid treatment reduces RAPTOR expression in keratinocytes.

Western blot analysis of anti-RAPTOR and anti-GAPDH on REKs treated with the retinoic acid receptor (RAR) agonist, all-trans retinoic acid (ATRA) and RAR antagonist AGN193109. Numbers indicate fold change (as a percentage) in RAPTOR expression in comparison with control (DMSO).

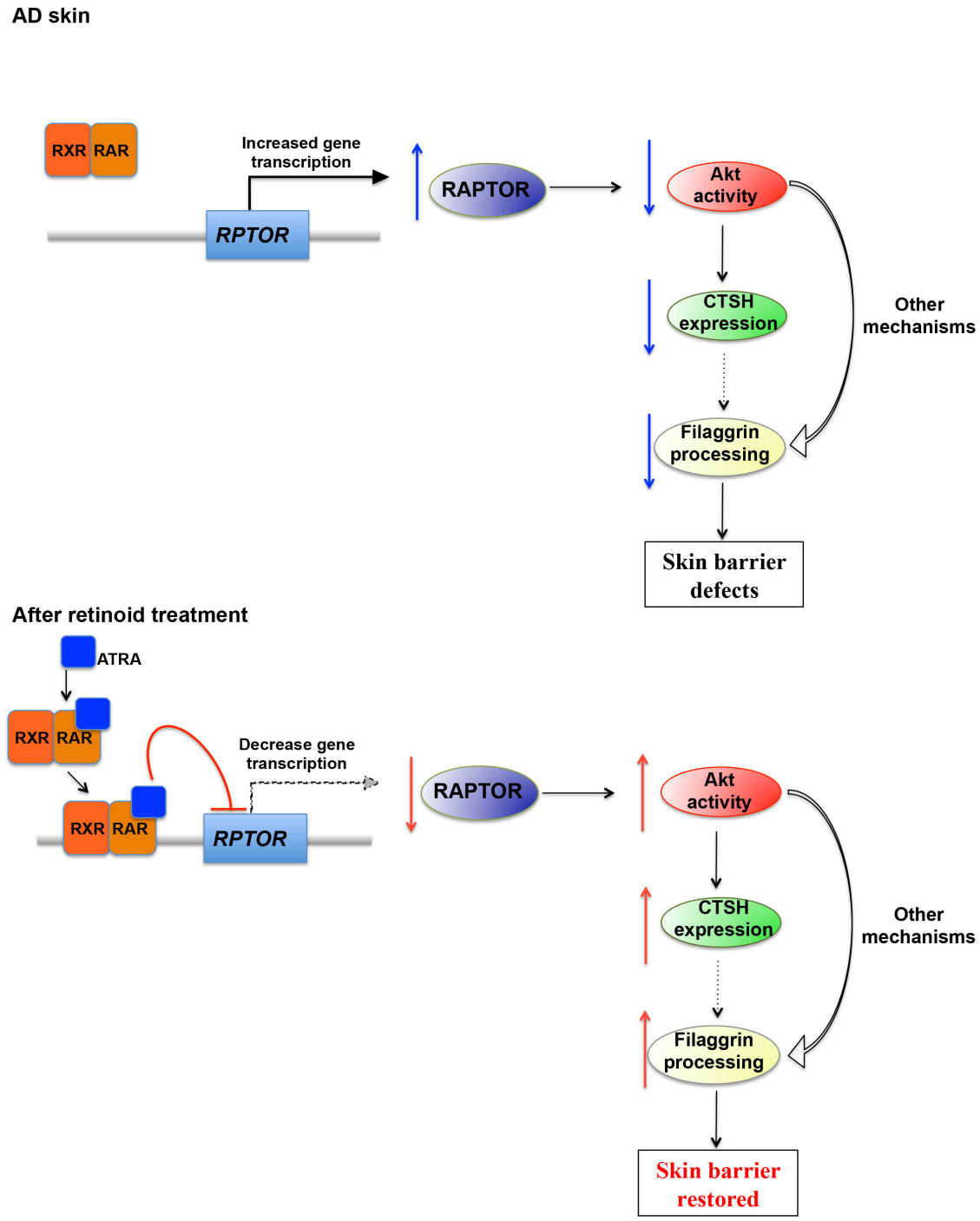

Figure 4.16 Model of a possible mechanism of how retinoic acid treatment can increase both Ctsh and filaggrin expression, and restore barrier defects.

4.3 Discussion

One of the most compelling arguments in support of barrier defect being a prerequisite for AD, comes from research into the function of the cornified envelope protein profilaggrin and how mutations in this gene have a profound association with AD (Presland and Dale, 2000; Smith *et al*., 2006; Brown *et al*., 2009). Since about 25 % of AD patients have been found to not have filaggrin mutations (reviewed in O'Regan *et al*., 2008) it is possible the observed barrier defect in AD can rise from misprocessing of profilaggrin to its functional subunit filaggrin. Akt null mice models display hyperkeratosis, have reduced cornified envelope strength and reduced filaggrin expression (Thrash *et al*., 2006; O'Shaughnessy *et al*., 2007), and over-activation of Akt results in hyperkeratosis and altered filaggrin expression demonstrating loss of Akt1 activity and hyperactive Akt1 both cause hyperkeratosis. The data presented here proposes Ctsh as a novel epidermal protease, which is required for filaggrin processing and epidermal barrier formation, and that in the skin, RAPTOR regulates Ctsh expression and filaggrin processing in an Akt1 mediated manner.

Microarray analysis of Akt1 knock down cells revealed Ctsh as the most differentially regulated gene with a down-regulation of over 4 fold. Cathepsin H belongs to the papain- like family of cysteine proteases consisting of 11 members in humans with orthologous counterparts present in mice (Puzer *et al*., 2004; Hagemann *et al*., 2004). Though Ctsh is expressed ubiquitously and involved in bulk protein degradation, it does display cell specific functions such as its role in the processing and secretions of pro surfactant protein C in type II pneumocytes (Brasch *et al*., 2002; Buhling *et al*., 2011). Ctsh deficient mice have reduced lung surfactant, which may interfere with breathing mechanisms causing respiratory complications (Buhling *et al*., 2011), and furthermore reduced Ctsh mRNA is reduced in airway smooth muscle cells in asthmatic individuals (Faiz *et al*., 2013). In the data presented here, a reduction in Ctsh in both Akt1 knock down cells and in cells treated with the PI3 kinase pharmacological inhibitor wortmannin demonstrates that Ctsh expression in keratinocytes is regulated by Akt signaling.

Immunofluorescence staining of the mouse embryo sections show Ctsh increase during the barrier acquisition stage at E17.5 and E18.5 suggesting Ctsh might be involved in late keratinocyte differentiation. Furthermore, Ctsh protein levels were only

detected in post-confluent differentiated cells and it co-localized with filaggrin in the upper granular layer. The overall reduction in filaggrin processing in day 5 Ctsh shRNA expressing cells and increase in overall filaggrin products upon over-expression of Ctsh in Akt1 knockdown cells is strong evidence that Ctsh is required for filaggrin processing. Ctsh functions predominantly as an aminopeptidase but may have limited substrate specific endo peptidase activity (Dodt & Reichwein, 2003), hence it may be directly involved in filaggrin processing, or it may be degrading an inhibitor and allowing another protease to process filaggrin. Interestingly there was a greater reduction in filaggrin intermediate bands and monomeric bands at day 6 suggesting filaggrin is getting degraded or proteolytically processed more rapidly in Ctsh shRNA knock down cells than the control. During the processing of profilaggrin to filaggrin, the large profilaggrin protein (> 400 kDa) is cleaved by numerous proteases to first yield the N- terminus and poly-filaggrin sequence, the latter is further processed to give different sized filaggrin intermediates which are eventually cleaved to give the monomeric filaggrin subunit (Pearton et al., 2002). Filaggrin is then degraded, a process involving Caspase-14, to release free amino acids that partially contribute to the production of natural moisturizing factors in stratum corneum (Harding *et al*., 2002; Rawlings and Matts, 2005). The proteolytic cascade in epidermis is executed by a careful balance between proteases and their inhibitors and is emerging as an important regulator of the keratinocyte differentiation program (reviewed in De Veer *et al*., 2014). An imbalance in the proteolytic cascade can have detrimental consequences such as reduction in proteases Elastase 2 or Matriptase (reviewed in De Veer *et al*., 2014) leading to reduced filaggrin processing and barrier defects, or as in the case of Netherton Syndrome where a reduction in the protease inhibitor LEKTI (lympho-epithelial Kazal type inhibitor) leads to excessive activity of serine protease causing increased degradation of epidermal constituents such as lipid processing enzymes and desmosomal proteins and increased filaggrin processing resulting in a thin and barrier-compromised stratum corneum (Hachem *et al*., 2006).

Ctsh deficient mice were hyperkeratotic and had reduced filaggrin expression. It was interesting that Ctsh heterozygous mice were more barrier compromised that the Ctsh knockout mice. It has been reported that frequency of $Ctsh^{-/-}$ mouse resulting from heterozygous mating is reduced resulting in dead newborns (Buhling *et al*., 2011). Although the cause was not determined, it is possible the $Ctsh^{-/-}$ skin phenotype is more severe hence prompting compensatory mechanisms resulting in the surviving mice to

have a milder phenotype than the heterozygous littermates. Reduction in Akt activity resulting in reduced filaggrin processing and barrier function has previously been demonstrated (Thrash *et al*., 2006; O'Shaughnessy *et al*., 2007). The data presented here shows for the first time that granular layer Akt phosphorylation is significantly reduced in AD uninvolved skin. Furthermore CTSH and filaggrin were also significantly reduced reinforcing the rationale that reduction in Akt phosphorylation reduces expression of its downstream target CTSH leading to a reduction in filaggrin levels and proteolytic processing.

RAPTOR, a regulatory component of the mTORC1 complex, is reported to modulate Akt activity (Sarbassov *et al*., 2005; Sully *et al*., 2013). In fact, over expression of RAPTOR in REKs decreased Akt phosphorylation, Ctsh expression and filaggrin processing. Furthermore, rapamycin treatment in RAPTOR overexpressing REKs caused an increase in Akt phosphorylation, Ctsh expression and mature filaggrin levels almost to those of the control. Rapamycin is a widely used immune suppressant and a dosage of 10 nM is currently used to maintain organ transplantation in children (Schachter *et al*., 2006; Goyal *et al*., 2013). Data presented here thus indicates that, at the same low dosage of 10 nM, rapamycin treatment is sufficient to restore Akt phosphorylation, Ctsh expression and correct the filaggrin processing defect. Taken together, these data suggest that Akt activity in keratinocytes can be modulated by RAPTOR, therefore RAPTOR should be increased in AD patients. Interestingly data indicates that high levels of epidermal RAPTOR expression are more common in AD than in normal population ($p \leq 0.01$, two-sided fisher exact test). Retinoids have been successfully used in treating skin conditions including Eczema. Results show that RAPTOR gene responds to retinoic acid treatment by decreasing its expression. It is possible that RAPTOR over-expression is caused by a SNP, potentially very weakly associated with AD, and barrier correction caused by retinoic acid treatment might be via a mechanism where retinoid-mediated decrease in RAPTOR is restoring both Ctsh expression and filaggrin processing.

In conclusion, these findings identify CTSH as a novel epidermal protease, and demonstrate that in AD an abnormal increase in RAPTOR reduces filaggrin processing via CTSH in an Akt1-mediated manner. Most importantly these data suggest that in those subset of patients without a filaggrin mutation, rapamycin treatment may be able

to restore the barrier defect by rescuing filaggrin protein levels and normal filaggrin proteolytic processing.

Chapter 5 : Reduced Akt activity alters Lamin A/C degradation resulting in skin barrier abnormalities

5.1 Introduction

The skin barrier functions as both a structurally and immunologically competent organ that is absolutely essential for an adult to survive the terrestrial environment (Proksch *et al*., 2008). Skin barrier competence is achieved by various intricate regulatory mechanisms involving multiple components; Notch and Wnt signaling pathway have been reported to regulate keratinocyte growth and commitment to differentiation (Fuchs & Raghavan, 2002), Protein kinase C and Rho/ROCK signaling pathway has been shown to regulate keratinocyte differentiation (Dotto, 1999; McMullan *et al*, 2003), and studies including the research conducted on p63-/- mice, where they are reported to have single layer of surface epithelium, have shown that p63 is required for keratinocyte stratification (Koster *et al*, 2002; Yang *et al*, 1999). Previous chapters have demonstrated the importance of the Akt signaling pathway in skin barrier formation. Apart from evidence of filaggrin misprocessing, the Akt1 shRNA expressing organotypic culture model displayed hyperkeratosis and parakeratosis, which are characteristic features of AD skin (Leung, 1995; Leung & Bieber, 2003). Parakeratosis, defined by nuclear retention in cornified layer, is often observed in AD and other inflammatory disorders such as psoriasis (Guttman-Yassky *et al*, 2011; Sakurai *et al*, 2002).

During stratum corneum formation, major changes of intracellular organization occur eventually resulting in the establishment of corneocyte that is reinforced by both lipid and protein components (Hohl *et al*, 1991; Rawlings & Harding, 2004; Steinert & Marekov, 1995). A key step of this cornification process is nuclear degradation although the processes governing denucleation still remain elusive (Presland *et al*., 1992; Lippens *et al*., 2005). Nuclear degradation during cornification is similar to apoptosis but is thought to involve different mechanisms (Takahashi *et al*., 2000). For example, p53 has important roles in inducing apoptosis, however p53-deficient mice do not display impaired epidermal formation (Li *et al*., 1998). Additionally UV induced cell death in keratinocytes is marked by DNA cleavage caused by an apoptotic DNase called Caspase-activated DNase (CAD), however CAD-deficient mice did not display nuclear degradation defects during keratinocyte differentiation (Kawane *et al*., 2003). In

the process of cornification nuclei are completely degraded with no nuclear fragments detected in corneocytes, where as in apoptosis DNA fragments are visible in apoptotic bodies (Lippens *et al*., 2000). Furthermore the pro-apoptotic caspases-1,6 and 7 remain unprocessed and are not activated during epidermal differentiation (Lippens *et al*., 2000) hence suggesting the mechanisms of nuclear degradation in keratinocyte differentiation is indeed different from pathways executed in apoptosis. Desquamin, a lectin-like glycoprotein that possess both trypsin-like serine protease and ribonuclease activity expressed in the upper granular layer, has been proposed to be involved in desquamation although its precise role remains to be established (Chen *et al*., 2000). *In vitro* experiments on human keratinocytes showed that this protease is capable of degrading nuclear matrix components in isolated nuclei leaving the nuclear inclusions intact (Selvanayagam *et al*., 1998; Chen *et al*., 2000). A study by Fischer *et al* identified the epidermal specific endonuclease, DNase-1-Like-2 (DNaseIL2), as degrading DNA during cornification (Fischer *et al*., 2007; Jager *et al*., 2007; Fischer *et al*., 2011).

In this chapter, we explored the hypothesis that a downstream effector of the Akt1 signaling pathway is involved in nuclear degradation during keratinocyte terminal differentiation, and reduction of Akt1 activity disrupts nuclear degradation, potentially disturbing barrier function. To elucidate potential downstream targets, we decided to profile differentially expressed proteins in the Akt1 shRNA expressing cells and carry out functional analysis on potential candidates to gain insight into the mechanisms involved in nuclear destruction during cornification.

5.2 Results

5.2.1 Parakeratosis is observed in AD uninvolved skin and the Akt-1 shRNA organotypic culture model

Parakeratosis has been previously reported in inflammatory skin disorders including atopic dermatitis (Sakurai *et al*, 2002). Consistent with these reports, DAPI staining of uninvolved AD patient skin sections indicated retained nuclei (white arrowheads) in the stratum corneum in comparison with the normal human skin (Fig 5.1, upper panels). Immunofluoresence indicated that granular layer pSerAkt activity was reduced in the AD uninvolved skin sections in comparison with the normal skin (Fig 5.1, lower panels).

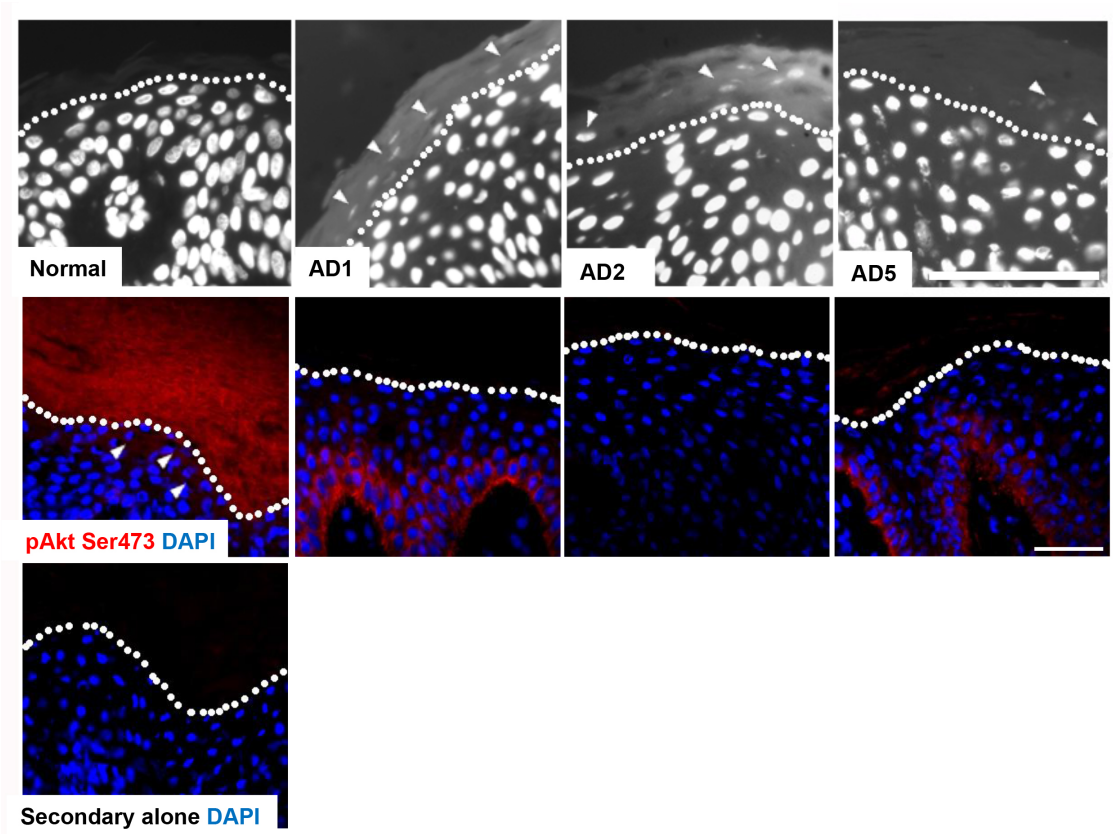

Figure 5.1 AD uninvolved skin is parakeratotic and displays reduced granular layer Akt phosphorlyation.

Upper panels: White arrows highlight the retained nuclei in the cornified layer of unaffected skin sections from three AD patients. The dotted line indicates the boundary between granular and cornified layer. Lower panels: granular Akt activity was reduced in the AD uninvolved skin sections in comparison with the normal skin. White arrows indicate the positive pAkt Ser473 staining detected in the suprabasal epidermis. Scale bar, 50 μM. The images of AD uninvolved skin sections with pSerAkt staining are also displayed in Fig 4.10

Similar to AD uninvolved skin, parakeratosis was also observed in the shRNA Akt1 knockdown cultures while the scrambled shRNA expressing controls appeared similar to normal skin (Fig 5.2 A). Previous studies have shown DNA degradation in the upper stratum granulosum to be mediated by DNase1L2 (Fischer *et al*., 2005; Jager *et al*., 2007; Fischer *et al*., 2011). Fischer and colleagues demonstrated by knockdown of DNase1L2 in a human skin equivalent *in vitro* model, that DNA degradation was reduced, leading to parakeratotic regions in the stratum corneum. However no change in protein expression levels of DNase 1L2 were observed in the both Akt1 shRNA expressing cell lines and in Akt-inhibited cells when compared to the controls (Fig 5.2 B). This suggests that the parakeratosis observed in our *in vitro* AD model was not due to altered levels of DNase1L2.

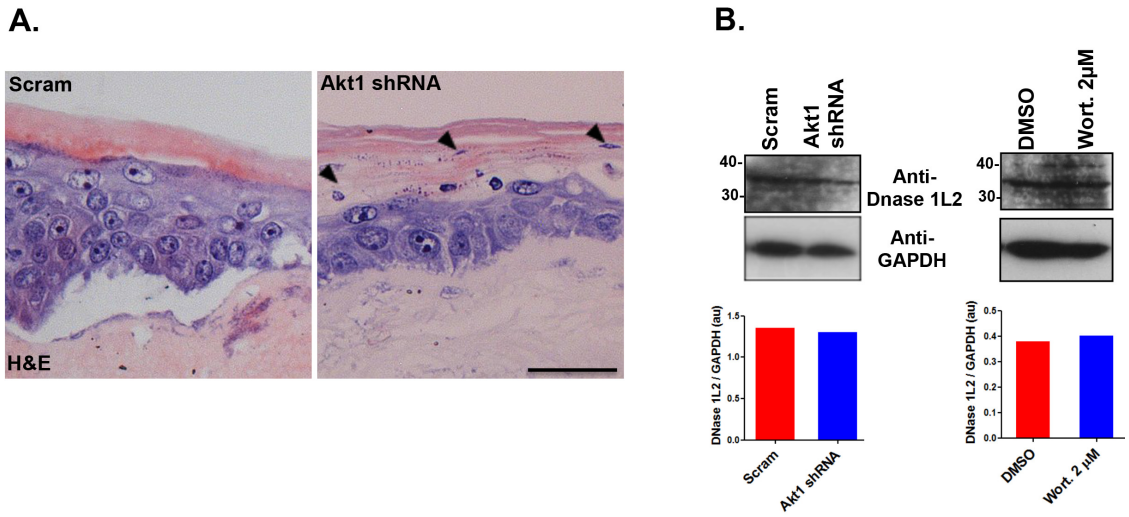

Figure 5.2 Parakeratosis in the Akt1 shRNA expressing culture model.

(A) H & E of Akt1 shRNA expressing organotypic cultures. Parakeratosis was observed. (B) Western blot of keratinocyte lysates for DNase1L2. Expression levels remained unchanged in both the Akt1 knock down and Akt inhibited cells in comparison with the controls. DNase1L2 protein expression levels relative to GAPDH were determined by densitometry using ImageJ software. Scale bar, 50 μM. au (arbitrary units). Westerns are representative images from n=2. Protein lysates were prepared using total lysis buffer.

5.3.2 Increased Lamin A/C expression in Akt-1 siRNA expressing cells

In order to gain insight into other potential mechanisms of nuclear degradation during cornified envelope formation, we set out to identify differentially expressed proteins as a means to determine possible downstream targets of the Akt1 signaling pathway that are potentially involved in nuclear degradation. To profile differentially expressed proteins in Akt1 shRNA expressing cells, equal amounts of total protein lysate of scrambled controls and Akt1 kd cells were separated on an SDS-PAGE gel, stained with colloidal coomassie blue which revealed a highly expressed 60 kDa band (Fig 5.3). This band was excised, de-stained overnight in distilled water and in-gel tryptic protein identification by mass spectrometry was performed (Biological Mass Spectrometry Centre, ICH, UCL).

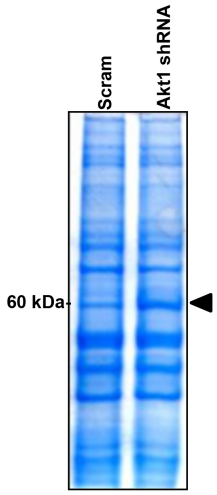

Figure 5.3 Coomassie stained gel showing the differentially expressed proteins in the Akt1 shRNA expressing cells at 60 – 80 kDa range.

Black arrow marks the differentially expressed band that was excised and sent for mass spectrometry analysis.

Table 5-1 lists the proteins within the target molecular weight range identified by ESI QTOF MS. Some of the identified proteins represent good candidates for further study; for example it has been reported that inhibition of Hsp90 prevented expression of keratin-1 and keratin-10 at both transcription and protein level, while no effect was observed on filaggrin (Miyoshi *et al.*, 2012). However, in view of the fact that retained nuclei was one of the AD phenotypic characteristic observed in our organotypic model, and since prelamin is a nuclear lamin protein (Dechat *et al.*, 2010; Lehner *et al.*, 1986), we decided to focus further investigations on this protein and determine its role in skin barrier formation.

Table 5-1 Proteins identified by ESI QTOF MS/MS

Accesion	Protein name	MW (kDa)	p*I* (pH)	PLGS score	Coverage %	Function
D4A4S3	Heat Shock Protein 70	67.1	5.47	4528.4	53.61	chaperone, stress response protein, protein folding and localization
LMNA_RAT	Prelamin A	74.2	6.55	359.66	30.67	structural protein, nucleus organization, transcription regulation
Q6AYI1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	69.1	9.1	342.13	26.83	RNA helicase, transcription regulation
K2C6A	Keratin 2	61.7	7.9	167.69	45	Structural protein, cytoskeletal
Q6IMZ3	Annexin	75.7	5.22	102	19.5	Calcium binding protein

*Table shows proteins identified by ESI QTOF MS/MS. Identified proteins are given a UniProt accession number. Coverage % indicates the percentage of peptides residues identified in each protein sequence. Reported PLGS score is -10*log (P), where P is the probability that the observed match between experimental and database sequence is a random event. Scores > 46 indicate protein identity at $p < 0.05$.*

5.3.3 Reduced Akt activity decreases Lamin A/C degradation and causes lamin A/C expression around retained nuclei in the cornified layer

Lamin A/C are type A lamins coded by a single gene *LMNA* that yields prelamin A and lamin C through alternate splicing. Prelamin is both farnesylated and cleaved to yield the mature lamin A (Lehner *et al.*, 1986). These A type lamins once translated are immediately transported into the nucleus where they are incorporated into the inner nuclear membrane (Dechat *et al.*, 2010; Lehner *et al.*, 1986). During mitosis, the complex nuclear lamin protofilaments form a dynamic structure that disassembles and reassembles following posttranslational modification (Ito *et al.*, 2007; Thompson *et al.*, 1997). During apoptosis however, the nuclear lamin is proteolytically degraded, which opens up the nucleus, subsequently leading to nuclear disintegration (Lazebnik *et al.*, 1995; Rao *et al.*, 1996). It has also been reported that Akt mediated lamin phosphorylation is required prior to lamin degradation (Cenni *et al.*, 2008).

Since terminal differentiation of keratinocytes has apoptosis-like events including the destruction of the nucleus, lamin degradation during keratinocyte differentiation was investigated by immunoblot and immunofluorescence in rat epidermal keratinocytes. In

the time course experiment of rat epidermal keratinocytes cultured over 6 days to post confluence, prelamin A (74 kDa), lamin A (72 kDa) and lamin C (70 kDa) protein expression was increased in cells in post confluent cultures coinciding with increase in pAkt activity and the differentiation marker keratin-1 (Fig 5.4 A). An increase in the lamin degradation products at 45 kDa and 28 kDa is also observed suggesting that lamin degradation may be associated with keratinocyte differentiation. Western analysis of post-confluent day 5 cells of Akt1 siRNA expressing cells and wortmannin treated pSerAkt reduced cells, revealed a reduction in the lamin degradation products (Fig 5.4 B). These findings are consistent with reported results that Akt mediated phosphorylation is a prerequisite for lamin degradation to proceed (Cenni *et al.*, 2008). To evaluate whether this reduced degradation results in altered lamin A/C expression patterns in the skin, immunofluorescence staining for lamin A/C on paraffin embedded Akt1 knock down organotypic culture sections were performed. On examination of the parakeratotic regions in the AD *in vitro* model, lamin A/C staining can be seen surrounding the retained nuclei, while the lamin A/C staining in scrambled was confined to the granular layer (Fig 5.4 C). Lamin A/C degradation was also reduced dose dependently in RAPTOR overexpressing REKs (Fig 5.4 D).

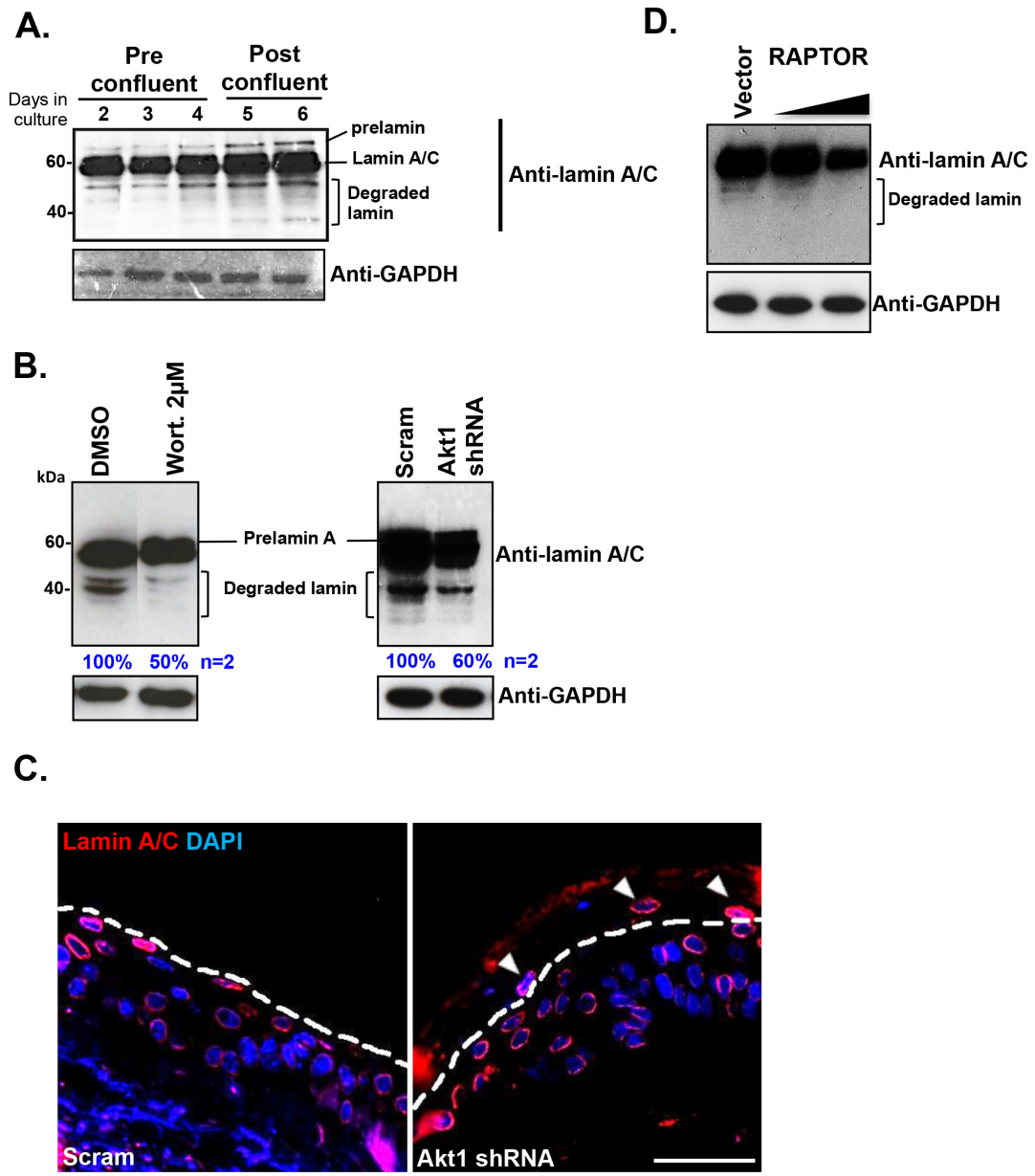

Figure 5.4 Lamin A/C expression is retained in the cornified layer and degradation was reduced when in Akt1 shRNA knockdown cultures

(A) Immunoblot of anti Lamin A/C and anti-GAPDH in both pre and post confluent rat epidermal keratinocyte cultures. Note the increase in prelamin, lamin A/C and the degraded products in days 5 and 6 post confluent cultures. (B) Immunoblot of prelamin and lamin A/C in both 2 μM wortmannin treated REKs and Akt1 shRNA expressing cells. Note the decrease in Lamin A/C degradation in Akt1 reduced samples in comparison with the controls. (C) Positive lamin staining in the parakeratotic regions in the Akt1 shRNA expressing organotypic culture model. White arrows highlight the retained nuclei with peripheral lamin A/C staining in the cornified layer. (D) Lamin degradation reduced in a dose dependent manner in RAPTOR overexpressing REKs. Dapi (blue) was used as a counter stain. The dotted line indicates the boundary between granular and cornified layer. Scale bar, 50 μM. All westerns and immunofluorescence images are representative images from n=2. Protein lysates were prepared using total lysis buffer.

To further examine the role of lamin A/C physiology in terminally differentiating cells, Akt1 shRNA expressing cells were grown to post confluence on coverslips, co-immunostained with the late keratinocyte differentiation markers Loricrin and lamin A/C, and analysed by confocal microscopy. Lamin A/C staining in the terminally differentiating cells, as indicated by positive loricrin staining, were significantly higher in the two biological replicates of Akt1 shRNA expressing cells when compared to the normal. Results also showed a significant reduction in loricrin protein levels in the Akt1 knock down cells in comparison with the normal (Fig 5.5A & B). This suggests in addition to the morphological changes and filaggrin misprocessing, reduced Akt activity may have an impact on other keratinocyte differentiation markers. Interestingly the nuclei volume in terminally differentiating cells was significantly smaller in both Akt1 shRNA expressing cell lines in comparison with the normal (Fig 5.6). Although the mechanisms governing nuclear volume changes remain unclear there is strong correlation between nuclear volume and RNA transcription suggesting a reduction in transcriptional activities in Akt1 shRNA expressing cells (Schmidt & Schibler, 1995; reviewed in Webster *et al*., 2009)

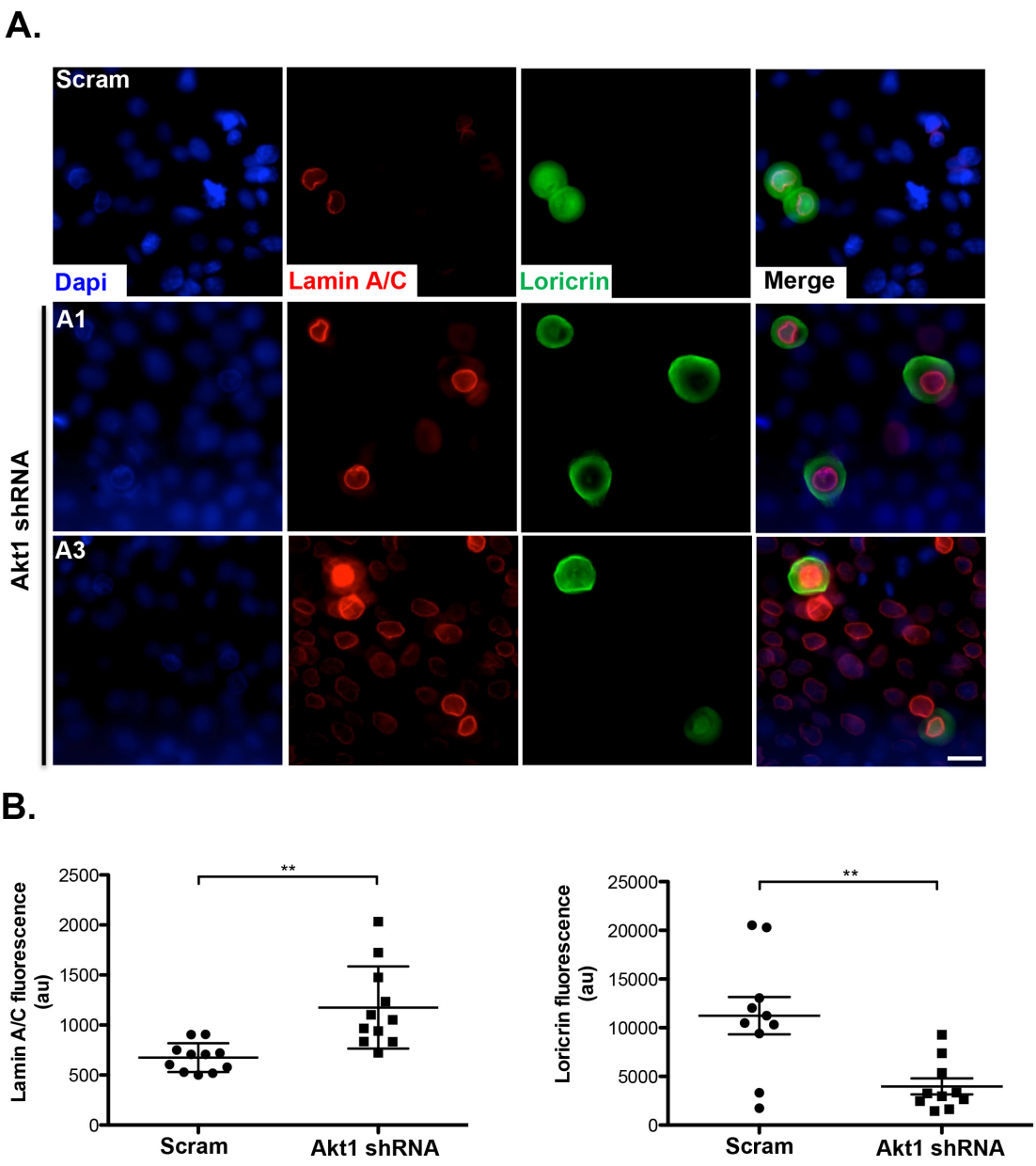

Figure 5.5 Reduced Akt activity increases lamin A/C protein levels in loricrin positive keratinocytes.

*(A) Immunolocalization studies of control and Akt1 shRNA expressing REKs using anti-loricrin and anti-lamin A/C antibodies. Note the elevated lamin A/C staining (red) in loricrin positive cells (green) in both biological replicates of Akt1 shRNA expressing cells (A1 & A3) in comparison with the control (scram). Images were taken by confocal microscopy. Dapi (blue) was used as a counter stain. Scale bar, 10 μM. (B) Scattergrams showing densitometry of both lamin A/C and loricrin immunofluorescence. Lamin A/C staining in loricrin positive Akt1 shRNA expressing cells (n=11) were significantly higher (p = 0.002) in comparison with the control. Loricrin protein expression was significantly lower (p = 0.003) in Akt1 shRNA expressing cells than the control (n=11). Densitometric quatification of immunofluorescence signals were determined using ImageJ software. Data expressed as mean ± SEM calculated from 11 different fields of view. Statistical analysis was performed using the non-parametric Mann-Whitney U-test. Significance was defined as $p<0.05$. ** $p \leq 0.01$. au (arbitrary units)*

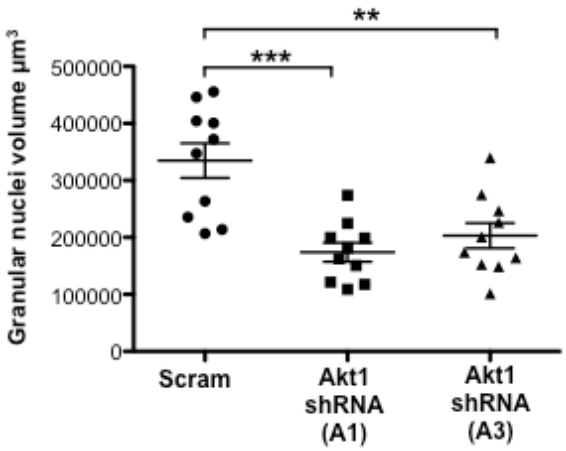

Figure 5.6 Granular nuclei volume significantly reduced in Akt1 shRNA expressing cells.

*Nuclei volume of loricrin positive cells was calculated using Volocity 3D Image Analysis Software. Data represents mean ± SEM calculated from 10 nuclei in every group. Statistical analysis was performed using the non-parametric Mann-Whitney U-test. Significance was defined as $p<0.05$. *** $p \leq 0.001$ ** $p \leq 0.01$. au, arbitrary units.*

5.3.4 Reduced Akt-1 mediated Lamin phosphorylation, leads to a reduction in keratinocyte terminal differentiation markers.

Lamin A/C has previously been established as a nuclear substrate of Akt (Cenni *et al.*, 2008). Marmiroli and colleagues have recently reported, using myoblasts of murine skeletal origin, that prelamin A degradation is triggered by Akt mediated phosphorylation at the sites serine 301 and serine 404 (Bertacchini *et al.*, 2013; Cenni *et al.*, 2008). To further explore lamin A phosphorylation by Akt in keratinocytes, flag-tagged wild type and mutant lamin A rat constructs where both serine residues were mutated to alanine, (gift, Professor Marmiroli, University of Bologna, Italy), were used to stably transfect REKs. Western analysis on post-confluent cultures revealed that S301A-mutated lamin A is degraded to the same extent as the wild type lamin A (Fig 5.7). Whereas lamin A degradation in cells transfected with S404A-mutated lamin A and double mutant (S301A/S404A) was much greater. There was also a reduction in endogenous lamin A/C degradation in all the mutant transfected cell lines in comparison with the wild type. In addition to affecting lamin degradation, there was a decrease in overall filaggrin processing in the single mutant transfected cells. Furthermore, the early differentiation markers keratin-1, keratin-10 and late differentiation marker loricrin was reduced in all of the mutant transfected cells, with the reduction greater in S404A-mutated lamin A expressing cells. The basal keratin-5 and pSer473Akt levels remained unchanged in all the mutant expressing cells in comparison with the wild type cells (Fig 5.7).

Immunohistochemistry was performed on wild type, single mutants and double mutant lamin A expressing cells grown on coverslips. Consistent with the western data, loricrin expression was reduced in all the mutant transfected cells (Fig 5.8A). Similarly, keratin-10 expression was also reduced in all mutant lamin A expressing cells, hence corroborating the western results (Fig 5.8 B). These results suggest that lack of lamin phosphorylation not only affects its degradation, but also consequently alters the program of keratinocyte terminal differentiation.

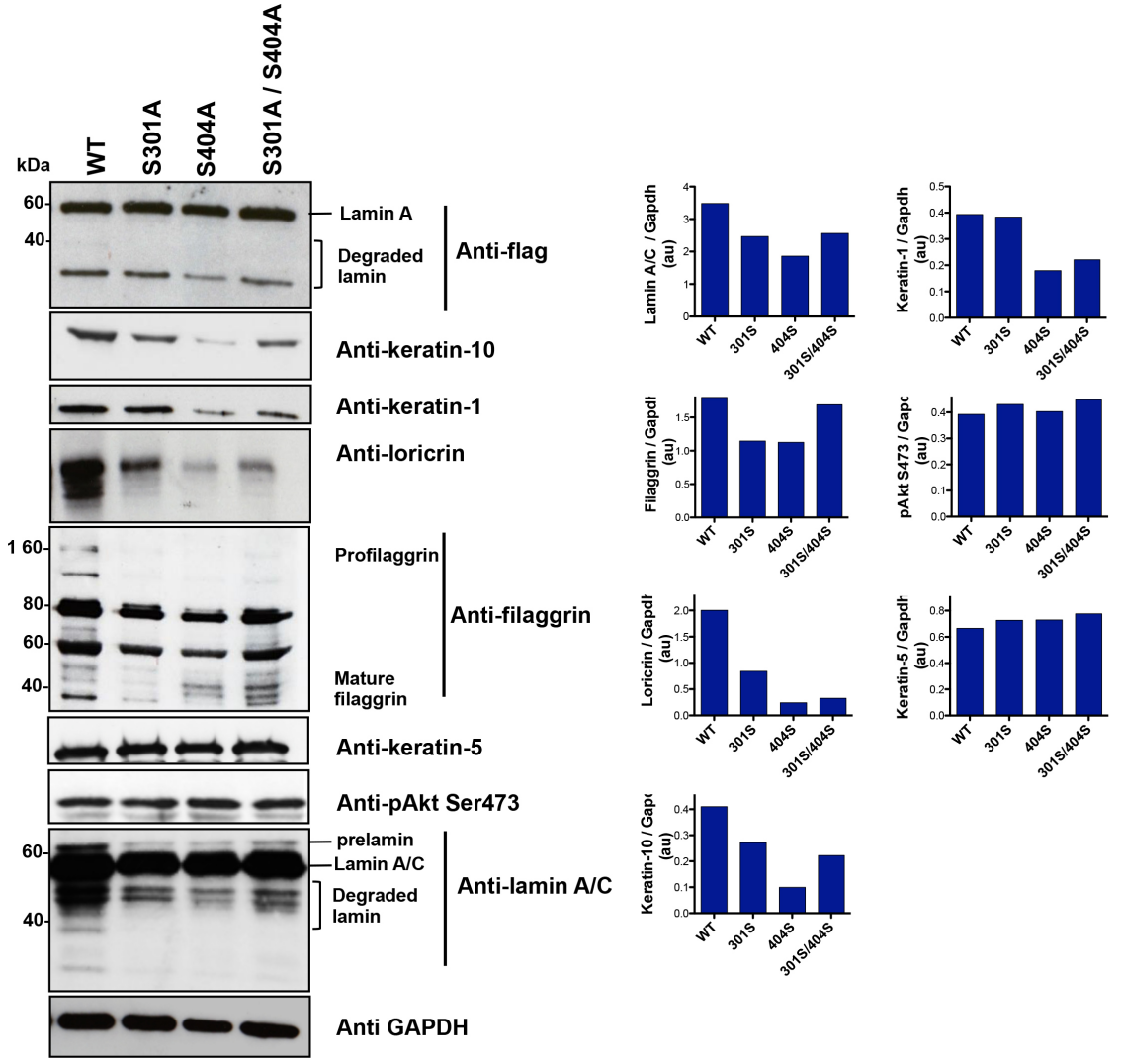

Figure 5.7 Reduced lamin A degradation associated with reduction in altered filaggrin processing and expression of other keratinocyte differentiation markers.

REKs were transfected with flag-tagged wild type lamin A rat constructs, and single S301A-mutant lamin A and S404A-mutant lamin A; and double mutant construct (S301A / S404A). All constructs express prelamin A and lamin A only. Immunoblot show a reduction in lamin A degradation products in laminA A404S expressing cells and double mutant construct expressing cells. An overall reduction in endogenous lamin A/C degradation products were observed in all lamin mutant construct expressing cells. The differentiation markers keratin-1, keratin-10, loricrin were also reduced in all of the lamin A mutant construct expressing cells. Western blot analysis of profilaggrin processing showed overall reduction in filaggrin processing in S301A-mutant transfected cells. Altered pattern of filaggrin intermediate products was observed in both S301A mutant and S301A/S404A double mutant construct expressing cells. Basal keratin-5 and pAkt levels remained unchanged. Protein expression levels relative to GAPDH were determined by densitometry using ImageJ software.

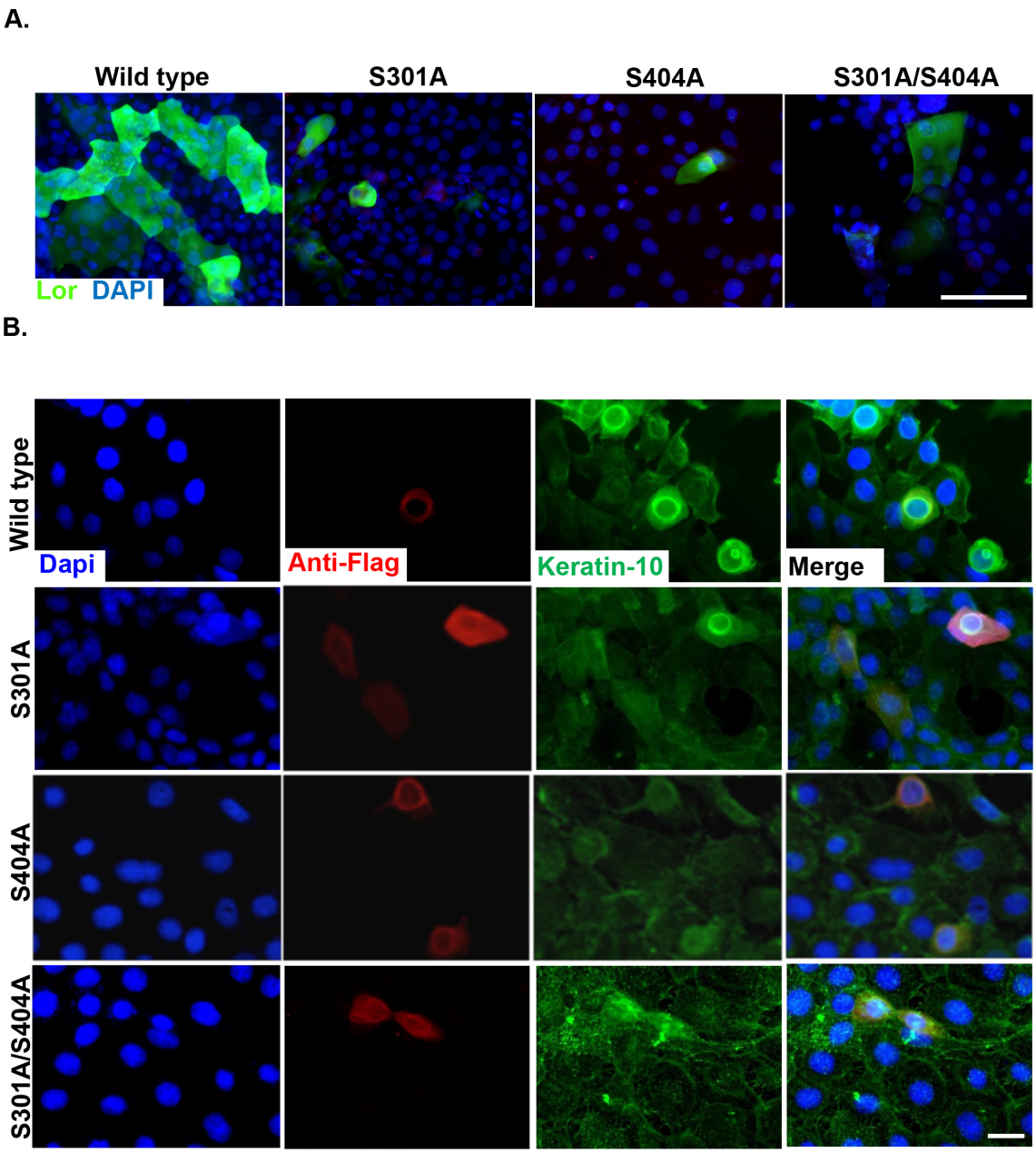

Figure 5.8 Reduced lamin A/C phosphorylion decreases expression of keratinocyte differentiation markers.

Co-immunolocalization studies of REKs transfected with flag tagged wild-type lamin A rat construct expressing prelamin A and lamin A, and single mutant constructs (S301A and S404A) and double mutant construct (S301A / S404A) using anti-loricrin, anti-flag and anti-keratin-10 antibodies. A. Loricrin protein levels were reduced in all the mutant construct expressing samples in comparison with the wild type expressing sample. B. Keratin-10 protein expression was also reduced in all mutant construct expressing samples with the reduction more obvious in the cells expressing lamin A S404A. Anti flag expression was increased in the mutant expressing terminally differentiated cells. Scale bar, 10 μM.

5.4 Discussion

A competent skin barrier depends on proper execution of the keratinocyte terminal differentiation program resulting in an anucleate cornified layer (Proksch *et al*., 2008). Cell death during terminal keratinocyte differentiation is a highly regulated specialized process with similarities to apoptosis where DNA is degraded and nucleus destroyed leading to a suicidal program that targets cells for elimination. Instead of being engulfed by macrophages as in the case of apoptosis, during cornification, these non-viable cells remain to serve a functional purpose by forming the skin barrier (reviewed in Lippens *et al*., 2005). Surprisingly little is understood about the mechanisms regulating terminal differentiation-associated epidermal cell death in the stratum epithelium. Failure of nuclear degradation results in parakeratosis which has been reported in inflammatory skin diseases such as AD and psoriasis. Consistent with this, we observed retained nuclei in the cornified layer in AD uninvolved skin sections and in the Akt1 shRNA expressing culture model. The data presented up to now establish the Akt1 shRNA expressing organotypic culture model as an attractive model of the parakeratotic aspects of AD and these data reveal lamin A/C as a downstream target of Akt1 signaling involved in nuclear degradation during cornification.

Studies have shown that migration of keratinocytes from basal to granular layer is accompanied with a gradual reduction in nuclei volume ending with complete loss of whole nucleus in the stratum corneum (Karasek *et al*., 1972; Gdula *et al*., 2013). These studies have revealed there is a general reduction in transcriptional events in the upper granular layer with cessation of all RNA activity just prior to formation of cornified layer (Karasek *et al*., 1972; Gdula *et al*., 2013). A strong correlation between nuclei volume and transcriptional activity has also been reported (Schmidt & Schibler, 1995; Webster *et al*., 2000). The data presented here shows a significant reduction in granular layer nuclei volume in Akt1 shRNA expressing cells in comparison with normal cells suggesting reduced transcriptional activity in these cells. The array analysis of Akt1 kd cells in chapter 3 showed that more than 50 % of the differentially expressed genes were down regulated with majority of these genes involved in lipid biosynthesis.

The keratinocyte specific endonuclease, DNase 1L2, has been reported to be essential for nucleus degradation during cornification, and knock down of DNase 1L2 have been reported to cause parakeratosis (Fischer *et al.*, 2007). However, our data

demonstrated that its expression remained unchanged in Akt1 knock down cells. Similar results were observed in Akt inhibited cells using the PI3 kinase pharmacological inhibitor, wortmannin. These results indicate that the lack of nuclear degradation observed in the *in vitro* AD model is most likely not due to DNase 1L2. Even though DNase 1L2 is reported to be an important mediator of nuclear degradation during cornification, it lacks a nuclear localization signal, hence can only gain access to DNA after break down of nuclear membrane, which in keratinocytes occurs during terminal differentiation in the transitional stage (Fischer *et al.*, 2007; Presland *et al.*, 1992; Shiokawa & Tanuma, 2001).

We show in Akt1 shRNA expressing cells, prelamin A protein levels were elevated. Prelamin A and lamin C are both alternate spliced products of the gene LMNA, where prelamin A undergoes modifications to yield lamin A. Lamin A/C is a major component of the inner nuclear membrane providing scaffolding for the cell nucleus and anchoring chromatin to the nuclear lamina (Goldman *et al.*, 2004; Lehner *et al*, 1986; Rao *et al.*, 1996). Studies have shown that lamin A/C degradation leads to nuclear dysregulation and apoptosis (Lazebnik *et al.*, 1995; Rao *et al.*, 1996). Furthermore, functions of lamin A/C also include maintenance of cell shape and mechanical properties; in fact studies have shown that ectopic expression of lamin A results in stiffness of nuclei and increased mechanical strength, hence making them more resistant to disintegration (Goldman *et al.*, 2008). Our data demonstrated lamin A/C degradation was reduced in both Akt1 shRNA expressing cells and Akt inhibited cells. Persistant lamin A/C staining was observed around retained nuclei in Akt1 shRNA expressing cultures. This is consistent with the immunofluorescence data which showed a significant increase in lamin A/C expression intensity in loricrin positive cells in comparison with the normal controls. These findings define a new role for Akt in lamin A/C degradation in keratinocytes, where lack of degradation leads to retention of lamin A/C positive nuclei in the cornified layer. In addition, these results can also explain why DNase 1L2 might not able to degrade the DNA, as persistent presence of lamin A/C protein in the nuclear lamina might delay nuclear envelope disintegration and hence will block DNase 1L2 from accessing the DNA.

Previous reports have demonstrated lamin A/C as a target of Akt signaling pathway (Barati *et al.*, 2006; Cenni *et al.*, 2008). It was reported that Akt phosphorylates lamin A/C at the sites S301 and S404, which upon phosphorylation

triggers lamin A/C degradation coinciding with nuclear lamina break up (Bertacchini *et al.*, 2013). Our data demonstrates that in cells transfected with the S404A-mutated lamin A and S301A/S404A double mutant lamin A, there was reduced lamin degradation. Degradation in cells transfected with S301A-mutated lamin A was comparable to wild-type expressing cells, similar to previous findings (Cenni *et al.*, 2008). Moreover, we observed a reduction in early and late keratinocyte differentiation markers with the effect more obvious in cells expressing S404A-mutated lamin A. These results were consistent with the immunoflourescence results where loricrin and keratin 10 levels were reduced in all of the cells expressing mutant lamin constructs. These results coupled with the observed overall reduction in filaggrin processing, suggested that phosphorylation at S404 is important for lamin degradation in keratinocytes, and also suggest a role for lamin A in regulating expression of important keratinocyte differentiation markers such as loricrin, keratin 10 and keratin 1. Lamin A/C expression is differentially regulated during development; in fact several studies have reported its involvement in cell differentiation (reviewed in Marmiroli *et al*., 2009). Therefore it is possible that in keratinocytes, apart from being involved in nuclear disintegration, lamin A/C play additional roles in the terminal differentiation program. The unusual filaggrin intermediate bands in the S404A and S301/404A mutant lamin A expressing cells were interesting. Filaggrin has been shown to be associated with nuclear degradation and keratin intermediate filament (IF) aggregation (Dale *et al*., 1997; Kuechle *et al*., 2000). Studies have reported that keratohyalin granules with profilaggrin aggregates localize to the perinuclear region sometimes forming deep invaginations at the areas of association (Dale *et al*., 1997; Kuechle *et al*., 2000). It is thought this may facilitate entry of cleaved N terminal domain of profilaggrin, into the nucleus where it has roles in nuclear degradation (Dale *et al*., 1997). It has also been reported that nucleus can engulf keratohyalin granules as N terminal-domain positive keratohyalin granules have been detected inside nucleus with intact nuclear envelope (Zhang *et al*., 2002). The data presented here shows an extra filaggrin intermediate band just above the 37 kDa monomeric band visible in both S404A and S301/404A mutant lamin A expressing cells. There was also accumulation of a band around 40 kDa in both mutant constructs expressing cells suggesting accumulation of intermediate forms. This suggests abnormalities in the end stage of filaggrin processing leading to accumulation of filaggrin intermediates. One possible explanation could be that since the nuclear lamina is linked to the actin cytoskeleton, a defective nuclear lamina can affect cytoskeletal dynamics (Lammerding *et al*., 2004) and interfere with the processing or

filaggrin. However Akt1 knock down cells had altered actin dynamics (discussed in chapter 3) and this particular filaggrin processing defect was not observed, it is therefore possible this may not be the reason. Another explanation is that since filaggrin has also shown to interact with nuclear lamins via the conserved helical rod domain, it is possible persistent presence of lamin A/C is increasing its association with filaggrin intermediates at the nuclear lamina preventing its processing. Alternatively, the increased association may allow it to be engulfed into the nucleus preventing access to cytoplasmic enzymes needed for end stage processing. It will be interesting to determine whether filaggrin or profilaggrin aggregates are detected either localizing with or inside the nuclei of S404 and S301/S404 lamin-A mutant construct expressing cells.

The indispensable role of A-type lamins in numerous cellular processes, including cell division, DNA replication and gene transcription may be attributed to their association in an expanding number of human diseases (reviewed in Marmiroli *et al*., 2009, Ivorra *et al*., 2005). These diseases collectively known as laminopathies include muscular dystrophy, lipodystrophy and progeria syndromes. Hutchinson-Gilford Progeria Syndrome (HGPS), a rare form of progeria is characterized by prelamin A accumulation (DeBusk, 1972). Symptoms of HGPS include accelerated ageing, alopecia and scleroderma (DeBusk, 1972; Merideth *et al*, 2008). Notably a link between increase of lamin A expression and parakeratosis has previously been reported in a transgenic mouse model replicating the skin phenotype of HGPS (Sagelius *et al*, 2008). When Sagelius and colleagues generated transgenic mice with tetracycline-inducible keratin-5 promoter to drive high levels of lamin A expression in epidermal keratinocytes, the observed skin abnormalities included hyperkeratosis and parakeratosis (Sagelius *et al*, 2008). Although no effective treatment currently exists for HGPS, Rapamycin treatment has been shown *in vivo* to decrease prelamin A levels, without altering lamin C, through a mechanism involving activation of mTOR dependent autophagic pathway (Cenni *et al*, 2011). Laminopathies have been associated with elevated mTORC1 signaling, ERK 1/2 signaling leading to reduced autophagy (Cenni *et al*, 2011; Choi & Worman, 2013; Ramos *et al*, 2013). Rapamycin, an mTORC1 specific inhibitor, have been reported to increase autophagy by reducing mTORC1 signaling and rescuing the disease phenotype (Cenni *et al*, 2011; Choi & Worman, 2013; Ramos *et al*, 2013). Data presented here showed a dose dependent increase in lamin A/C degradation in RAPTOR overexpressing cells. It is therefore

possible the rescue in disease phenotype by reduction of prelamin A levels with rapamycin treatment is mediated through Akt1. For future work, it will be noteworthy to determine whether rapamycin treatment reduces lamin A in our *in vitro* AD model and eliminates the parakeratotic phenotype.

The obvious reduction in expression of important early and late keratinocyte differentiation markers in the S404A-mutated lamin A transfected cells suggest a possible transcriptional regulation role for lamin A during cornification process. The transcription factor activator protein 1 proteins (AP-1), composed of jun and fos proteins that form homodimers (jun/jun) and heterodimers (jun/fos) which bind to AP-1 factor DNA binding sites have been implicated in keratinocyte proliferation and differentiation (Han *et al*, 2012; Mehic *et al*, 2005; O'Shaughnessy *et al*, 2009). Interestingly it has been reported that c-fos is sequestered in the nuclear envelope in a lamin A/C dependent manner, which effectively reduces AP-1 factor DNA binding activity (Ivorra *et al*, 2006). Since filaggrin, keratin 1, keratin 10 and loricrin have been reported to have AP-1 factor binding sites in their promoter regions this might suggest an explanation for the reduction in their protein expression levels (Rossi et al., 1998; Jang & Steinert, 2002).

In this study we show Akt-mediated lamin A/C phosphorylation is essential for nuclear degradation and possible transcriptional regulation of key keratinocyte differentiated-associated target genes, hence clearly defining a role for Lamin A/C in skin barrier formation.

Chapter 6 : General discussion

AD is a common disorder with a prevalence that may have increased considerably over the last three decades (Bieber, 2010; Asher *et al.,* 2006). It has been established that a skin barrier defect is a major cause of AD development, with *FLG* mutations being an important risk factor (Presland *et al*., 2000; Palmer *et al*., 2006). However the mechanisms involved in processing of profilaggrin to filaggrin still need to be fully understood (List *et al*., 2003). The Akt signaling pathway is important for barrier formation as reduced Akt activity leads to reduced filaggrin levels and hyperkeratosis (Thrash *et al*., 2006; O'Shaughnessy *et al*., 2007 and this work). It has been reported that treatment of keratinocytes with the drug rapamycin, which specifically inhibits RAPTOR-mTORC1, increases Akt activity via inhibition of a negative feedback loop. This thesis therefore explored the hypothesis that in AD, a potential upregulation of epidermal RAPTOR causes a reduction in granular Akt phosphorylation that leads to misprocessing of profilaggrin and barrier defects. To address this an Akt1 shRNA expressing REK organotypic culture model was established. This thesis has identified downstream effectors of Akt signaling involved in key processes for forming strong cornified envelopes. The work presented here provide evidence that Akt1 controls filaggrin processing, via different mechanisms, mediated through HspB1 and cathepsin H, which is identified here as a novel epidermal protease. The study also shows Akt controls epidermal Lamin A/C degradation leading to nuclear degradation, an event crucial for barrier formation. The thesis reveals a novel mechanism where RAPTOR mediated down regulation of Akt activity can cause the barrier disruption observed in AD through misprocessing of filaggrin and disruptive nuclear degradation.

6.1 Akt1 is activity required for filaggrin processing and the normal cornification process

The skin of atopic dermatitis patients has features of both parakeratosis and hyperkeratosis. Histological examination of the Akt1 shRNA REK culture model revealed presence of both features along with reduced filaggrin expression hence establishing this as an appropriate AD *in vitro* model. Lack of Akt-dependent phosphorylation of HspB1 and shRNA knockdown of HspB1 in keratinocytes leads to hyperkeratosis and impaired filaggrin processing (O'Shaughnessy *et al*., 2007) therefore

suggesting a role for pSerHspB1 in profilaggrin processing and cornification that may be Akt-mediated via mechanisms that remain to be elucidated. Consistent with these findings, the data presented here suggests HspB1 phosphorylation and its association with filaggrin is Akt1 dependent. Furthermore inhibition of Akt1 activity using the PI3 kinase inhibitor, Wortmannin, not only decreased the association between HspB1 and filaggrin association but also revealed a HspB1 staining pattern at the cell periphery similar to actin staining. HspB1 and actin interaction is well established (Lavoie *et al*., 1993; Guay *et al*., 1997; Graceffa, 2011), an action that is favoured by monomeric non-phosphorylated form and prevented by the dimeric and trimeric phosphorylated HspB1 forms. To further explore the potential HspB1 and actin interaction, co-immunoprecipation assays were performed, and results revealed an increased interaction of actin with HspB1 in both Akt1 knock down and Akt inhibited REKs, which was not observed in the control cells. Actin dynamics alter during cell differentiation and it has been reported that in stratified cultures actin staining at the cell periphery is more diffuse in comparison to basal cells (Kubler *et al*., 1991; Lewis *et al*., 1987). It is therefore possible increased HspB1 binding to actin in Akt inhibited cells, stabilises the actin cytoskeleton leading to persistent actin staining around the cell periphery. The data presented here suggests, in the absence of Akt activity, non-phosphorylated monomeric HspB1 interacts with actin instead of filaggrin, hence interfering with filaggrin processing. The exact role HspB1 plays in profilaggrin processing remains to be determined. Since HspB1 is a well described chaperone protein that interacts with many proteins, it is therefore reasonable to assume that HspB1 is acting in its chaperone capacity by binding to filaggrin and facilitating the proteolytic processing of filaggrin. It is also interesting that HspB1 interacted with specific filaggrin intermediates (Fig 3.7 A). Association of HspB1 with a subset of filaggrin intermediates has previously been observed in Akt induced tamoxifen treated REK culture model (O'Shaughnessy *et al*., 2007). HspB1 has also been reported interacting with filaggrin inside keratohyalin granules (Jonak *et al*., 2005; O'Shaughnessy *et al*., 2007). Given the role of filaggrin in aggregating keratin filaments and nuclear degradation (Presland *et al*., 1992; Pearton *et al*., 2002; Lonsdale-Eccles *et al*., 1982), it is important to ensure mature filaggrin is released at the correct point in terminal differentiation. Profilaggrin is found sequestered in keratohyalin granules of which it forms the main constituent, an action which is thought to prevent its premature access to other cellular contents (Presland *et al*., 1992). Taken together this suggests HspB1 binding to filaggrin may assist in sequestering it inside the keratohyalin granules

or the binding may facilitate proteolytic events at a specific stage of filaggrin processing.

6.2 Granular layer pSer473Akt and cathepsin H, an Akt1 dependent epidermal protease in profilaggrin processing, is reduced in eczema

To elucidate further downstream targets of Akt signaling that may be involved in skin barrier function, microarray analysis was performed on Akt1 knock down REKs and genes altered with a fold change of ≥ 1.5 were analysed. The array analysis revealed that many genes involved in lipid synthesis and retinoic acid signaling pathways were differentially expressed. Lipids play important roles in maintenance of water and permeability barrier of the epidermis (Furuse *et al*., 2002), and retinoid pathways are important regulators of keratinocyte differentiation (Fowler *et al*., 2003; Shen et al., 2011). The most differentially regulated gene down-regulated more than 4-fold was the lysosomal cysteine protease, cathepsin H (Ctsh). Reports have indicated Ctsh is found in high concentrations in specific tissues such as kidney, spleen and in type II pneumocytes, although Ctsh expression has previously not been characterized in the epidermis (Guha & Padh, 2008; Buhling *et al*., 2011). The data presented here showed an increase in Ctsh levels during mouse embryo barrier acquisition (Fig 4.4). In keratinocyte culture the Ctsh expression was detected only in post confluent differentiated keratinocytes coincident with upregulation of both pSer473Akt and the keratinocyte terminal differentiation marker keratin 1 (Fig 4.5A) therefore suggesting a terminal differentiation related role for Ctsh in the epidermis. Studies have indicated tissue specific functions for Ctsh (Lu *et al*., 2007; Guha & Padh, 2008; Buhling *et al*., 2011). For example, the most well characterized role of Ctsh is its involvement in processing of lung surfactant B in type II pneumocytes (Buhling *et al*., 2011). Additionally Ctsh expression pattern in lung development is both temporally and spatially regulated with an important role in lung branching morphogenesis (Lu *et al*., 2007). Data presented here also showed Ctsh co-localized with filaggrin in the granular layer both in REK organotypic cultures and normal human skin (Fig 4.6). The reduction in filaggrin products in Ctsh shRNA expressing cells in day 5 and increase in overall filaggrin processing in Akt1 kd REKs overexpressing Ctsh, strongly suggests that Ctsh is required for the normal processing of filaggrin. The increase in processing of filaggrin intermediate products in day 6 Ctsh shRNA expressing cells suggests an increase in proteolytic activity of another enzyme leading to increased clearing of

filaggrin products. This implies an over activation of another protease involved in profilaggrin-filaggrin processing pathway, whose proteolytic activity may otherwise be controlled either directly or indirectly by Ctsh. Previous studies have indicated inhibition of Ctsh activity during lung branching morphogenesis lead to increased levels of mature bone morphogenetic protein 4 (Bmp4) which prevents lateral lung branching (Lu *et al*., 2007; Kishimoto & Itoi, 2013). Tight regulation of Bmp4 is required for proper lung development, where different levels of Bmp4 are required at different stages and this is achieved via local induction of Ctsh which degrades Bmp4 hence regulating its availability (Lu *et al*., 2007). Tight regulation of protease activity during keratinocyte differentiation is also important as evidenced by pathological conditions caused by dysregulation of proteolytic activity (Hachem *et al*., 2006; De Veer *et al*., 2014). It will be interesting to investigate whether Ctsh affects BMP signaling in the epidermis. The importance of BMP signaling in hair morphogenesis is extensively studied (Guha *et al*., 2004; Kan *et al*., 2011; Rishikaysh *et al*., 2014) but less is known about its role in epidermal differentiation (Guha *et al*., 2004; Kan *et al*., 2011; Rishikaysh *et al*., 2014). An *in vivo* 2010 study by Yu *et al*., however showed that over expression of BMP signaling pathway caused reduced filaggrin expression and hyperkeratinization leading to Ichthyosis Vulgaris (IV) like skin disease (Yu *et al*., 2010). Although this study reported a transcriptional down regulation in filaggrin levels (Yu *et al*., 2010), which was not observed in the AD *in vitro* model, it is worth investigating whether BMP signaling has an effect on filaggrin processing as well. Interestingly BMP2 was upregulated and Inhibin, an antagonist of BMP signaling (Wiater & Vale, 2003) was downregulated in the AD in *in vitro* model.

Although the Ctsh-deficient mouse was reported as being phenotypically normal in comparison with their wild type littermates (Buhling *et al*., 2011), histological examination of both homozygous ($Ctsh^{-/-}$) and heterozygous ($Ctsh^{+/-}$) neonatal mice skin showed reduced filaggrin expression and hyperkeratosis. Additionally isolated cornified envelopes from Ctsh deficient mice are significantly weaker in comparison with wild type. These results define an important role for Ctsh in skin barrier function. Previous reports have established the importance of Akt in skin barrier formation with Akt1 null mice displaying cornified envelope fragility, reduced filaggrin processing and hyperkeratosis (Thrash *et al*., 2006; O'Shaughnessy *et al*., 2007), however epidermal Akt activity in AD skin has not been previously described. Data presented here shows a significant reduction in granular pSer473Akt in non-affected AD skin. Additionally

both filaggrin and Ctsh expression in the granular layer was also reduced significantly in non-affected AD skin sections. These data thus identify Ctsh as a novel epidermal protease that is a downstream target of the Akt1 signaling pathway and is involved in filaggrin processing and skin barrier function. Results also indicate that reduced Akt1 activity, resulting in reduced Ctsh expression, may contribute to pathology of AD via impaired processing of filaggrin and subsequent skin barrier dysfunction.

6.3 Reduced Akt activity alters Lamin A/C degradation resulting in skin barrier abnormalities

As parakeratosis was another feature observed in our AD *in vitro* model and in AD skin (Sakurai *et al*., 2002), we wanted to examine whether Akt signaling is involved in the nuclear degradation process during cornification. Examination of keratinocyte specific nuclease, DNase 1L2, revealed no apparent change in protein levels suggesting retention of nuclei in the parakeratotic regions was not due to changes in DNase 1L2, which is the main nuclease identified in nuclear degradation during cornification (Fischer *et al*., 2005; Jager *et al*., 2007; Fischer *et al*., 2011). To profile differentially expressed proteins with functions potentially involved in nuclear degradation, protein lysates were run on an SDS-PAGE gel and stained with colloidal coomassie blue. Mass spectrometric analysis of highly expressed 60 - 80 kDa protein band identified nuclear lamin protein, Prelamin. Lamin A/C are type A lamins coded by the *LMNA* gene that yelds prelamin A and lamin C through alternate splicing (Dechat *et al*., 2010). Prelamin A is further processed to produce the mature lamin A (Lehner *et al*., 1986). It has been reported that lamin A/C degradation, is required prior nuclear disintegration during apoptosis (Lazebnik *et al*., 1995). It has also been shown that Akt mediated lamin phosphorylation is needed before lamin A/C degradation can occur (Cenni *et al*., 2008). Data presented here shows persistence of lamin A/C staining around the retained nuclei in the parakeratotic regions and a reduction in lamin A/C degradation products in the AD *in vitro* model. Reduced degraded lamin A/C products were also observed in Akt inhibited REKs treated with the PI3 kinase inhibitor, wortmannin. Quantification of immunofluorescence signals indicated lamin A/C expression in loricrin positive cells is significantly higher in comparison with normal cells (Fig 5.5B). Since DNase 1L2 does not have a nuclear localization signal, indicating it can only get access to nucleus once nuclear envelope is disrupted, this suggests that persistent lamin A/C presence around

the nuclear would hinder the nuclear degradation process and therefore may result in parakeratotic regions as observed in the AD *in vitro* model.

Reports have identified serine 301 and serine 404 as specific Akt phosphorylation sites on lamin A which when phosphorylated triggers degradation of lamin A (Bertacchini *et al*., 2013; Cenni *et al*., 2008). Mutant lamin A constructs where the serine residue at these sites have been substituted for alanine were obtained (generous gift from Professor Marmiroli, University of Bologna, Italy) to further investigate effect of lamin A phosphorylation in keratinocyte differentiation process. Data showed a reduction in lamin A degradation in the REKs expressing S404A (single mutant) and S301A/S404 (double mutant) with greater reduction observed in S404A single mutant expressing cells. Interestingly an overall reduction in protein levels of both early and late differentiation markers was also observed in these same lamin A-mutant construct expressing REKs again with a greater reduction observed in S404A mutant expressing cells. Aberrant filaggrin processing was observed in all lamin A-mutant expressing lines. The unusual filaggrin intermediate banding pattern observed in both S404A and S301A/S404A lamin A mutant expressing cells is suggestive of abnormalities at the end stage of filaggrin processing in these cells. Since filaggrin associates with nuclear lamins via the conserved helical rod domain (Kuechle *et al*., 2000), an increased association with lamin due to its persistant presence may interrupt with the proteolysis of filaggrin. Additionally, nucleus have been shown to engulf keratohyalin granules while keeping nuclear envelope intact, in which case, lack of nuclear envelope degradation would prevent filaggrin intermediate products from accessing cytosolic enzymes (Zhang *et al*., 2002).

The overall reduction in keratinocyte differentiation markers including filaggrin suggests a possible transcriptional regulation role for lamin A. Several transcriptional factors have been implicated as being important regulators at different stages of keratinocyte differentiation (Eckert *et al*., 1997; Eckert *et al*., 2013), out of these Ap-1 which is important for keratinocyte proliferation and differentiation, is found in the promoter regions of filaggrin, K1, K10 and loricrin (Jang & Steinert, 2002; Han *et al*., 2012; Mehic *et al*., 2005). It has been reported that lamin A/C dependent sequestering of c-fos at the nuclear envelope reduces Ap-1 factor DNA binding activity, which may explain the reductions in the protein levels of the keratinocyte markers (Ivorra *et al*., 2006). The data presented here suggests Akt-mediated phosphorylation of lamin A,

particularly at the residue S404, is important for nuclear degradation. Data also suggests apart from abnormal nuclear disintegration, lack of lamin A degradation may also affect other aspects of keratinocyte differentiation by altering the expression and processing of key epidermal proteins.

6.4 Increased RAPTOR leads to decreased filaggrin expression

The results discussed so far have identified possible mechanisms where reduced epidermal Akt activity can give rise to an AD-like phenotype. Data also showed granular pSerAkt activity is reduced in a subset of AD patients. To address whether RAPTOR can modulate Akt activity and its downstream targets, REKs were transfected with myc-tagged RAPTOR overexpressing plasmid. Data showed that low pSer473Akt and low filaggrin expression correlated with high RAPTOR expression. Additionally a reduction in lamin A/C degradation was also observed in RAPTOR over-expressing cells. Rapamycin treatment of RAPTOR-over expressing cells not only restored pSer473Akt levels, but also rescued filaggrin and Ctsh expression to an extent. This strongly suggests that the decrease in Akt activity and its altered barrier function related downstream effects are mediated by an increase in RAPTOR. Examination of normal skin sections and non-affected AD skin indicated RAPTOR increase can occur in both populations although data suggests the increase in RAPTOR levels in AD skin is greater compared to normal skin. Interestingly, granular RAPTOR increase was associated with a decrease in filaggrin expression. Altered filaggrin processing in AD skin have been identified independent of filaggrin mutations in an African population suggesting possible mechanisms leading to misprocessing of profilaggrin (Thawer-Esmail *et al*., 2014). Reports have identified SNPs in the *RAPTOR* gene under climate selection and interestingly increased RAPTOR expression levels have been reported in sub-saharan African areas (Sun *et al*., 2010). Although Thawer-Esmail *et al*. did not report a cause for reduced filaggrin products in the African AD population, it is possible filaggrin expression and processing is impaired due to an increase in RAPTOR.

Treatment of human keratinocytes with retinoic acid agonist resulted in a decrease in RAPTOR levels where treatment with an antagonist increased RAPTOR expression. The retinoid signaling pathway is dysregulated in AD and retinoic acid based therapy has been successful in hand eczema patients (Gericke *et al*., 2013; Bissonnette *et al*., 2010). It is possible the improvement in skin barrier with retinoic

acid treatment in the hand eczema patients is due to a mechanism where decrease in RAPTOR leads to increase in Akt activity and restoration of barrier-related downstream effects (Fig 6.1)

6.5 Final conclusions and future work

Barrier dysfunction is reported as the major cause of AD pathogenicity. Understanding key mechanisms that lead to barrier disruption should lead to better therapeutic approaches. This thesis has identified key roles of Akt1 in regulating filaggrin processing and nuclear degradation, which are two vital events involved in the normal cornification process.

- The presented data showed how reduced *in vitro* Akt activity decreased the HspB1-filaggrin interaction while increasing HspB1-actin interaction. The exact role of HspB1 in filaggrin processing is unclear, however data presented here and in literature suggests that the HspB1-filaggrin interaction is required for normal filaggrin processing. Since HspB1 is a chaperone protein which can bind to numerous proteins (O'Shaughnessy *et al*., 2007; Jonak *et al*., 2005; During *et al*., 2007; Ojha *et al*., 2011), it may be interesting to do a co-immunoprecipitation assay followed by mass spectrometry to identify novel HspB1 binding partners that may be involved in filaggrin processing. HspB1 was also reported to co localize with the keratin filaments during differentiation (O'Shaughnessy *et al*., 2007). Further investigations will be needed to clarify whether HspB1 may have a role in cytoskeletal break down during cornification process.

- The data also showed that reduced Akt activity led to decreased lamin A/C degradation disrupting nuclear disintegration process giving rise to parakeratosis. It would be interesting to evaluate how decreased lamin degradation and the lack of nuclear degradation alters filaggrin processing. Investigating the fate of filaggrin products by immunofluorescence staining in these lamin mutant expressing cells may further our understanding of end stage filaggrin proteolysis process. It will also be worthwhile to determine whether rapamycin treatment can rescue parakeratotic phenotype in the AD *in vitro* model. Increased BMP signaling in the epidermis of transgenic mice causes decrease in filaggrin expression and IV-like symptoms (Yu *et al*., 2010); and

leads to psoriasis like symptoms with hyperkeratosis and parakeratosis (Blessing *et al*., 1996). Additionally overexpression of BMP signaling in murine epidermis leads to downregulation of AP-1 (jun/fos) transcription factors (Wach *et al*., 2001), which are key regulators of keratinocyte differentiation (Eckert *et al*., 2013). As BMP signaling was increased in the AD *in vitro* model, it is therefore possible the observed parateratosis and down regulation of keratinocyte differentiation markers may be influenced by altered BMP signaling pathway. Further studies are needed to determine the contribution of BMP signaling towards skin barrier formation.

- This study also demonstrated for the first time that granular pSer473Akt is reduced in non-affected AD skin. *In vivo* and *in vitro* results presented in this thesis strongly lends support to the hypothesis that increase in RAPTOR can lead to a reduction in epidermal granular Akt1 phosphorylation leading to impaired filaggrin processing and barrier defects in AD. The data presented here suggests a possible therapeutic route where in the subset of patients with impaired filaggrin processing, drugs aimed at reducing epidermal RAPTOR expression may restore the barrier defects in AD.

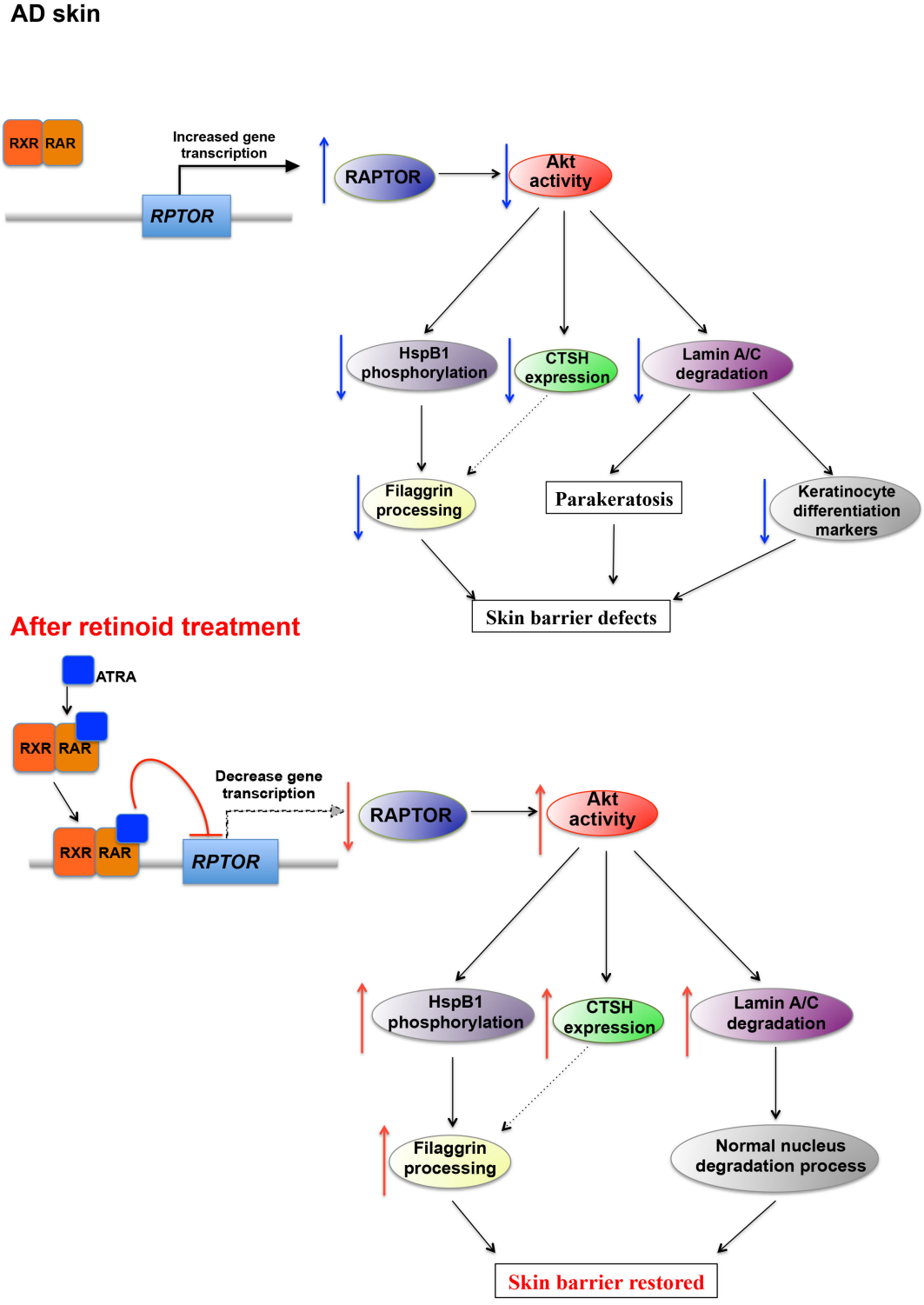

Figure 6.1 Proposed mechanism of how retinoic acid treatment may lead to skin barrier restoration in AD

In AD, RAPTOR increase is associated with reduced Akt phosphorylation which subsequently decreases HspB1 phosphorylation, Ctsh expression and lamin degradation. This leads to reduced filaggrin processing, parakeratosis and reduction of keratinocyte differentiation markers resulting in skin barrier defects. All trans retinoic acid (ATRA) treatment binds to retinoic acid receptor (RAR) which is in complex with retinoic x receptor (RXR), that binds to RAPTOR promoter region causing a reduction in RAPTOR expression. This leads to an increase in Akt activity and its downstream signaling pathways leading to restoration of skin barrier defects.

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Publications

Contributed towards:

Youssef G, Gerner L, **Naeem AS**, Ralph Oliver, Ono M, O'Neill C, O' Shaughnessy RF (2013) Rab3Gap1 mediates exocytosis of Claudin-1 and tight junction formation during epidermal barrier acquisition. *Developmental Biology* **380**: 274-85.

Sully K, Akinduro O, Philpott MP, **Naeem AS**, Harwood CA, Reeve VE, O'Shaughnessy RF, Byrne C (2013) The mTOR inhibitor rapamycin opposes carcinogenic changes to epidermal Akt1/PKBα isoform signaling. *Oncogene* **32**: 3254-62.

Publications in preparation arising from work presented in this thesis:

Naeem AS, Brown S, Zhu Y, Moffat M, Cookson B, Harper JI, Di W L, Reinheckel T, O'Shaughnessy RF. An mTORC1/AKT1/Cathepsin H axis controls epidermal filaggrin levels in skin, a novel mechanism for skin barrier disruption in Atopic Dermatitis. *(In revision)*

Naeem AS, Marmiroli S, & O'Shaughnessy RF. Akt1-mediated Lamin A/C degradation is required for nuclear degradation and normal epidermal terminal differentiation. *(In preparation)*

Appendix: Akt1 shRNA microarray data

Transcripts Cluster Id	FC (abs) ([Scrambled control] vs [Akt1 shRNA])	Log FC ([Scrambled control] vs [Akt1 shRNA])	Change in AKT1 shRNA	[Scrambled control](raw)	[Akt1 shRNA](raw)	[Scrambled control] (normalized)	[Akt1 shRNA] (normalized)	gene symbol	gene description
7339010	3.867894	1.9515482	DOWN	898.4988	230.90825	1.8980517	-0.05349648	Ctsh	cathepsin H
7070419	2.9233336	1.5476145	DOWN	313.1256	107.22988	1.4646826	-0.08293188	Slfn3	schlafen 3
7374777	2.7440472	1.4563053	DOWN	983.09546	367.04636	1.1348367	-0.3214686	S100g	S100 calcium binding protein G
7159536	2.7373846	1.4527981	DOWN	1946.4788	712.8574	1.3376613	-0.11513686	Calml3	calmodulin-like 3
7338934	2.3844414	1.2536514	DOWN	892.10974	374.08124	1.2226024	-0.03104901	Nt5e	5' nucleotidase, ecto
7261177	2.2645502	1.1792245	DOWN	190.98816	84.37275	1.1087635	-0.07046104	Sema3a	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A
7041124	2.1282182	1.0896461	DOWN	1546.4495	737.1759	0.85994434	-0.22970176	Il33	interleukin 33
7219186	2.1190374	1.0834091	DOWN	430.58868	201.31915	1.0209947	-0.06241441	Pkib	protein kinase (cAMP-dependent, catalytic) inhibitor beta
7039650	2.1007307	1.0708911	DOWN	2152.4736	1026.4053	1.0095105	-0.06138063	Fads1	fatty acid desaturase 1
7190182	2.0649838	1.0461304	DOWN	651.19617	314.96286	0.96922636	-0.07690406	Sepp1	selenoprotein P, plasma, 1
7166170	2.0062873	1.0045283	DOWN	693.51355	347.1023	0.8863859	-0.11814237	Idi1	isopentenyl-diphosphate delta isomerase 1
7145003	1.9431511	0.9583981	DOWN	676.86975	353.19824	0.7183852	-0.24001288	Galnt7	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 (GalNAc-T7)
7163897	1.9213254	0.94210196	DOWN	831.1727	432.3076	0.87843037	-0.06367159	Nqo2	NAD(P)H dehydrogenase, quinone 2
7199114	1.9142269	0.93676186	DOWN	3740.1987	1973.0505	0.698895	-0.23786688	Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)
7144691	1.8814454	0.9118414	DOWN	2326.4019	1237.0355	0.8772564	-0.034585	Sc4mol	sterol-C4-methyl oxidase-like
7190101	1.8680103	0.9015024	DOWN	1781.6633	953.9816	0.83419704	-0.06730533	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
7219866	1.8675976	0.9011836	DOWN	2270.1812	1210.6006	0.8640437	-0.03713989	Cd24	CD24 molecule
7188573	1.8345416	0.8754196	DOWN	468.9148	258.14017	0.71388817	-0.16153145	F2rl2	coagulation factor II (thrombin) receptor-like 2
7204890	1.8228955	0.8662319	DOWN	1149.8516	631.62256	0.8031602	-0.06307173	Slc1a3	solute carrier family 1 (glial high affinity glutamate transporter), member 3
7375305	1.773934	0.82695234	DOWN	381.8687	213.62616	0.7487569	-0.07819545	Dmd	dystrophin
7216123	1.7580512	0.813977	DOWN	638.0548	363.97003	0.7199464	-0.09403062	Cth	cystathionase (cystathionine gamma-lyase)
7163946	1.7292497	0.79014623	DOWN	351.08698	205.01962	0.5894518	-0.20069444	Serpinb9	serine (or cysteine) peptidase inhibitor, clade B, member 9
7190623	1.7195625	0.78204155	DOWN	202.85176	118.605316	0.6758816	-0.10615993	Prlr	prolactin receptor
7162611	1.7194976	0.7819871	DOWN	292.98584	170.47264	0.7273979	-0.05458915	Aspn	asporin

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7198615	1.7053828	0.7700956	DOWN	436.36862	256.54135	0.6829815	-0.0871141	F3	coagulation factor III (thromboplastin, tissue factor)
7265111	1.6925765	0.7592211	DOWN	230.9136	136.95712	0.64894557	-0.11027551	Pde1c	phosphodiesterase 1C
7109887	1.6727378	0.74221134	DOWN	582.0183	348.17383	0.61544514	-0.1267662	Serpinb3	serine protease inhibitor B3 | serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 3A
7202569	1.6598706	0.73107076	DOWN	2717.8147	1634.5928	0.71206474	-0.01900601	F2r	coagulation factor II (thrombin) receptor
7291785	1.6591924	0.73048127	DOWN	326.81833	197.52448	0.6311164	-0.09936488	Ptafr	platelet-activating factor receptor
7282767	1.6576132	0.7291074	DOWN	371.44678	224.55573	0.62715626	-0.10195112	Sulf1	sulfatase 1
7119590	1.6365954	0.71069765	DOWN	163.93881	100.18532	0.68575144	-0.02494621	Prom1	prominin 1
7177248	1.632235	0.70684886	DOWN	288.29684	176.5693	0.67661476	-0.0302341	Lipg	lipase, endothelial
7048681	1.5996624	0.6777675	DOWN	955.55676	596.32526	0.5943904	-0.08337712	Apoe	apolipoprotein E
7169575	1.5891198	0.6682279	DOWN	228.32175	144.533	0.5483906	-0.11983728	Pcdhb19	protocadherin beta 19
7059148	1.5876905	0.6669297	DOWN	461.86316	284.68225	0.60414314	-0.06278658	Mrgpre	MAS-related GPR, member E
7269448	1.587062	0.6663585	DOWN	1834.5374	1163.044	0.5061655	-0.16019297	C1s	complement component 1, s subcomponent
7117811	1.5861855	0.66556144	DOWN	1007.06335	635.72687	0.5667529	-0.09880853	Ppat	phosphoribosyl pyrophosphate amidotransferase
7165193	1.5830573	0.6627134	DOWN	359.685	227.18631	0.5984101	-0.06430328	Inhba	inhibin beta-A
7288540	1.582866	0.6625391	DOWN	217.43701	137.54758	0.549464	-0.11307514	Elavl2	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)
7381109	1.5639746	0.64521706	DOWN	269.86768	174.55577	0.4753523	-0.16986477	Ace2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2
7179997	1.5612137	0.642668	DOWN	859.33386	544.24774	0.53987217	-0.10279584	Scoc	short coiled-coil protein
7060258	1.5602093	0.6417396	DOWN	3600.508	2308.2927	0.6157942	-0.02594543	Fads2	fatty acid desaturase 2
7215874	1.5532016	0.6352451	DOWN	2204.6375	1416.6733	0.6071472	-0.02809787	Rabggtb	Rab geranylgeranyltransferase, beta subunit
7070338	1.5450618	0.62766457	DOWN	250.92374	162.87256	0.51036215	-0.11730242	Ccl2	chemokine (C-C motif) ligand 2
7339851	1.5449033	0.6275165	DOWN	300.16937	194.2227	0.58404016	-0.04347634	Slco2a1	solute carrier organic anion transporter family, member 2a1
7300127	1.536898	0.62002146	DOWN	381.12244	247.9853	0.5399437	-0.08007777	Ptgr2	prostaglandin reductase 2
7171430	1.5150789	0.5993929	DOWN	962.0304	627.2106	0.52523804	-0.07415485	Sec11c	SEC11 homolog C (S. cerevisiae)
7178192	1.5140852	0.59844637	DOWN	1624.7483	1055.9048	0.5521593	-0.04628706	Nae1	NEDD8 activating enzyme E1 subunit 1
7057283	1.5118145	0.59628105	DOWN	1023.2451	665.4905	0.54397964	-0.05230141	Nupr1	nuclear protein, transcriptional regulator, 1

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7189968	1.5113683	0.59585524	DOWN	75.7495	50.075203	0.5593066	-0.03654862	Emb	embigin homolog (mouse)
7094780	1.5075654	0.59222054	DOWN	375.82718	249.47827	0.5382509	-0.05396962	St6gal1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1
7214100	1.5016582	0.58655643	DOWN	606.53	404.12137	0.54350185	-0.04305458	Gar1	GAR1 ribonucleoprotein homolog (yeast)
7257717	1.5043546	-0.5891447	UP	333.34668	503.93585	-0.4479513	0.14119339	LOC5003	similar to hypothetical protein MGC6835
7304448	1.507118	-0.59179235	UP	715.38245	1079.2058	-0.5388336	0.05295873	Rhob	ras homolog gene family, member B
7225246	1.5096408	-0.5942054	UP	605.1352	913.5659	-0.5364223	0.05778313	Entpd2	ectonucleoside triphosphate diphosphohydrolase 2
7133190	1.520396	-0.6044471	UP	476.9381	728.9518	-0.4569273	0.14751983	Hr	hairless
7182917	1.5211831	-0.60519385	UP	7053.8037	10672.923	-0.5061021	0.09909177	Mt1a	metallothionein 1a
7217458	1.522646	-0.6065805	UP	838.5768	1276.96	-0.5888238	0.0177567	Abcg1	ATP-binding cassette, sub-family G (WHITE), member 1
7271580	1.5377868	-0.62085557	UP	226.59244	348.64508	-0.5584781	0.06237745	Tuba8	tubulin, alpha 8
7130740	1.5492314	-0.6315527	UP	860.88916	1329.6708	-0.5316157	0.09993696	Dnase1l3	deoxyribonuclease 1-like 3
7216312	1.5518134	-0.633955	UP	347.08575	542.70935	-0.5108953	0.12305975	Olr1748|(	olfactory receptor 1748 | olfactory receptor 1746 | olfactory receptor 1749
7032552	1.5688354	-0.64969397	UP	600.88275	944.98566	-0.5497842	0.09990978	Pcsk6	proprotein convertase subtilisin/kexin type 6
7233989	1.5690848	-0.6499233	UP	1235.152	1937.1852	-0.6141515	0.03577185	Id1	inhibitor of DNA binding 1
7286266	1.5755988	-0.65590024	UP	429.57043	676.9406	-0.6317473	0.02415299	Abca1	ATP-binding cassette, sub-family A (ABC1), member 1
7357647	1.5872831	-0.66655946	UP	1251.0774	1985.7639	-0.6187563	0.04780316	Ramp1	receptor (G protein-coupled) activity modifying protein 1
7302739	1.5926888	-0.67146444	UP	177.85187	284.07025	-0.5709324	0.10053206	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1
7160176	1.6122543	-0.6890793	UP	7254.3164	11649.869	-0.6169653	0.07211399	Mt1a	metallothionein 1a
7269505	1.6359761	-0.7101517	UP	1129.0217	1868.1594	-0.4635787	0.24657297	Eno2	enolase 2, gamma, neuronal
7232862	1.6525651	-0.7247071	UP	396.63812	657.57556	-0.583838	0.14086914	Bmp2	bone morphogenetic protein 2
7232578	1.6614695	-0.7324598	UP	260.94574	435.14258	-0.6230853	0.10937452	Vps16	vacuolar protein sorting 16 homolog (S. cerevisiae)
7226331	1.6818787	-0.7500737	UP	102.528564	174.77339	-0.4812396	0.2688341	Angptl2	angiopoietin-like 2
7307499	1.690948	-0.7578323	UP	1149.4105	1945.4691	-0.6932159	0.06461644	Pygl	phosphorylase, glycogen, liver
7268847	1.7163279	-0.77932525	UP	529.87024	916.01697	-0.6188517	0.16047359	Fxyd4	FXYD domain-containing ion transport regulator 4
7199859	1.7332342	-0.79346657	UP	842.4178	1491.5641	-0.4999518	0.29351473	Tspan5	tetraspanin 5

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7326269	1.7779582	-0.8302214	UP	662.1633	1153.788	-0.7003441	0.12987733	Nxph4	neurexophilin 4
7139731	1.7795435	-0.8315072	UP	214.0715	383.16122	-0.7008321	0.13067508	Sorbs3	sorbin and SH3 domain containing 3
7070500	1.7924464	-0.8419299	UP	920.65686	1614.3041	-0.7657719	0.07615805	Expi	extracellular proteinase inhibitor
7237440	1.8444185	-0.8831661	UP	434.5183	800.90875	-0.8128576	0.07030845	Ccbl1	cysteine conjugate-beta lyase, cytoplasmic
7060230	1.8448814	-0.8835281	UP	152.96866	289.5097	-0.561532	0.3219961	Asrgl1	asparaginase like 1
7103514	1.8684394	-0.9018338	UP	703.5388	1320.2849	-0.7776003	0.12423348	Cldn3	claudin 3
7139747	2.241544	-1.1644928	UP	1107.2075	2479.8892	-1.0805402	0.08395267	Ppp3cc	protein phosphatase 3, catalytic subunit, gamma isoform
7133151	2.3436143	-1.2287352	UP	633.63745	1481.9663	-1.1905875	0.03814769	Bin3	bridging integrator 3
7315441	2.353636	-1.2348912	UP	153.67033	372.19412	-0.8850913	0.34979987	Tmbim4	transmembrane BAX inhibitor motif containing 4
7231820	2.372969	-1.2466931	UP	583.0729	1383.8896	-1.2044177	0.04227543	Ckmt1	creatine kinase, mitochondrial 1
7139712	2.8560135	-1.5140028	UP	447.93988	1279.8582	-1.4472628	0.06674004	Pdlim2	PDZ and LIM domain 2
7317826	2.8922126	-1.5321736	UP	327.38562	948.4475	-1.3754025	0.15677118	Khdrbs3	KH domain containing, RNA binding, signal transduction associated 3