Analysis of the Role of Rho GTPases in Epithelial Cells

by

Alanna Stanley B.Sc M.Sc

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Research Supervisor: Dr. Fabio Quondamatteo
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Authors Declaration

I declare that all of the work presented in this thesis was carried out in accordance with the regulations of the National University of Ireland, Galway. This is original work carried out by myself, except where indicated by reference in the text. This thesis has not been submitted previously for any other academic award.

Signed: ___________________________ Date: ______________________

______________________________
Alanna Stanley
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There was a tension to the thing, a feeling of much straining and striving towards some distant and incomprehensible goal. As a wizard, it was something that Ponder had only before encountered in acorns, a tiny soundless voice which said, yes, I am but a small, green, simple object – but I dream about forests.

-Terry Pratchett
Abstract

Rho GTPases are a family of small GTP binding proteins that function as molecular switches for the control of a variety of fundamental cellular processes, and therefore represent potentially vital proteins for skin homeostasis.

Through ultrastructural analysis of murine skin bearing specific gene deletions in keratinocytes, the involvement of Neural Wiskott-Aldrich syndrome protein (N-WASP) in maintaining cell-cell and cell-matrix adhesion, of RhoA in maintaining epidermal integrity including the dermal epidermal junction (DEJ), and of Rac1 in maintaining the structure of the permeability barrier, was examined as part of wider projects and discussed in the appropriate cell biological context. It was found that these proteins are dispensable for maintaining the above aspects of the integrity of the interfollicular epidermis (IFE).

The effects of Rac1 deletion in keratinocytes were also studied in relation to the maintenance of the DEJ, to the tissue localisation and arrangements of collagen fibrils and of decorin, and to the distribution and the level of protein expression of Nox1. These aspects were also investigated in the presence of irritant contact dermatitis. Primarily, these studies were conducted in 11-month-old mice, however, the DEJ was also studied in younger mice.

These studies mainly showed: sporadic duplication of the basement membrane and increased thickness of the Lamina lucida due to the lack of Rac1, which were more marked in older mice; alteration in the size distribution of the diameter of collagen fibrils in relation to a higher inflammatory status; and clear expression of Nox1 in IFE under normal conditions, which was not affected by deletion of Rac1 or inflammation.

Other than adding important ultrastructural knowledge to the cell biology of the N-WASP, Rac1 and RhoA, this thesis revealed unknown functions of Rac1 in the homeostasis of the DEJ and dermal collagen, and contributed to clarify the role of Rac1 in the regulation of Nox1 function in keratinocytes in vivo.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AJ</td>
<td>Adherens Junction</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin related protein 2/3</td>
</tr>
<tr>
<td>BM</td>
<td>Basement Membrane</td>
</tr>
<tr>
<td>DEJ</td>
<td>Dermal Epidermal Junction</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyleneiodonium chloride</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ECS</td>
<td>Extracellular Space</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
</tr>
<tr>
<td>FACIT</td>
<td>Fibril associated collagens with interrupted triple-helices</td>
</tr>
<tr>
<td>GAPs</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>GAS</td>
<td>γ-activated sequence</td>
</tr>
<tr>
<td>GDIs</td>
<td>Guanine nucleotide dissociation inhibitors</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HD</td>
<td>Hemidesmosomes</td>
</tr>
<tr>
<td>ICD</td>
<td>Irritant Contact Dermatitis</td>
</tr>
<tr>
<td>IFE</td>
<td>Interfollicular Epidermis</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>JAMs</td>
<td>Junctional adhesion molecules</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LD</td>
<td>Lamina Densa</td>
</tr>
<tr>
<td>LL</td>
<td>Lamina Lucida</td>
</tr>
<tr>
<td>MDBK</td>
<td>Madin-Darby Bovine Kidney</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>Nox</td>
<td>NADPH Oxidase</td>
</tr>
<tr>
<td>NOXA1</td>
<td>Nox activator 1</td>
</tr>
<tr>
<td>NOXO1</td>
<td>Nox organiser 1</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal Transducers and Activators of Transcription 1</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TER</td>
<td>Transepithelial Resistance</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junction</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultra violet B</td>
</tr>
<tr>
<td>ZO</td>
<td>Zona Occludens</td>
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Chapter 1

General Introduction
1.1 General aspects of Rho GTPases

Rho GTPases are a family of more than 20 small intracellular proteins grouped into 8 subfamilies, namely Rho, Rac, Cdc42, Rnd, RhoD, RhoH/TTF, RhoBTB and Miro (Table 1.1). The best studied members of this family are RhoA, Rac1 and Cdc42 and will be the primary focus in this thesis.

<table>
<thead>
<tr>
<th>Rho GTPase Subfamily</th>
<th>Members of the Subfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho</td>
<td>RhoA, RhoB, RhoC</td>
</tr>
<tr>
<td>Rac</td>
<td>Rac1, Rac2, Rac3, RhoG</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cdc42, TC10, TCL, Chip, Wrch-1</td>
</tr>
<tr>
<td>Rnd</td>
<td>Rnd1, Rnd2, Rnd3/RhoE</td>
</tr>
<tr>
<td>RhoD</td>
<td>RhoD, Rif</td>
</tr>
<tr>
<td>RhoH/TTF</td>
<td>RhoH/TTF</td>
</tr>
<tr>
<td>RhoBTB</td>
<td>RhoBTB1, RhoBTB2</td>
</tr>
<tr>
<td>Miro</td>
<td>Miro1, Miro2</td>
</tr>
</tbody>
</table>

Table 1.1 Rho GTPase Subfamilies and Members

In response to numerous types of different extracellular signals, Rho GTPases function as molecular switches affecting large groups of different downstream effector proteins of which over a hundred have been identified (Hall, 2012, Chaineau et al., 2013, Kuhn and Geyer, 2014, Bishop and Hall, 2000). It is, therefore, not surprising that this family of proteins thereby control a wide variety of fundamental cellular processes. These functions of Rho GTPases have been elucidated primarily through in vitro studies, and include actin cytoskeletal reorganisation, of which they are historically known for, but also gene transcription, proliferation, apoptosis, cell cycle progression, vesicular transport, microtubule dynamics and regulation of NADPH oxidase complexes to name but a few (Hall, 2012, Heasman and Ridley, 2008, Pedersen and Brakebusch, 2010, Spiering and Hodgson, 2011, Stanley et al., 2012). This molecular switch is mediated by
the ability of Rho GTPases to cycle between two conformational forms, an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound state (Fig. 1.1). This cycle is basically regulated by three types of cellular proteins, namely Guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and Guanine nucleotide dissociation inhibitors (GDIs). GEFs stimulate the exchange of GDP with GTP by causing the release of GDP from a nucleotide binding loop within the protein. This renders the nucleotide binding site free for GTP, which in a cellular system would occur spontaneously given the high amount of GTP compared to GDP. GAPs stimulate the hydrolysis of intrinsic bound GTP thus inactivating the protein. GDIs were originally found to prevent the disassociation of GDP, and thought to be inhibitors of its activation. However, it has since been determined that their primary function is to maintain a stable soluble cytosolic pool of inactive prenylated Rho GTPases (Cherfils and Zeghouf, 2013, Garcia-Mata et al., 2011).

Figure 1.1 The Rho GTPase Cycle. (See opposite page for description)
In addition to the GTPase cycle, guanine nucleotide exchange for members of the Rho GTPases family has been found to be redox regulated (Heo and Campbell, 2005, Heo et al., 2006). This mechanism of redox regulation has been elucidated for Rac1, Cdc42 and RhoA (Heo and Campbell, 2005, Heo et al., 2006). In brief, oxidation of a redox sensitive cysteine within the guanine nucleotide binding loop leads to a cascade of events resulting in the withdrawal of an electron from GDP and its subsequent release. As during the GTPase cycle, this renders the nucleotide binding site free for GTP. Other members of the Rho GTPase family have also been found to contain a redox sensitive cysteine, it is predicted that they could also be redox regulated through this mechanism (Heo, 2011). RhoA, in contrast to Rac1 and Cdc42, has a second redox sensitive cysteine within the guanine nucleotide binding loop. Following GDP release, if this second cysteine is oxidised an intramolecular disulphide bond is formed between the two cysteines. This prevents the protein from binding GTP, thereby rendering it inactive (Heo et al., 2006). This disulphide bond can be reduced in the presence of a reducing agent, restoring the ability of RhoA to bind nucleotides (Heo, 2011, Heo et al., 2006, Mitchell et al., 2013, Stanley et al., 2014).

**Figure 1.1 The Rho GTPase Cycle.** (Diagram on page opposite) In response to extracellular stimuli Rho GTPases cycle between inactive GDP-bound and an active GTP-bound state. The cycle is controlled by Guanine Nucleotide Exchange Factor (GEF) which exchanges GTP for GDP, and by GTPase Activating Proteins (GAP) which promote GTPase activity i.e. promote hydrolysis of GTP with the result of changing the Rho GTPase into a GDP bound state. Most of the Rho GTPase family members are prenylated* (red tail protruding from the Rho GTPases), therefore in order to be removed from the membrane they have to be bound by Guanine Nucleotide Dissociation Inhibitors (GDI) which solubilises these Rho GTPases. In this form GDIs function to maintain a stable cytosolic soluble pool of inactive prenylated Rho GTPases. In addition to this classic method of Rho GTPase regulation, an alternative mechanism mediated by reactive oxygen species has been found for some Rho GTPase proteins (not depicted in figure, see text above for detailed description). CM = cell membrane, EX = extracellular space, CY = cytoplasm, Pi= Phosphate. Figure adapted according to (Cherfils and Zeghouf, 2013, Garcia-Mata et al., 2011, Jaffe and Hall, 2005).

* Not all Rho GTPase proteins are prenylated. The Rho BTB and Miro subfamilies, in addition to Chp/Wrch-2 of the Cdc42 subfamily are not prenylated (Wennerberg and Der, 2004).
As outlined previously, Rho GTPase proteins are involved in the regulation of numerous cellular functions. This regulation is mediated through their activation or inhibition of downstream targets. However, elucidating the pathways and functions of different Rho GTPase proteins is made difficult by the fact that they can affect numerous different downstream targets. This is made even more complicated as in some cases downstream targets can be affected by more than one Rho GTPase protein (Fig. 1.2).

Figure 1.2 Overlap of Downstream Targets of the Major Rho GTPases. This diagram provides an example of some of the main downstream effectors that can be mediated by more than one Rho GTPase. Adapted from (Citi et al., 2011, Iden and Collard, 2008, Stanley et al., 2014).

Several different in vitro methods have been employed in order to elucidate the functions of Rho GTPases over the last two decades. The use of dominant negative or constitutively active mutants, pharmacological inhibitors, and bacterial toxins that activate or inactivate the proteins have enabled great strides to be made in understanding the functions of Rho GTPases (Wang and Zheng, 2007, Pedersen and Brakebusch, 2010). However, such methods are not without limitations, most importantly being that their effects are not restricted to individual Rho GTPase proteins. For example,
dominant negative mutants work by binding to and sequestering Rho GEFs. The effect in the cell would therefore be unspecific due to the fact that many Rho GEFs activate a number of different Rho GTPase proteins (Debreceni et al., 2004, Wang and Zheng, 2007). Another example is the pharmacological inhibitor, C3 exoenzyme, which affects all members of the Rho subfamily (Boquet, 1999, Vogelsgesang et al., 2007). This has been used extensively to elucidate the functions of these proteins; however, its use cannot provide information on the effects of individual members of the subfamily.

The advent of genetic manipulation studies that enable specific targeting of individual Rho GTPase proteins has become the ideal method to study the function of these proteins (Wang and Zheng, 2007, Pedersen and Brakebusch, 2010). The generation of knockout (KO) models, in which individual genes can be inactivated, has led to the ability to study the function of individual proteins during embryonic development, normal physiological homeostasis and in the progression of diseases (Hall et al., 2009). Such models have been generated for several Rho GTPase proteins, and have progressed knowledge of Rho GTPase functions greatly, in many cases revealing the involvement of these proteins in a diverse array of physiological functions. This topic has been excellently reviewed (Wang and Zheng, 2007, Heasman and Ridley, 2008, Pedersen and Brakebusch, 2010).

As mentioned above, of all the Rho GTPase proteins, RhoA, Rac1 and Cdc42 are the most studied. These proteins are highly conserved from yeast to mammal. In mammals they are found to be ubiquitously expressed and indeed the importance of these proteins is highlighted by the fact that KO mouse models for these proteins leads to embryonic lethality (Chen et al., 2000, Sugihara et al., 1998, Wang and Zheng, 2007). This prevents the study of the function of such clearly important proteins during normal physiological homeostasis. This problem has been overcome with the development of the Cre/LoxP based technology which enables selective tissue specific deletion of target genes (Branda and Dymecki, 2004, Wang and Zheng, 2007, Hall et al., 2009, Pedersen and Brakebusch, 2010).

Work carried out for this thesis utilises such a model where mice bear specific deletion of different Rho GTPases and their effectors in the keratinocytes of the skin. Many of
the vital cellular processes regulated by Rho GTPases are central to maintaining the physiological function and structure of the skin therefore making this an excellent model to analyse the effects of deletion of Rho GTPase proteins.

1.2 Structure and function of skin

The skin is a complex organ that envelops the entire body. It functions primarily in protecting the body from the external environment, but also in sensation, immunological response, thermoregulation, excretion, and absorption. The skin is comprised of two layers, the epidermis which overlies the dermis (Quondamatteo, 2014, Ross and Pawlina, 2011) (Fig. 1.3). At the interface between the epidermis and dermis is an anatomically distinct functional unit, the dermal epidermal junction (DEJ) which is responsible for anchoring the epidermis to the dermis (Briggaman and Wheeler, 1975, Burgeson and Christiano, 1997). This junction has an undulating pattern which can clearly be seen at the microscopic level. This pattern is due to fingerlike projections known as dermal papillae, where the dermis projects into the epidermis, and rete ridges, where the epidermis projects into the dermis (Ross and Pawlina, 2011). At the ultrastructural level, a similar pattern can be seen. In this case the undulating pattern is formed by fingerlike projections of the plasma membrane of the basal keratinocytes into the dermis. These structures, termed serrations, are found principally in the dermal papillae (Lavker and Sun, 1982). Such an arrangement provides extra adhesive power at this junction (Lavker and Sun, 1982, Ross and Pawlina, 2011).

1.2.1 The Epidermis

The epidermis is a specialised stratified squamous epithelium which forms a stable cohesive sheet on the dermis. The major cell type of this layer is the keratin producing keratinocyte. The epidermis can be subdivided into the interfollicular epidermis (IFE)
and epidermal derivatives, such as hair follicles, sweat glands, and sebaceous glands (Ross and Pawlina, 2011) (Fig. 1.3).

In relation to the epidermis, for the purposes of this project it is the barrier function, which is provided by the ability of this layer to maintain a stable cohesive sheet, which is the main focus. This epidermal barrier function which protects against abrasion, mechanical insults, chemicals, toxins, microbes and dehydration of the body is primarily provided by the IFE (Ross and Pawlina, 2011). Therefore the description and discussions in this thesis will focus mainly on this region of the epidermis.

**Figure 1.3 Schematic Representation of the Skin Structure.** The epidermis (purple) forms the outer surface of the skin and can be subdivided into the interfollicular epidermis (IFE) and epidermal derivatives, such as hair follicles, sebaceous glands and sweat glands. Underlying the epidermis is the dermis (green), which is an irregular dense connective tissue and is rich in collagen fibrils. At the interface between these two layers is the dermal epidermal junction (DEJ). This follows an undulating pattern due to the dermal papillae (DP) and rete ridges (RR) which provide extra stability to the DEJ. Beneath the dermis is the subcutis, consisting primarily of fat tissue. Blood vessels (blue and red), lymphatics and nerves (not depicted here) traverse this region. It is important to note that the schematic representation here is of human skin. Some histological differences exist between human and mouse skin, most notably the fact that in mouse skin sweat glands are only found in the foot pads of mice (Treuting et al., 2012). Figure adapted from (McGrath et al., 2008).
Maintaining the structural integrity of the epidermis is key to the barrier function and is enabled principally by three histological features of the epidermis. Firstly, the ability of the epidermis to undergo constant renewal of its building blocks i.e. the keratinocytes. Secondly, is the ability of the epidermis to maintain mechanical strength, which is provided by both strong cell-matrix junctions at the DEJ, and mechanical cell-cell junctions, namely desmosomes and adherens junction (AJs). These junctions are either directly or indirectly connected to the actin cytoskeleton of the keratinocytes. These junctions therefore ultimately join the cytoskeleton of all the keratinocytes together, thus being central to the formation of a stable cohesive mechanically strong sheet. The third structural feature of the epidermis key to maintaining its barrier function is its ability to function as both an outside-in and an inside-out permeability barrier.

1.2.1.1 Keratinocytes and renewal of the epidermis

The keratinocytes of the epidermis originate in the mitotically active basal layer (stratum basale). From here they undergo a process of terminal differentiation and migrate outwards where they are shed from the surface giving rise to a number of different layers, namely the stratum spinosum, stratum granulosum, and stratum corneum in the epidermis (Fig 1.4).

Figure 1.4 Schematic Representation of Epidermal Layers. Keratinocytes of the epidermis are arranged into layers namely stratum basale, which is attached to the basement membrane, stratum spinosum, stratum granulosum and stratum corneum. Figure adapted from (McGrath et al., 2008).
Within the stratum basale are stem cells and transient amplifying cells that are responsible for renewal of the IFE (Dahl, 2012, Watt, 2001, Watt et al., 2006, Fuchs, 2008). It should be noted that epidermal stem cells also exist in the sebaceous glands and the bulge region of the hair follicles (Dahl, 2012, Watt, 2001, Watt et al., 2006, Fuchs, 2008). However, it is becoming increasingly clear that stem cells from these niches do not contribute to self-renewal of the IFE under normal physiological conditions, but do however contribute during wound healing (Plikus et al., 2012, Senoo, 2013, Watt et al., 2006). Therefore, here, the discussion focuses on the IFE stem cell population. These stem cells can self-renew by undergoing symmetric division giving rise to two daughter stem cells. The stem cells can also undergo asymmetric division thereby giving rise to one daughter stem cell and one daughter cell destined for differentiation. However, before the latter cell type withdraws from the cell cycle to differentiate it is known as a transient amplifying cell. The transient amplifying cells can undergo a few rounds of division and are the cell type that contributes most to the numbers of cells required for renewal of the epidermis (Dahl, 2012, Watt, 2001, Watt et al., 2006). These cells withdraw from the cell cycle and detach from the DEJ to undergo terminal differentiation. Keratinocytes of the stratum corneum are called corneocytes and have undergone terminal differentiation. These cells are now keratin filled dead cells that are stacked on top of each other and will eventually be shed from the surface (Ross and Pawlina, 2011).

1.2.1.2 Dermal Epidermal Junction

The dermal epidermal junction (DEJ) functions primarily as an anchoring complex consisting of a continuum of molecules that begin in the basal keratinocytes and extend into the dermis. This unit also functions as a barrier between the epidermis and dermis thereby impeding cell transmigration and passively regulating exchange of macromolecules (Briggaman and Wheeler, 1975, Burgeson and Christiano, 1997, Quondamatteo, 2014). The central component of the DEJ is the epidermal basement membrane (BM). The basal keratinocytes are attached to the BM primarily through specialised junctional complexes called hemidesmosomes (HDs), but also through focal
adhesions between the HDs. The BM in turn is attached to the dermis (Fig. 1.5). The DEJ can only be visualised at the ultrastructural level, and indeed, analysis by transmission electron microscopy (TEM) is the only method that can reveal structural disturbances that can subsequently have detrimental effects on the stability and integrity of the skin.

**Figure 1.5 Ultrastructure of the Dermal Epidermal Junction.** Image showing a keratinocyte (K) adhered to the basement membrane (BM). The lamina lucida (LL) and the lamina densa (LD) are clearly evident. The LD is connected with the underlying dermis (D) via anchoring fibrils (AF), which form the lamina fibroreticularis. Hemidesmosomes (white arrow) and focal adhesion (white arrowhead) are the main structures responsible for keratinocyte adhesion to the BM. While HDs are clearly recognisable as individual structures, the focal adhesions are not distinguishable at the ultrastructural level. However, these are known to be located in the region between hemidesmosomes. Scale bar = 500nm.

The epidermal BM, considered to be a typical BM, is a 50-100nm thick sheet-like layer of specialized extracellular matrix (Hashmi and Marinkovich, 2011, Inoue, 1989). It functions as the adhesive interface and the barrier between the two layers of the skin, as well as influencing cell behaviour and differentiation of keratinocytes (Breitkreutz et al., 2013, Quondamatteo, 2002, Breitkreutz et al., 2009). Using traditional chemical ultrastructural preparation techniques, it appears as two distinct layers, the Lamina
Densa (LD) and Lamina Lucida (LL) (Hashmi and Marinkovich, 2011, Quondamatteo, 2002). It should be noted that with the use of freeze fracture methods, no LL is detected. It is therefore suggested that the presence of this structure may just be an artefact of the dehydration process (Breitkreutz et al., 2013, Chan and Inoue, 1994, Hashmi and Marinkovich, 2011). Work carried out for this project utilises traditional chemical ultrastructural preparation techniques and therefore here the LL is considered.

The epidermal BM, like all BMs, consists of a complex network of proteins, the key components being collagen type IV isoforms, laminins, nidogens (1 & 2) and perlecan (Breitkreutz et al., 2013, Breitkreutz et al., 2009, Hashmi and Marinkovich, 2011, Quondamatteo, 2002). For the epidermal BM, the collagen type IV isoforms are \((\alpha_1(IV))_2\alpha_2(IV)\) and \((\alpha_5(IV))_2\alpha_6(IV)\) heterotrimers (Hasegawa et al., 2007). The laminin isoforms of the epidermal BM are laminin-332, laminin-321 and laminin-511 (Breitkreutz et al., 2013, Breitkreutz et al., 2009, Hashmi and Marinkovich, 2011).

The supramolecular structure of the BM is comprised of two separate networks that become intertwined together. One network is composed of collagen type IV, and the other network is composed of laminin. After their formation they are joined together by nidogen and perlecan and essentially become intermeshed (Breitkreutz et al., 2013, Breitkreutz et al., 2009, Quondamatteo, 2002). This is the traditional and accepted view of how BMs are structured. However, a more recent paper on the supramolecular structure of the epidermal BM may change this view (Behrens et al., 2012). In this paper it is reported that nidogen proteins become incorporated into laminin and collagen type IV networks. Once incorporated into either network they lose their affinity for the other network. Therefore it was found that nidogen proteins were not responsible for uniting the two networks. This function was attributed to perlecan only (Behrens et al., 2012). Irrespective of how the supramolecular structure of the BM is formed, it is basically the combined networks of laminins and collagen type IV that constitutes the LD.

A number of structures are involved in the adhesion of the epidermis to the BM (Fig 1.6). These are hemidesmosomes (HDs) which connect keratin intermediate filaments to the BM, and focal adhesions (FA) which connect the actin microfilaments to the BM (Hopkinson et al., 2014, Zhang and Labouesse, 2010).
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Basal keratinocytes are attached via Hemidesmosomes (HD) and Focal Adhesions (FA) to the underlying basement membrane which is composed of two layers the Lamina lucida (LL) and Lamina densa (LD). The central components of HDs and FAs are integrins α6β4 and α3β1 respectively. These integrins mediate the attachment to the basement membrane via Laminin-332, this interaction constitutes the anchoring filaments. CD151 interacts and regulates the activity of integrins α6β4 and α3β1. The basement membrane in turn is attached to the underlying dermis via anchoring fibrils. Inner plaque of HD (IQ), Outer plaque of HD (OQ). Adapted from (Tsuruta et al., 2011).

There are two types of HDs, those found in the skin are termed type I HDs and are typical in tissues that are exposed to abrasion and mechanical stresses. Epithelial tissues, such as the intestinal lining, contain type II HDs which are more fundamental in structure (de Pereda et al., 2009). For the purposes of this project only type I HDs are of interest, therefore, when the nomenclature HDs is used in the text it is generically referring to type I HD only.

In the skin, the HDs can be seen at the ultrastructural level studded along the plasma membrane of basal keratinocytes (Briggaman and Wheeler, 1975, Burgeson and Christiano, 1997, Quondammatteo, 2014). They are composed of inner and outer electron dense attachment plaques separated by an electron lucent region. Each HD serves as an attachment site intracellularly for intermediate filaments, and extracellularly for
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anchoring filaments that traverse the LL and insert into the LD of the BM (Briggaman and Wheeler, 1975, Burgeson and Christiano, 1997).

The major components of the inner plaque are two plakin family cytoskeletal linker proteins; plectin and bullous pemphigoid antigen 230 (BP230). These proteins are responsible for mediating attachment of intermediate filaments to the HD (de Pereda et al., 2009, Hopkinson et al., 2014, Zhang and Labouesse, 2010). In turn, these proteins bind to transmembrane proteins of the outer plaque, namely α6β4 integrin and BP180 [also termed type XVII collagen or bullous pemphigoid antigen 2 (BPAG2)] (Borradori and Sonnenberg, 1999, Hopkinson et al., 2014). These transmembrane proteins maintain strong adhesion to the BM through their extracellular domain which interacts with laminin-332 found at the upper part of the LD (Masunaga et al., 1996). It is these interactions, which traverse the LL, that constitute the anchoring filaments (Burgeson and Christiano, 1997). The cytoplasmic tail of the β4 subunit of the α6β4 integrin interacts with the other proteins of the HD i.e. plectin, BP230 and BP180. This interaction is important for maintaining the overall molecular integrity of the HD as well as playing a role in signalling pathways associated with cell growth, migration and survival (Borradori and Sonnenberg, 1999, Hopkinson et al., 2014). The cytoplasmic domain of BP180 is thought to function in the recruitment and stabilization of the other HD proteins and thus plays a major role in the assembly of this junction (Borradori and Sonnenberg, 1999, Hopkinson et al., 2014).

Between the HDs, the basal keratinocytes are adhered to the BM via FAs, with α3β1 integrin forming the major transmembrane component. FAs function to link the actin cytoskeleton of the basal keratinocytes, indirectly through actin binding proteins, to the BM via its interaction with laminin-332 (Carter et al., 1990, Watt, 2002, Xia et al., 1996). This integrin has been found to contribute to the overall integrity of the DEJ (DiPersio et al., 1997, DiPersio et al., 2000).

Another protein must be mentioned when discussing HDs and FAs, namely CD151, a member of the tetraspanin family of transmembrane proteins. This protein, which consists of four transmembrane domains, two short cytoplasmic tails, and two extracellular loops, interacts and regulates the activity of α6β4 and α3β1 during cell

The BM, in turn, is attached to the dermis, and this adherence is enhanced by anchoring fibrils. The anchoring fibrils extend from the BM into the reticular layer where they terminate either in anchoring plaques within the ECM or loop around to insert back into the BM. These fibres interact strongly with interstitial collagen fibrils thus providing anchorage of the epidermis to the dermis (Villone et al., 2008). The anchoring fibrils are composed of type VII collagen and attach to type IV collagen and laminin-332 in the BM (Burgeson and Christiano, 1997).

1.2.1.3 Desmosomes and Adherens Junctions

The mechanical adhesion between the keratinocytes of the epidermis is mediated by two types of intracellular junctions; desmosomes and adherens junctions (AJs) (Delva et al., 2009, Ivanov and Naydenov, 2013). Desmosomes are strong intracellular junctions that resist shearing forces placed on the tissue and are therefore typical of tissues, such as the skin, that are exposed to mechanical stress (Delva et al., 2009, Desai et al., 2009). AJs function in maintaining physical contact between cells and are essential for the formation and maintenance of cell-cell adhesion (Ivanov and Naydenov, 2013, Niessen, 2007). Both desmosomes and AJs work in synergy to maintain the overall cohesiveness of the epidermis. Their strength is provided by a complex of proteins that not only link the plasma membrane of neighbouring cells but also mediate a link to the cytoskeleton. In the case of desmosomes, this is to keratin intermediate filaments (Delva et al., 2009, Garrod and Chidgey, 2008), and in the case of AJs, this is to the microfilaments of the actin cytoskeleton (Ivanov and Naydenov, 2013, Niessen, 2007). It is the difference in the cytoskeletal components that these junctions attach that accounts for the higher mechanical competence of desmosomes.

Both these junctions can be clearly viewed ultrastructurally (Fig. 1.7a). Desmosomes, in the basal layer of keratinocytes, are located on the lateral and apical sides of the cells,
whereas in suprabasal layers they are found on all sides of the cells. AJs are found between desmosomes and appear simpler in structure than desmosomes when viewed ultrastructurally (Quondamatteo, 2014).

Figure 1.7 Ultrastructure of desmosomes and adherens junctions. a) Neighbouring keratinocytes (K) showing the presence of desmosomes (black arrow) and adherens junction (black arrowhead) Scale bar=500nm. b) High magnification image of a desmosome showing extracellular core region (red arrow), intracellular dense plaque (blue arrow) and inner plaque (yellow arrows). Scale bar=100nm. c) High magnification image of adherens junction (black arrowhead) showing extracellular core region (red arrow) and intracellular dense plaque (blue arrow). Scale bar=500nm.

Desmosomes are quite an obvious structure when viewing the ultrastructure of epidermis as they appear as dense button like structures between the cells. Each desmosome consists of two halves that are mirrored in the neighbouring cells. Each half of this junction consists of a dense plaque just inside the plasma membrane that is surrounded by a less dense region that constitutes the inner plaque. Extracellularly, the two halves of the desmosome sandwich an extracellular core region in which a dense midline and transverse striations can be seen (Delva et al., 2009, Garrod and Chidgey, 2008, Stokes, 2007) (Fig 1.7b).
The overall structural arrangement of AJs parallels that of the desmosome, consisting of two halves sandwiching an extracellular region, however, they are much less dense in appearance. In AJs the intracellular plaque is singular and much less obvious than that of desmosomes. Indeed this is also the case with the extracellular domain as it is electron lucent. As such AJs are ultrastructurally characterized by a pair of opposing plasma membranes with a distance of 10-20nm (Meng and Takeichi, 2009) (Fig 1.7c).

The proteins that make up the molecular structure of the desmosomes and AJs (Fig. 1.8) can be divided into three categories. These are; the transmembrane adhesion protein family of cadherins, the armadillo family of structural proteins, and cytoskeletal linker proteins (Delva et al., 2009, Garrod and Chidgey, 2008, Stokes, 2007, Ivanov and Naydenov, 2013, Niessen, 2007). While both junctions are constructed to a large extent by proteins of the same families of proteins, there is a high degree of specificity in terms of components of each junction.

![Figure 1.8 Schematic Representation of the Molecular Structure of Desmosome and Adherens Junctions](image)

Desmosomes and Adherens Junctions provide strong mechanical adhesion between cells by linking to the cytoskeleton, intermediate and actin filaments respectively. Desmosomes consist of transmembrane cadherins Desmocollin and Desmoglein, armadillo structural proteins, Plakoglobin and Plakophilin, and the cytoskeletal linker protein Desmoplakin. Adherens Junctions of keratinocytes consist of cadherin family protein E-cadherin, armadillo structural proteins p120-catenin and β-catenin and the cytoskeletal linker protein α-catenin. Adapted from (Garrod and Chidgey, 2008, Meng and Takeichi, 2009).
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Two subfamilies of cadherins specific to desmosomes, namely; desmogleins and desmocollins, mediate the adhesion of this junction. Their extracellular domains occupy the central core region where they bind with their counterparts from the neighbouring keratinocyte, thereby coupling the two halves of the desmosome. It is this interaction that constitutes the transverse striations and dense midline in the extracellular core region of the desmosome. The cytoplasmic tails of desmoglein and desmocollin proteins interact with the armadillo proteins plakoglobin and plakophilin. In turn, these armadillo proteins bind the cytoskeletal linker protein desmoplakin, a member of the plakin family of proteins. It is this complex of protein-protein interactions that comprise the outer dense plaque of the desmosome. Desmoplakin mediates the linking of intermediate filaments (Delva et al., 2009, Garrod and Chidgey, 2008) and microtubules (Lechler and Fuchs, 2007) to the desmosome. It is this interaction that constitutes the inner dense plaque of the desmosome.

The cadherin family protein at the core of AJs varies from tissue to tissue. In terms of the epidermis, E-cadherin is most prominent and is found to be expressed in all layers of the epidermis. Similar to the arrangement in desmosomes, the extracellular domain of E-cadherin binds with their counterpart in the neighbouring keratinocyte. Intracellularly, the cytoplasmic tails bind with armadillo proteins p120-catenin, which is essential for stabilising the junction at the surface, and β-catenin, which is essential for full adhesive power in the junction (Meng and Takeichi, 2009, Niessen, 2007, Takeichi, 2014). Interestingly, although plakoglobin is primarily associated with desmosomes, it can substitute for β-catenin within AJs (Niessen, 2007). The linkage of this junction to the cytoskeleton is mediated by the actin binding protein α-catenin which binds to the AJ via β-catenin.

The importance of desmosomes and AJs in maintaining the overall integrity of the epidermis is highlighted by human diseases that affect the different proteins that make this junction, as well as mouse models bearing genetic deletions (Brooke et al., 2012, Lai-Cheong et al., 2007). Defects in the proteins that contribute to the formation of these junctions can result in fragility of the epidermis, in many cases resulting in severe blistering (Lai-Cheong et al., 2007, Brooke et al., 2012).
1.2.1.4 Permeability Barrier

The permeability barrier of the skin functions primarily in preventing the loss of water and other components from the body (inside-out permeability), but also to protect against uptake of substances or pathogens from the external environment (outside-in permeability). This barrier is provided principally by the stratum corneum but also, to a lesser extent, by TJs present in the stratum granulosum (Baroni et al., 2012, Madison, 2003, Menon et al., 2012).

The stratum corneum consists of a number of layers of terminally differentiated keratinocytes, termed corneocytes, held together by modified desmosomes, termed corneodesmosomes (Madison, 2003, Menon et al., 2012). The corneocytes are dead cells filled with water and fibrillar keratin surrounded by a thick cornified envelop. The extracellular space between the corneocytes is occupied by lipids giving an overall ‘bricks and mortar’ arrangement to the stratum corneum (Fig. 1.9) (Menon et al., 2012).

Figure 1.9 Ultrastructure of ‘bricks and mortar’ of permeability barrier. Image shows stratum corneum (SC) held together by corneocytes (black arrowhead). Lamellar bodies (LB) extrude lipids (white arrows) that surround the corneocytes. Scale bar = 500nm. SG = Stratum granulosum.
The lipids of the stratum corneum are stored in specialised secretory organelles, lamellar bodies, in the keratinocytes of the stratum granulosum. Lamellar bodies can only be seen at the ultrastructural level where they can be characteristically recognised by their stacked lamellar internal structure (Madison, 2003, Menon et al., 2012). They extrude lipids from the apical surface of the outermost layer of the stratum granulosum into the space between this layer and the stratum corneum (Fig. 1.9). This ensures that as the keratinocytes differentiate into corneocytes they become surrounded by lipid layers (Menon et al., 2012).

The structure and function of TJs has been largely elucidated in simple epithelial and endothelial cells. They are the most apical cell-cell junction where they provide a continuous seal around the cells thereby preventing the paracellular passage of solutes, water and pathogens (Kirschner et al., 2010, Kirschner and Brandner, 2012). They also provide a fence function, preventing the mixing of apical and basolateral membrane proteins. Ultrastructurally these junctions are defined as regions where the plasma membranes of neighbouring cells come so close together that they appear as if they are fused (Kirschner et al., 2010, Kirschner and Brandner, 2012). These points are referred to as ‘kissing points’.

While the existence and the importance of TJs in simple epithelial tissue has long been established, their existence in the epidermis has only recently been identified (Brandner, 2009, Langbein et al., 2002, Morita et al., 1998, Pummi et al., 2001, Yoshida et al., 2001). Although early ultrastructural studies utilising electron dense tracers indicated the presence of a barrier provided by the stratum granulosum (Elias and Friend, 1975, Logan et al., 1978) it was thought that this was due to lipids secreted by the lamellar bodies (Brandner, 2009, Elias et al., 1977). However, it is now well established that TJs are formed in the epidermis, primarily in the stratum granulosum, and that they contribute to the permeability barrier of the skin (Brandner, 2009, Kirschner et al., 2010, Kirschner and Brandner, 2012).

The adhesive component of the TJs are composed of three families of transmembrane proteins; claudins, occludins and junctional adhesion molecules (JAMs) (Kirschner et al., 2010, Shin et al., 2006) (Fig 1.10). Claudins and occludins are the larger proteins of
this junction both consisting of four transmembrane strands, thereby having two extracellular domains and two intracellular domains. JAMs, which are members of the immunoglobulin superfamily, only consist of one transmembrane domain. All these proteins interact with their counterparts in the neighbouring cell through their extracellular domains, and with junctional scaffolding proteins through their intracellular domains (Kirschner et al., 2010, Shin et al., 2006).

The key junctional scaffolding proteins found in TJs are zonula occludens (ZO) proteins and cingulin (Kirschner et al., 2010). ZO proteins are scaffolding proteins within this junction. They have the capability of binding to the three transmembrane proteins, other peripheral junctional proteins, as well as directly to the actin cytoskeleton. Cingulin also functions in connecting the actin cytoskeleton to TJs through its interaction with the ZO-proteins and JAMs (Kirschner et al., 2010, Shin et al., 2006).

**Figure 1.10 Structure of Tight Junctions in Epidermis.** a) Shows ultrastructure of a tight junction in mouse skin (black box) located above a desmosome (black arrow) between neighbouring keratinocytes (K) in the upper layer of the stratum granulosum (SG). SC = Stratum corneum, Scale bar = 500nm. b) Schematic representation of the molecular structure of tight junctions. Adapted from (Niessen, 2007).
1.2.2 The Dermis

The dermis is the connective tissue layer of the skin that provides support to the epidermis. It is composed mainly of collagen fibrils, providing strength, and elastic fibres, providing flexibility, embedded in an interstitial matrix (Cleary, 1996, Quondamatteo, 2014). Interspersed within this matrix are three main cell types namely fibroblasts, mast cells and macrophages (Ross and Pawlina, 2011, Quondamatteo, 2014) (Fig. 1.11).

Collagens account for the vast majority, roughly 80%, of the total dermis (Ross and Pawlina, 2011). Of all the collagen types found in the dermis, collagens I, III, and V, which are fibril forming collagens, are most abundant. These collagen fibres aggregate into parallel bundles thereby forming fibrils. The more fibres within the fibril, the thicker and stronger it becomes (Cleary, 1996, Krieg and Aumailley, 2011). Other collagens of the dermis include FACIT (fibril associated collagens with interrupted triple-helices) collagens XII and XIV, and micro-fibrillar forming collagen VI, which function in organisation and stability of the extracellular matrix (ECM) (Kielty and Shuttleworth, 1997, Krieg and Aumailley, 2011, Shaw and Olsen, 1991). Elastin forms
the core of elastic fibres within the dermis. The outer core of these structures is formed by microfibrils which are predominantly composed of fibrillins (Kielty et al., 2002). These elastic fibres are found interwoven between collagen fibrils where their flexibility complements the tensile strength of collagen (Cleary, 1996, Kielty et al., 2002). Based on the size of collagen fibrils and elastic fibres, the dermis can be divided into two layers. The more superficial layer, the papillary dermis, contains finer collagen and elastic fibres, and the deeper reticular layer, contains fibres that are denser (Cleary, 1996, Quondamatteo, 2014).

The interstitial matrix is a clear viscous amorphous material composed primarily of proteoglycans and glycoproteins which are primarily involved in the organisation of the ECM (Bosman and Stamenkovic, 2003, Iozzo and Schaefer, 2010, Jarvelainen et al., 2009). The most prominent proteoglycan in the dermis is decorin, a member of the small leucine rich proteoglycan family. Decorin is involved in the deposition and organisation of collagen fibres (Bosman and Stamenkovic, 2003, Neill et al., 2012, Schaefer and Iozzo, 2008, Kennedy and Thorley, 2000). This function is mediated by direct binding of the horseshoe shaped decorin to collagen fibrils, and this interaction is critical to maintaining the structural and mechanical integrity of the skin (Iozzo and Schaefer, 2010, Neill et al., 2012, Fleischmajer et al., 1991, Scott, 1996). In addition to its role in collagen fibrillogenesis, decorin has also been found to directly bind a variety of cytokines and growth factors and is now recognised to be involved in signalling in many cellular functions including adhesion, migration, proliferation, differentiation and the inflammatory response (Bosman and Stamenkovic, 2003, Iozzo and Schaefer, 2010).

Fibroblasts are metabolically active cells that are responsible for the production of all the ECM components, proteases for ECM degradation, and monitoring the interstitial fluid volume and pressure (McAnulty, 2007, Ross and Pawlina, 2011, Sorrell and Caplan, 2004). These cells are therefore vital for the regulation and maintenance of the ECM under normal physiological conditions. They are also critical in the repair of tissue following injury, where in response to inflammatory signals they differentiate into myofibroblasts and are recruited to the injury site in order to lay down new ECM (Eckes et al., 2010, McAnulty, 2007, Sorrell and Caplan, 2004). In addition to their role in
ECM maintenance, fibroblasts also secrete cytokines and growth factors, therefore, they influence the activity of other mesenchymal cells as well as keratinocytes for both normal physiological homeostasis and tissue repair (Abraham et al., 2007, Eckes et al., 2010, Ghahary and Ghaffari, 2007, Rubinchik and Levi-Schaffer, 1994, Sorrell and Caplan, 2009).

Mast cells are granule containing cells found distributed within the dermis primarily in the region of small blood vessels, hair follicles, sebaceous and sweat glands (Ross and Pawlina, 2011). These cells are important for the regulation of inflammation and immunity during allergic and anaphylactic reactions and pathogen invasion. They are also thought to play a role in all stages of wound healing (Harvima and Nilsson, 2011, Tay et al., 2014, Wilgus and Wulff, 2014), even though new data suggest they are dispensable for this (Antsiferova et al., 2013, Willenborg et al., 2014). Upon activation mast cells degranulate and secrete their substances, which include cytokines, growth factors, proteases and lipid and vasoactive mediators, into the ECM (Bischoff, 2007, Wilgus and Wulff, 2014). Therefore, in addition to their role in inflammation and in the immune response, these cells may also influence a wide variety of biological functions including tissue homeostasis, remodelling, and angiogenesis (Bischoff, 2007, Reber et al., 2012, Voehringer, 2013).

Macrophages are large phagocytic cells that play vital roles during wound healing, infection, tissue resorption and immune response (Mahdavian Delavary et al., 2011, Ross and Pawlina, 2011). Resident macrophages function primarily to engulf and thereby remove dead and dying cells from the tissue for normal homeostasis and during wound healing. They also play a major role in the immune response. In response to invasion, macrophages are recruited to engulf and digest the pathogens and other foreign materials. They also function as proinflammatory cells by recruiting more macrophages if needed, recruiting other inflammatory cells and also working as antigen presenting cells. Macrophages are therefore essential for the normal homeostasis of the skin under normal conditions, under invasion and during wound healing (Mahdavian Delavary et al., 2011, Ross and Pawlina, 2011).
Chapter 2

Rho GTPases and the Maintenance of the Ultrastructural Integrity of the Interfollicular Epidermis in vivo
2.1 Introduction

As discussed in Chapter 1, a critical aspect of the structural integrity of the interfollicular epidermis (IFE) is the ability of keratinocytes to form a stable cohesive sheet which is fundamentally achieved through cell-cell and cell-matrix junctions. The key role of Rho GTPases in regulating the integrity of the IFE is through maintaining these cell-cell and cell-matrix junctions. Such junctions are not stagnant structures as in response to the needs of the tissue, the formation and deformation of junctions and indeed reorganisation of existing junctions is constant (Nekrasova and Green, 2013, Petit and Thiery, 2000, Takeichi, 2014, Tsuruta et al., 2003, Zhang and Labouesse, 2010). This is not limited to the actual junction, but also with the interaction of the junction and the cytoskeleton, which in turn itself also undergoes constant reorganisation (McCormack et al., 2013, Wehrle-Haller, 2012). The molecular switch function provided by Rho GTPase proteins is central to providing cells with the ability to adapt these junctions in response to their environment (McCormack et al., 2013, Petit and Thiery, 2000, Takeichi, 2014, Wehrle-Haller, 2012, Citi et al., 2011).

Work contained in this thesis forms part of a larger project conducted in collaboration with Professor Cord Brakebusch, of the Biotech and Innovation Centre (BRIC) in the University of Copenhagen, investigating the role of Rho GTPases in the development, maintenance, wound healing, inflammation and cancer in skin in vivo. The models utilised for this function are mice bearing deletion of different Rho GTPases and their effectors using the Cre/LoxP system driven by Keratin 5 as a promoter. Keratin 5 is expressed in stratified squamous epithelium (Chu and Weiss, 2002) and therefore this model results in targeted gene deletion in the keratinocytes of the skin. During this analysis, specific questions were raised regarding structural aspects of the IFE that can only be answered using transmission electron microscopy (TEM). To this end, firstly the involvement of N-WASP, a downstream effector of Cdc42, in maintaining the overall integrity of the IFE is examined. Secondly, the role of RhoA in maintaining the integrity of desmosomes, AJs and the DEJ is investigated. Thirdly, the role of Rac1 in maintaining the integrity of the permeability barrier of the IFE is examined.
2.2 N-WASP and the Ultrastructural Integrity of the Interfollicular Epidermis

In this section ultrastructural work on the involvement of N-WASP in maintaining the overall integrity of the IFE is presented.

Neural Wiskott-Aldrich Syndrome Protein (N-WASP) is a member of the WASP/WAVE family of proteins which regulate actin polymerisation through the Actin related protein 2/3 (Arp2/3) complex (Pollitt and Insall, 2009). The interaction between N-WASP and the Arp2/3 complex has been linked to the maintenance of endothelial and epithelial AJs in vitro and in vivo (Lie et al., 2010, Rajput et al., 2013, Kovacs et al., 2011). There is also indication that N-WASP may also be involved in maintaining the structure of desmosomes. N-WASP has been shown to mediate the connection to the cytoskeleton in desmosome-like junctions in the seminiferous epithelium of the rat testis (Lee et al., 2004). One of the key proteins involved in the activation of N-WASP, and subsequently Arp2/3, is the Rho GTPase protein Cdc42 (Symons et al., 1996).

Cdc42 has been linked to a variety of cellular functions, including, but not limited to, actin cytoskeleton reorganisation, cell polarity, proliferation, and apoptosis (Jaffe and Hall, 2005, Pedersen and Brakebusch, 2010, Spiering and Hodgson, 2011). Of particular interest in terms of its role in maintaining epidermal integrity is work carried out in mice bearing a keratinocyte restricted deletion of Cdc42 in the skin (Wu et al., 2006a, Wu et al., 2006b). In this model, Cdc42 was shown to play a role in both the proper deposition of BM components and the maintenance of its structure in the skin (Wu et al., 2006a). This Cdc42 deletion also resulted in defects in polarized deposition of laminin-332, and a pathological accumulation of its unprocessed chains in many regions of the DEJ. At a later stage misdirected collagen type IV and nidogen deposition were found at the DEJ, which the authors suggest may be a knock on effect from the defects in proper deposition of laminin-332 (Wu et al., 2006a). Deletion of Cdc42 also resulted in altered expression of α6, β4, and β1 integrins, which showed expression restricted to basal keratinocytes in the control mice, but were also found to be expressed in suprabasal layers in these mice bearing a keratinocyte restricted deletion of Cdc42 in the skin mice.
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However, the appearance and distribution of HDs was comparable to controls with no evidence of blistering at the DEJ (Wu et al., 2006a).

The effect of deletion of Cdc42 in keratinocytes was not restricted to the DEJ. It was also shown that this Rho GTPase protein was necessary for the formation (Du et al., 2009) and maintenance (Wu et al., 2006b) of mature cell-cell junctions. In terms of maintaining mature cell-cell junctions, keratinocyte restricted deletion of Cdc42 resulted in decreased expression of E-cadherin, plakoglobin, β-catenin and α-catenin in 4.5 month old mice, which caused reduced cell-cell adhesion and thus intraepidermal blistering (Wu et al., 2006b). It was determined that the underlying cause of these defects was due to Cdc42 stabilisation of β-catenin levels through a pathway involving aPKCζ, and therefore in the absence of Cdc42 there was increased degradation of β-catenin (Wu et al., 2006b). Cdc42 regulation of aPKCζ was also found to be necessary for the formation of mature cell-cell junctions in keratinocytes, although in this case this was independent of β-catenin (Du et al., 2009).

The necessity for Cdc42 in maintaining junctional structure through this pathway was also reported for AJs in Drosophila (Georgiou et al., 2008). Interestingly this study found that N-WASP and Arp2/3 were the downstream effectors of this pathway for AJs maintenance (Georgiou et al., 2008). Like Cdc42, N-WASP has not only been linked to the maintenance of epithelial junctions but also to their formation. Two independent in vitro studies carried out in intestinal epithelial cells link N-WASP to the normal accumulation of E-cadherin and F-actin during the formation of cell-cell junctions (Ivanov et al., 2005, Otani et al., 2006).

To date, no studies indicate an involvement of N-WASP in either the formation or the maintenance of the BM, however, there are studies that indicate that N-WASP could play a role in maintaining junctions of the DEJ. N-WASP was found to co-localise with plectin, which in turn co-localised with epithelial integrins α6, α3, β1 and β4 in a colon carcinoma cell line (McInroy and Maatta, 2011). This co-localisation was found in the basal region of podosomes (McInroy and Maatta, 2011) which are cell protrusions that function in adhesion, migration and invasion (Linder and Kopp, 2005). In mouse embryonic fibroblasts bearing an N-WASP deletion, more dispersed localisation of the
β1 integrin was reported (Misra et al., 2007). This resulted in reduced adhesion of the fibroblasts to fibronectin, a component of the ECM, and a subsequent increase in the speed of migration (Misra et al., 2007). As discussed, deletion of Cdc42 in keratinocytes of the skin resulted in substantial defects at the DEJ, however, the downstream pathway involved was not elucidated (Wu et al., 2006a). These studies provide evidence that N-WASP may be a potential downstream target in the Cdc42 function in maintenance of the DEJ.

Taken together, N-WASP, like its activator Cdc42, has the potential to play a role in maintaining the overall structural integrity of the epidermis. The importance of this protein is highlighted, like with Cdc42, by the fact that N-WASP KO mice showed embryonic lethality (Lommel et al., 2001, Snapper et al., 2001). In order to investigate the role of N-WASP function in maintaining the structural integrity of the IFE mice bearing a keratinocyte-restricted deletion of N-WASP in the skin driven by the K5 promoter are utilised (Lefever et al., 2010). Immunofluorescent staining carried out in the lab of Professor Cord Brakebusch found these mice bearing a N-WASP deletion in keratinocytes of the skin showed normal expression of α6 integrin and laminin-332. Also, the distribution of E-cadherin and F-actin in these mice was found to be comparable to controls (Lefever et al., 2010). This provided indication that N-WASP was not affecting the integrity of the IFE. In order to confirm this finding ultrastructural analysis was applied, as this method is the only way of determining if any subtle defects not detectable at the light microscopic level are present.

### 2.2.1 Materials & Methods

#### 2.2.1.1 Mice

(Generated by the lab of Professor Cord Brakebusch (Lefever et al., 2010))

To obtain mice with a keratinocyte-restricted deletion of N-WASP, transgenic mice expressing Cre recombinase under the control of the K5 promoter (Ramirez et al., 2004) were intercrossed with mice homozygous for a floxed N-WASP allele (N-WASP (fl/fl)) (Lommel et al., 2001). Here after these mice will be referred to as K5-N-WASP
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deficient mice. Littermates (N-WASP (fl/+)) K5Cre and N-WASP (fl/fl) no Cre) were
used as controls. All mice were on a 129Sv/C57Bl6 outbred background. All animal
studies were carried out according to Danish rules of animal welfare.

2.2.1.2 Sampling, fixation and embedding

(These procedures were carried out in the Department of Histology in the Centre of
Anatomy, University Göttingen)

Following sacrifice small samples of back skin from 2 week old N-WASP control (n=2)
and K5-N-WASP deficient (n=2) mice were taken and fixed overnight in 3%
paraformaldehyde, 3% glutaraldehyde in 0.2M Sörensen’s buffer pH 7.4. Tissue
samples were washed for 20min in 0.2M Sörensen’s buffer before secondary fixation in
1% Osmium tetroxide (OsO₄) in 0.15 mM phosphate-buffered saline (PBS) pH 7.4 for 2
hours (X2 with 20min PBS wash in between). Following secondary fixation samples
were dehydrated through a graded series of ethanol and embedded in epon resin.

2.2.1.3 Sectioning, staining and image capture

All sectioning was carried out on a Reichert-Jung ultramicrotome. Semithin sections,
(1μm) for orientation purposes, were taken using a glass knife. These were collected and
placed on glass slides and then dried on a hotplate. Sections were stained with Toludine
blue for 10 seconds, rinsed with distilled water and dried on a hotplate. Ultrathin sections
(90-100nm) were cut using a 45° DiATOME diamond knife and
collected on 250 mesh copper grids. These sections were stained with uranyl acetate for
35min and lead citrate for 20min in a Leica EM AC20 stainer. Sections were examined
with a Hitachi H-7000 electron microscope fitted with a 1K Hamamatsu Digital Camera.
Images were captured using AMTV542 Image Capture Engine software.
This is the standard protocol of sectioning, staining and image capture for ultrastructural
analysis and will be referred to as such elsewhere in this thesis.
2.2.1.4 Qualitative Analysis of the Interfollicular Epidermis

Sections from control (n=2) and K5-N-WASP deficient (n=2) mice were taken and analysed for the overall integrity of the IFE, and the presence of normally structured desmosomes, AJs and the DEJ.

2.2.2 Results

Qualitative analysis of the IFE at low magnification did not reveal any substantial ultrastructural differences between control and K5-N-WASP deficient mice. The thickness and general morphology of the keratinocytes was comparable between control and K5-N-WASP deficient mice (Fig. 2.1).

![Ultrastructural Overview of the Interfollicular Epidermis of K5-N-WASP deficient mice](image)

**Figure 2.1 Ultrastructural Overview of the Interfollicular Epidermis of K5-N-WASP deficient mice.** Images show comparable ultrastructural morphology of the epidermis (E) overlying the dermis (D) in control and K5-N-WASP deficient mice. Scale bars = 2μm

At the DEJ, the epidermis was seen as a well demarcated layer overlying the dermis (Fig. 2.2 a & b). Keratinocytes appear closely adhered to the underlying BM. Electron dense HDs were clearly present studded along the basal plasma membrane, with no
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evidence of microblistering between these junctions in both groups. (Fig. 2.2 c &d). For the most part, the BM appeared ultrastructurally defined with a continuous electron dense layer; the LD, underlying an electron lucent layer; the LL. There were regions where an ultrastructurally defined BM was not present and appeared blurred in both groups (Fig 2.2 e & f). No regions of clear breakage or discontinuation of the BM was observed.

Figure 2.2 Ultrastructure of the DEJ in control and K5-N-WASP deficient mice. (a&b) show the epidermis (E) as a well demarcated layer overlying the dermis (D). (c&d) show high magnification images where keratinocytes (K) are adhered to an ultrastructurally defined BM (blue arrow). HDs (yellow arrow) are seen studded along the BM. Also seen are keratin filaments (black arrowhead) extending from HDs. (e&f) show images of undefined BM where it appears blurred due to tangential cut (green arrow). Scale bars = 500nm.
The presence of normally structured desmosomes in K5-N-WASP deficient mice, comparable to controls, was clearly evident when intercellular areas were viewed at high magnification (Fig. 2.3a & b). Evidence of the presence of AJs was also found in both groups (Fig. 2.3c & d). Intercellular spacing was also seen in large portions of the IFE in both control and K5-N-WASP deficient mice (Fig. 2.3c&d).

**Figure 2.3** Ultrastructure of desmosomes and AJs in control and K5-N-WASP deficient mice. (a&b) show neighbouring keratinocytes with clearly evident desmosomes (black arrows). (c&d) show neighbouring keratinocytes with desmosomes and AJs (red arrows). Also evident in these images are intercellular spacing in regions between desmosomes (*). Keratin filaments (black arrowhead). Scale bars = 500nm.
2.2.3 Discussion

N-WASP has been linked to the maintenance of AJs in epithelial cells in vitro (Ivanov et al., 2005, Kovacs et al., 2011, Otani et al., 2006). There is also indication that N-WASP may also be involved in maintaining the structure of desmosome-like junctions in the seminiferous epithelium of the rat testis (Lee et al., 2004). In addition there are indications from in vitro studies that this protein may play a role in maintaining cell-ECM attachment at the DEJ (McInroy and Maatta, 2011, Misra et al., 2007). This evidence indicates that N-WASP would play a role in formation and maintaining the structural integrity of the IFE. Indeed, the deletion of Cdc42, a known activator of N-WASP, in keratinocytes in vivo resulted in defects in formation and maintenance of mature cell-cell junctions, as well as the polarised deposition of BM components at the DEJ (Du et al., 2009, Wu et al., 2006a, Wu et al., 2006b). Here, the ultrastructural examination of the IFE of mice bearing a keratinocyte restricted deletion of N-WASP did not reveal any such defects in the integrity of cell-cell junctions and, of the DEJ in two week old mice.

The epidermis appeared as a well demarcated layer overlying the dermis, with no evidence of any changes in thickness or general morphology. This would indicate that keratinocytes bearing a N-WASP deletion are undergoing normal differentiation and proliferation. This result is consistent with findings from immunofluorescent stainings, carried out in the lab of Professor Brakebusch, for differentiation and hyperproliferation markers in the IFE of 9 day old K5-N-WASP deficient mice (Lefever et al., 2010). However, in another study, utilising a similar in vivo model, but where the deletion of N-WASP occurs more slowly, thickening of the IFE, due to increased proliferation of keratinocytes was reported (Lyubimova et al., 2010). Although keratinocyte proliferation rate was increased in these K5-N-WASP deficient mice, the cells still underwent normal differentiation (Lyubimova et al., 2010). Interestingly, although these studies showed different results in the effects of N-WASP deletion on the rate of proliferation of keratinocytes of the IFE, they both showed defects in entering the anagen phase of hair follicle proliferation (Lefever et al., 2010, Lyubimova et al., 2010). This would indicate a possible effect of N-WASP on proliferation during
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hyperproliferative conditions, such as during hair growth. However, no effects on the rate of wound healing were found by either group (Lefever et al., 2010, Lyubimova et al., 2010). In addition to this, as part of this project on N-WASP, reduced proliferation in primary K5-N-WASP deficient keratinocytes in vitro, along with a defect in entering the anagen phase of hair growth due to an increase in TGFβ activity, was found by other members of the group, (Lefever et al., 2010).

At the DEJ, an ultrastructurally defined BM was present with keratinocytes closely adhered. HDs were present studded along the plasma membrane of the basal keratinocytes at the DEJ with no evidence of microblisters in the region between HDs. This result provides indication that N-WASP is not essential for maintaining the structural integrity of the DEJ. This is substantiated by immunofluorescent staining carried out in the lab of Professor Brakebusch showing normal expression of α6 integrin and laminin-332 in these K5-N-WASP deficient mice (Lefever et al., 2010).

The presence of normally structured desmosomes was evident in both control and K5-N-WASP deficient mice. Of note, although work carried out in another study utilising mice bearing a keratinocyte restricted deletion of N-WASP in the skin did not investigate the effects of this deletion on cell-cell junctions, they do report ulceration of the epidermis (Lyubimova et al., 2010). This began in a few individuals at 4-5 weeks, but by 20 weeks all of the mice with a N-WASP deletion in the keratinocytes of the skin had multiple ulcers (Lyubimova et al., 2010). These authors discuss that this may be attributed to defects in desmosomes, as mice bearing targeted deletion of desmocollin-1 show thickening of epidermis, alopecia and ulceration in the skin (Chidgey et al., 2001, Lyubimova et al., 2010). Interestingly, in the study on desmocollin-1 KO, the presence of ultrastructurally normal desmosomes is reported. However, ultrastructural examination of lesions led this group to conclude these were caused by weakened adhesion power in the desmosomes (Chidgey et al., 2001). Indeed, the appearance of ultrastructurally normal desmosomes was also reported for mice bearing a keratinocyte deletion of Cdc42 in the skin even though the expression of plakoglobin, β-catenin and α-catenin was reduced. In these mice ulceration of the skin was not reported, but the presence of microblisters in mice older than 4 months in most regions of the epidermis
was found (Wu et al., 2006b). Of particular interest is that in the study carried out by Lyubimova et al., 2010, a decrease in Glycogen synthase kinase 3 beta (GSK-3β) phosphorylation and subsequent decrease in the nuclear localisation of β-catenin was attributed to hair follicle progenitor cell differentiation. As discussed above, this pathway was found to cause defects in the hair follicles of the mice bearing a keratinocyte restricted deletion of Cdc42 in the skin, where eventually the total cellular reduction in β-catenin caused defects in the cell-cell junctions (Wu et al., 2006b).

In these studies, the phenotype resulting from targeted deletions becomes more severe with age. Therefore, while a protein may be sufficiently deleted from a tissue, it may take time before the effects on that tissue are obvious. Therefore, while no ultrastructural defects were observed in desmosomes in 2-week-old mice due to the deletion of N-WASP in keratinocytes, it is conceivable that like in the mice bearing a keratinocyte restricted deletion of Cdc42 in the skin, with increasing age defects may develop. However, there was no evidence of microblistering or ulceration found in the skin in the K5-N-WASP deficient mice used for this study, even up to 1 year old mice (Lefever et al., 2010, Brakebusch, 2014). Also, while changes in GSK-3β were found in the K5-N-WASP deficient mice used in this study, no other similarity to the mice bearing a keratinocyte restricted deletion of Cdc42 was found (Brakebusch, 2014, Wu et al., 2006b). Therefore taken together this indicated that the deletion of N-WASP does not affect the integrity of the desmosomes.

The ultrastructural analysis of AJs was not conclusive here due to widening of the intercellular spaces in regions between desmosomes in the majority of the IFE. This was attributed to effects of tissue processing and not the effect of the lack of N-WASP, as this intercellular spacing was found in both groups. Indeed, the presence of calcium chloride in fixation buffers provides a much better result with regard to the ultrastructure preservation of cell junctions. Nevertheless, in some regions, closely opposed cell membranes, typical of AJs, was observed. Therefore, although the processing of the tissue was not optimal, AJs of normal ultrastructural appearance in the absence of N-WASP were observed. This result in conjunction with immunofluorescent staining, performed in the lab of Professor Brakebusch, showing normal distribution of E-
cadherin and F-actin (Lefever et al., 2010) indicates that N-WASP is dispensable for maintenance of AJs.

These results show no obvious ultrastructural effects in the IFE due to the lack of N-WASP in two week old mice, although this does not rule out the possibility that ultrastructural defects may develop with age. However, in older mice, through light microscopy analysis in the lab of Professor Brakebusch, no indications of the development of defects in the IFE were evident (Lefever et al., 2010, Brakebusch, 2014). Taken together this indicates that N-WASP is dispensable for the maintenance of the ultrastructural integrity of the IFE.

2.3 RhoA and the Ultrastructural integrity of Desmosomes, Adherens Junctions and the Dermal Epidermal Junction

In this section, ultrastructural work clarifying the biological role of RhoA in maintaining the integrity of desmosomes, AJs and the DEJ is carried out. The exact role of RhoA in the formation and maintenance of desmosomes and AJs has still to be fully elucidated as to date contradictory evidence has been reported, primarily from in vitro studies. Inhibition of RhoA in keratinocytes in vitro was found not to have an effect on the formation and maintenance of desmosomes (Braga et al., 1997). In contrast to this, a study on pemphigus, an autoimmune disease that affects desmoglein resulting in skin blistering, found that the disruption of desmosomes was through interference with RhoA signalling (Waschke et al., 2006). Here, incubation of human skin explants, i.e., an ex-vivo model, with pemphigus IgG resulted in epidermal splitting, an effect that could be prevented in the presence of Cytotoxic Necrotizing Factor of Yersinia (CNFy), a toxin for the selective activation of RhoA. This group also found the same effect of CNFy in a human keratinocyte cell line. These in vitro experiments revealed that active RhoA could prevent pemphigus IgG induced cellular dissociation, keratin filament retraction, and the alteration of desmoglein, desmoplakin, and plakoglobin expression. Indeed incubation of these cells with pemphigus IgG alone
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reduced the activity of RhoA by 50%. This group therefore reported that RhoA is crucial for maintaining the integrity of desmosomes, most probably through the regulation of cytoskeletal anchorage to these junctions (Waschke et al., 2006). Interestingly, in another report it was found that RhoA was necessary for the formation of desmosomes, but that sustained activation prevented their maturation (Godsel et al., 2010). Plakophilin, via RhoA, induced actin cytoskeletal reorganisation necessary for the recruitment to and formation of desmoplakin-intermediate filament association for the maturation of the desmosome. While RhoA was necessary for the initial steps in this process, its sustained activation prevented complete maturation of the desmosome (Godsel et al., 2010). Therefore, RhoA has been implicated in being both essential and detrimental to the maintenance of the integrity of desmosomes. It appears that this is also the case for AJs.

It has been reported that inhibition of RhoA, or indeed RhoB or RhoC, with dominant negative mutants, did not affect the formation of AJs in two keratinocytes cell lines. Also cell-cell adhesion in this study could not be induced by the expression of dominant active forms of these proteins (Kee et al., 2002). Interestingly, Kee et al., 2002, discusses that the reason for failure of RhoA inhibition to block AJ formation, could be due to the level of inhibition. They report that the level was enough to block stress fibre formation, and that there is the possibility that the level of RhoA needed to maintain junctions is lower (Kee et al., 2002). On the other hand, in the study on pemphigus, incubation of keratinocytes in vitro with pemphigus IgG caused alteration in E-cadherin expression which could be prevented in the presence of active RhoA (Waschke et al., 2006). When keratinocyte cell lines were microinjected with C3 coenzyme, which inhibits all Rho subfamily members, the formation of AJs was prevented (Braga et al., 1997, Kee et al., 2002).

The involvement of RhoA in maintaining the structural integrity of the DEJ has not been investigated prior to this study. However, there is evidence that RhoA plays a role in BM maintenance in other systems. Loss of RhoA activity in vivo and subsequent destabilization of microtubules in the basal region of epiblast cells, were found to be necessary for BM breakdown for epithelial mesenchymal transition (EMT) during
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gastrulation in chick embryos (Nakaya et al., 2008). Exogenous expression of RhoA leads to retention and maintenance of the BM and subsequent defects in gastrulation (Nakaya et al., 2008). Similar results for RhoA preventing BM breakdown during neural crest cell EMT has been reported (Wu et al., 2011). In an epithelial cell line, integrin α6β4 mediated signalling through RhoA was found to be involved in the remodelling of both reconstituted and native BM during migration (Rabinovitz et al., 2001). This provides indication that RhoA is involved in maintaining the BM, and therefore the deletion of RhoA in keratinocytes could result in the breakdown of the BM.
To this end, the exact role of RhoA in the development and maintenance of keratinocyte cell-cell junctions, and the maintenance of the DEJ are still to be elucidated.

2.3.1 Materials & Methods

2.3.1.1 Mice

(Generated by the lab of Professor Cord Brakebusch (Jackson et al., 2011))

To obtain mice with a keratinocyte-restricted deletion of RhoA, transgenic mice expressing Cre recombinase under the control of the K5 promoter (Ramirez et al., 2004) were intercrossed with mice homozygous for a floxed RhoA allele (RhoA (fl/fl) K5Cre) (Jackson et al., 2011). Here after these mice will be referred to as K5-RhoA deficient mice. Littermates (RhoA (fl/+)) K5Cre and RhoA (fl/fl) no Cre) were used as controls.
Hosting and sacrifice of mice, harvesting and fixation of tissue samples was carried out in the Animal Facility of the BRIC (Copenhagen University), in accordance with all local ethical and legal requirements. For ultrastructural analysis small samples of back skin from RhoA control (n=6) and K5-RhoA deficient (n=6) mice, aged 2-7 months, were taken and fixed in 4% paraformaldehyde, 2% glutaraldehyde, in 0.1M N-acacodylate buffer, p.H 7.4, supplemented with 2mM CaCl2 (TEM fixation solution). Samples were then shipped to Anatomy, NUI Galway for further processing and analysis.
2.3.1.2 Secondary fixation

- Rinse in 0.1M Na-cacodylate buffer, p.H 7.4, supplemented with 2mM CaCl₂ (TEM Buffer) X 3.
- Secondarily fix in 1% OsO₄ in TEM buffer for 2 hours

2.3.1.3 Infiltration and embedding

- Dehydrate samples through a graded series of ethanol (30%, 50%, 70%, 90% 100%) 2 X 10min
- Incubate in propylene oxide for 1 hour
- Infiltrate samples with graded series of Agar low viscosity resin mixed with propylene oxide as follows
  - 50%-50% for 5 hours
  - 75%-25% overnight
  - 100% resin for 8 hours
- Place samples with labels in moulds containing 100% Agar low viscosity resin and incubate at 60°C for a minimum of 48 hours

2.3.1.4 Sectioning, staining and image capture

Samples were processed, sectioned and imaged for TEM as per standard protocol described previously. (See page 49)

2.3.1.5 Qualitative Analysis of the Interfollicular Epidermis

For each RhoA control (n=6) and K5-RhoA deficient (n=6) mouse 3 samples were taken and analysed for the presence of normally structured desmosomes, AJs and the DEJ.
2.3.1.6 Quantitative Analysis of the integrity of the DEJ

Both morphometric and stereological methods were employed to analyse the integrity of the DEJ. For each control (n=5) and K5-RhoA deficient (n=5) mouse 3 tissue samples were analysed. From each tissue sample 10 non-overlapping images of the interfollicular DEJ were sequentially taken at 20,000X magnification. All measurements, for parameters listed below, were taken using ImageJ software (Schneider et al., 2012) and data was collected and analysed using Microsoft Excel®. (Approximately 200μm of DEJ per mouse was analysed)

To evaluate the integrity of the DEJ the following parameters were evaluated.

1. **The presence of defined BM** at the DEJ as characterised by the presence of a clearly definable LD and LL. The length of this region was measured along with the length of regions of undefined BM. This was then totalled for each sample, and percentage of defined BM was calculated by dividing this total by the total length of DEJ analysed for each sample.

2. **Frequency of HD at the DEJ** was calculated by dividing the total number of HD by the total length of DEJ analysed for each sample, then multiplied by 10 to give the frequency per 10μm.

3. **Percentage of DEJ occupied by HDs** was determined by measuring the length of each HD and dividing their total length by the total length of DEJ analysed for each sample.

4. **Thickness of the defined BM, LD and LL** was determined by calculating arithmetic mean thickness. Sectioning of samples results in the BM being cut at different angles and therefore the apparent thickness tends to be overestimated (Dockery et al., 1998). In order to overcome this problem a stereological method based upon orthogonal intercepts was developed (Jensen, 1979) and is employed here.

To this end, using Adobe® Photoshop® cs2, a stereological square grid, was superimposed with accurate alignment onto the images in order to provide test lines (Fig. 2.4 (red lines)). Where the lower surface of the LD crosses a test line, the length of an orthogonal line (ln), drawn from this point to the plasma
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membrane (or midpoint of HD), and a second line to the upper surface of the LD was measured to determine BM and LD thickness respectively (Fig. 2.4 (green and blue lines)). By subtracting the value for LD from that for BM, the thickness of LL was calculated.

Figure 2.4 Measurement Method for Basement Membrane Thickness. Red lines = test lines. Green line = orthogonal line (ln), drawn from the lower surface of the LD to the plasma membrane (or midpoint of HD) to measure thickness of BM. Blue lines = orthogonal line (ln), drawn from the lower surface of the LD to the upper surface of the LD to measure thickness of LD. E = epidermis, D = dermis, Scale bar = 500nm.

The BM arithmetic mean thickness ($Ta$) is calculated using the following formula (Dockery et al., 1998, Jensen et al., 1979):

$$Ta = \frac{la}{4} \quad \text{where} \quad la = \sum \frac{ln}{n}$$

$la$ and subsequently $Ta$ was calculated per sample.

(Measurements for the thickness of the BM, LD and LL were carried out by Ms. Sinead Whyte, a final year BSc. student who performed these measurements under my co-supervision)
2.3.1.7 Statistics

Mean and standard deviation are shown for groups. For all parameters coefficient of variation (standard deviation/mean), expressed as percentage, was calculated for each group (interindividual variation) and also for each mouse in the group (intraindividual variation). Results were analysed using one way ANOVA and subsequent Tukey’s test in Mintab version 16 taking \( p = <0.05 \) as the level of statistical significance.

2.3.2 Results

2.3.2.1 Qualitative Analysis of the Interfollicular Epidermis in K5-RhoA deficient mice

Ultrastructural analysis of the interfollicular epidermis at low magnification revealed that the overall structure of the epidermis in K5-RhoA deficient mice was comparable to that of the control. All epidermal layers were found to be clearly defined and keratinocytes appeared to form a cohesive sheet in both groups (Fig. 2.5).

Figure 2.5 Ultrastructural Overview of the Interfollicular Epidermis in K5-RhoA deficient mice. Images show general overview of the IFE where the epidermis (E) appears as a well demarcated layer overlying the dermis (D). Scale bars = 2µm.
Figure 2.6 Ultrastructure of the Interfollicular Epidermis in K5-RhoA deficient mice. All images show neighbouring keratinocytes (K) with clearly evident normally structured desmosomes (black arrows). In (a&b) normally structured AJs (red arrows) are evident. Keratin filaments (black arrowhead). Scale bars = 500nm.
High magnification analysis of intercellular regions revealed normally structured desmosomes and AJs in K5-RhoA deficient mice (Fig. 2.6). The arrangement and localisation of these junctions do not appear to be altered due to RhoA deletion. No evidence of microblisters due to the lack of RhoA was found in the epidermis.

At the DEJ, keratinocytes appeared closely adhered to the underlying BM (Fig. 2.7). For the most part, the BM appeared ultrastructurally defined with a continuous electron dense layer; the LD, underlying an electron lucent layer; the LL. There were regions where an ultrastructurally defined BM was not present and appeared blurred in both groups (Fig. 2.7 e&f). No regions of obvious breakage or discontinuation of the BM was observed due to the deletion of RhoA. One occasion of BM discontinuation was observed in a control mouse due to the passage of an immune cell (Fig. 2.7 g). Electron dense HDs were clearly present studded along the basal plasma membrane, with no evidence of microblistering between these junctions in both groups.
Figure 2.7 Ultrastructure of the DEJ in K5-RhoA deficient mice. (a,b,c&d) show normally structured DEJ in both control and K5-RhoA deficient mice. Keratinocytes (K) are adhered to an ultrastructurally defined BM (blue arrow). HDs (yellow arrow) are seen studded along the BM. In (c&d) anchoring fibrils (orange arrow) can been seen extending from the BM into the underlying dermis (D). (e&f) show images of undefined BM where it appears blurred due to tangential cut (green arrow). Scale bars = 500nm. In (g) an immune cell passing through the BM (white/black arrow) in a control mouse can be seen. Scale bar = 2μm.
Quantitative analysis on the percentage of ultrastructurally defined BM at the DEJ revealed there was no statistically significant difference between controls and K5-RhoA deficient groups (Fig. 2.8). Interindividual and intraindividual variation for this parameter revealed a slight decrease in the K5-RhoA deficient group compared to the controls (Table 2.1). The frequency and percentage of DEJ occupied by HDs also showed no significant difference between the two groups (Fig. 2.8). Interindividual and intraindividual variation for these parameters showed similar levels, with only a slight increase in interindividual variation seen for the percentage of DEJ occupied by HDs in K5-RhoA deficient mice (Table 2.1).

The thickness of the defined BM at the DEJ was also calculated. No statistically significant difference was found in the thickness for the whole BM (Fig. 2.9). Consistent with this, is that no statistically significant difference was found for the individual layers of the BM, i.e. the LD and LL (Fig. 2.9). Interindividual variability for this parameter shows a slight decrease for the thickness of the LD in the K5-RhoA deficient group (Table 2.1). For intraindividual variability an increase is seen for the BM and LD in the K5-RhoA deficient mice, but the LL shows a similar level to the control (Table 2.1).

<table>
<thead>
<tr>
<th></th>
<th>Interindividual Variation</th>
<th>Intraindividual Variation</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>K5-RhoA deficient</td>
</tr>
<tr>
<td>% Defined BM</td>
<td>11%</td>
<td>6%</td>
</tr>
<tr>
<td>Frequency of HD</td>
<td>13%</td>
<td>15%</td>
</tr>
<tr>
<td>% of DEJ occupied by HD</td>
<td>13%</td>
<td>17%</td>
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<tr>
<td>BM Thickness</td>
<td>8%</td>
<td>7%</td>
</tr>
<tr>
<td>LD Thickness</td>
<td>12%</td>
<td>6%</td>
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<tr>
<td>LL Thickness</td>
<td>7%</td>
<td>9%</td>
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Table 2.1 Interindividual and Intraindividual Variation at the Dermal Epidermal Junction
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Figure 2.8 Quantitative analysis of the integrity of the DEJ in K5-RhoA deficient mice. (n=5).

- Presence of Defined Basement Membrane at DEJ:
  - Control
  - K5-RhoA deficient
  - p = 0.109

- No. of Hemidesmosome/10μm Defined Basement Membrane:
  - p = 0.586

- % of Defined DEJ Occupied by Hemidesmosome:
  - p = 0.195
Figure 2.9 Thickness of the DEJ in K5-RhoA deficient mice. (n=5).
2.3.3 Discussion

The function of RhoA in the maintenance of desmosomal structural integrity has still to be fully elucidated. It has been reported that this protein is vital for the development of this junction (Godsel et al., 2010). However, its role in maintaining the integrity of mature desmosomes is contradictory, being found to be necessary (Waschke et al., 2006), not essential (Braga et al., 1997) and indeed detrimental (Godsel et al., 2010). Contradictory results from in vitro studies are also reported in the case of AJs, being found to be both dispensable (Kee et al., 2002) and necessary (Waschke et al., 2006).

As previously discussed, these junctions are essential for the overall integrity of the IFE. Here ultrastructural analysis of the IFE in mice bearing a keratinocyte restricted deletion of RhoA in the skin, did not reveal any indication of pathological alterations. This is substantiated by histological and immunofluorescent work carried out within this project by other members of the group in the lab of Professor Cord Brakebusch. These findings revealed no obvious morphological differences between the control and K5-RhoA deficient mice, and also no indication of effects on differentiation or proliferation (Jackson et al., 2011).

Ultrastructural examination of desmosomes found that they were of normal structured appearance in K5-RhoA deficient mice. Although as discussed with the K5-RhoA deficient mice, desmosomes can appear to be structurally normal but still have reduced adhesive power (Chidgey et al., 2001) or alterations in desmosomal components (Wu et al., 2006b). This does not appear to be the case here as no evidence of blistering was found at the microscopic level (Jackson et al., 2011) and indeed in all specimens examined ultrastructurally at low magnification revealed that the keratinocytes appeared to form a cohesive epithelial sheet. This finding is also substantiated by immunofluorescent staining which showed normal expression of desmoplakin in K5-RhoA deficient mice (Jackson et al., 2011).

Similar to the results for desmosomes, the integrity of AJs did not appear to be affected by the deletion of RhoA. The ultrastructural appearance of AJs in K5-RhoA deficient
mice was comparable to that of the controls. Indeed immunofluorescent staining carried out within this project by other members of the group in the lab of Professor Cord Brakebusch showed normal expression of E-cadherin in the K5-RhoA deficient mice (Jackson et al., 2011). Interestingly, in the study carried out by Kee, et al., 2002, where RhoA was found to be dispensable for AJ integrity, the same effect was found when either RhoB or RhoC were inhibited (Kee et al., 2002), but when all Rho subfamily members (RhoA, RhoB and RhoC) were inhibited together the formation of AJs was prevented (Braga et al., 1997, Kee et al., 2002).

This would suggest a redundant function of RhoA, i.e. that the other members of the subfamily can compensate when RhoA is absent. Indeed, RhoA, RhoB and RhoC show 85% sequence homology, and they overlap greatly in their GEFs and downstream effectors, although their affinity for these effectors is different, and therefore they are responsible for different biological functions (Wheeler and Ridley, 2004). Given this consideration, many of the functions attributed to RhoA from in vitro studies utilising dominant negative or constitutively active mutants or the use of C3 exoenzymes may actually be the result of inhibiting all three Rho subfamily members (Zhou and Zheng, 2013).

In fact, while western blot analysis, carried out in the lab of Professor Brakebusch, could not detect expression of RhoC in either the control or K5-RhoA deficient mice, a four-fold increase in the expression of RhoB in the absence of RhoA was found (Jackson et al., 2011). Indeed RhoB has been reported to regulate AJs in Sertoli cells (Lui et al., 2003). Therefore, the fact that no defects in AJs were evident in this study may be due to the ability of RhoB to compensate for the lack of RhoA under normal physiological conditions. Mice bearing a constitutive deletion of RhoB, and indeed also those bearing a constitutive deletion of RhoC, do not display a developmental phenotype (Liu et al., 2001, Hakem et al., 2005), whereas constitutive deletion of RhoA in mice results in embryonic lethality (Wang and Zheng, 2007). This may suggest that RhoB or RhoC can compensate for RhoA to a certain extent, but not completely. To date, neither RhoB nor RhoC have been suggested to play a role in desmosome development and maintenance, but this does not preclude that the lack of an effect on desmosomes here is not due to compensation from RhoB. It would therefore be interesting to look at the
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effects on cell-cell junctions under stress conditions in these K5-RhoA deficient mice, or in mice bearing a double or triple deficiency of Rho subfamily members.

In terms of the DEJ, it was expected that deletion of RhoA in keratinocytes would result in the breakdown of the BM due to two studies which showed its inhibition to be involved in this process for EMT (Nakaya et al., 2008, Wu et al., 2011). Qualitative ultrastructural analysis revealed that the epidermis appeared as a well demarcated layer overlying the dermis in K5-RhoA deficient mice. An ultrastructurally defined BM was seen at most of the DEJ in both groups. Undefined regions were seen in both groups to a similar extent and are thought to be due to a tangential cut in these regions. Quantification of the presence of a defined BM revealed that this was not significantly changed due to the deletion of RhoA. A slight decrease in the interindividual and intraindividual variation for the percentage defined BM was seen for K5-RhoA deficient mice. This could suggest tighter regulation of the integrity of the BM in the RhoA deficient group. A tight control of variance suggests biological importance of that feature (Clegg, 1983, Dockery et al., 1988). Analysis of variance can be a very useful tool, particularly when studying models bearing deletions of proteins, in determining effects of this deletion. While the overall quantification of certain features may be the same, the variance may differ due to an alternative mechanism of regulation of that feature. However, the variation values here for both groups are below 15%, a low percentage of variability, with roughly 4% difference between the groups, which would indicate that this difference may be due to chance rather than deletion of RhoA.

HDs of normal appearance were found studded along the basal side of keratinocytes of the stratum basale with no evidence of microblisters between them in K5-RhoA deficient mice. Quantification of their number was carried out to determine the frequency of HDs and percentage of DEJ occupied by HDs. Any reduction or increase in the frequency of HDs due to RhoA deficiency would indicate the loss or gain respectively of junctions. The reason for also measuring the percentage of DEJ occupied by HDs was because while the frequency may stay the same, the size of the junctions may be affected. Actually, therefore the extent of BM attached by HDs is a better parameter in determining alterations in the integrity of these junctions. To this end,
using both methods to determine the integrity of HDs, it was found that there was no statistically significant difference in integrity of HDs due to the deletion of RhoA in keratinocytes. Interindividual and intraindividual variation for these parameters show similar levels with only a slight increase in interindividual variation seen for the percentage of DEJ occupied by HDs. Like with the percentage of defined BM, this level of variability is low and is more likely to be accounted for by chance than the RhoA deletion.

It is conceivable that measuring the thickness of the BM could provide indication for any changes in the amount of components forming the BM, i.e. it would become thicker with more components and thinner with less. This would be particularly evident in the thickness of the LD. Likewise, an increase in thickness of the LL would reflect more separation of the keratinocytes from the BM, which would provide indication for defects in the adhesive power of HDs or FAs. Here, this qualitative analysis revealed no statistically significant difference in the thickness of the BM or its layers, the LD and LL. Again the interindividual and intraindividual variability for these parameters is low. This indicates that deletion of RhoA in keratinocytes does not result in breakdown of the DEJ.

Taken together these results indicate that RhoA is dispensable for the maintenance of the integrity of desmosomes, AJs and the DEJ, and subsequently the integrity of the IFE.

2.4 Rac1 and the ultrastructural integrity of TJs in the interfollicular epidermis

In this section, an ultrastructural analysis of the role of Rac1 in maintaining the integrity of the permeability barrier of the IFE is examined.

Initial studies carried out in a mouse model bearing a keratinocyte restricted Rac1 deletion in the skin revealed no major disruption of the IFE, but did show severe defects in the hair follicles (Chrostek et al., 2006). Also reported was an increase in the numbers of macrophages within the dermis. As the numbers of macrophages decreased following
the removal of hair follicles, their presence in the dermis was attributed to the defects in hair follicles (Chrostek et al., 2006). However, subsequent studies in this model revealed that these mice have a heightened inflammatory response, even without stimulation (Pedersen et al., 2012). A number of possible reasons for this heightened inflammation were raised. Was this simply a remnant of the inflammatory response initiated during the breakdown of the hair follicles? Did the deletion of Rac1 in the keratinocytes result in aberrant crosstalk between keratinocytes and the immune system? Or, could this heightened inflammation be due to a defect in the permeability barrier of the skin due to Rac1 deficiency? In this context, the work carried out to analyse the third hypothesis is the one presented here.

As discussed previously the permeability barrier of the skin is provided principally by the ‘bricks and mortar’ arrangement of the stratum corneum and the TJs of the stratum granulosum (Menon et al., 2012, Quondamatteo, 2014). However, it has been recently demonstrated in claudin1-/- deficient mice that the TJs of the stratum granulosum contribute to the establishment and maintenance of the stratum corneum barrier function (Sugawara et al., 2013). Therefore, TJs may be playing a greater role in this barrier function of the epidermis than previously thought. Indeed, defects in the TJs have been shown to be a contributing factor to the barrier dysfunction characteristic of the chronic inflammatory skin disease, atopic dermatitis (De Benedetto et al., 2011). This therefore provides a precedent that defects in the TJs may contribute to the overall disruption of the permeability barrier of the epidermis. The structural components that make up this permeability barrier can only be examined ultrastructurally.

Studies utilising dominant negative or constitutively active mutants of Rac1 in epithelial cell lines reported contradictory results regarding the involvement of this protein in formation and activity of TJs (Bruewer et al., 2004, Eisen et al., 2006, Jou and Nelson, 1998, Jou et al., 1998). Indeed either activation or inhibition resulted in defects in the TJ structure and function corresponding with increased paracellular permeability, reorganization of the actin cytoskeleton and alteration in TJ protein localisation (Bruewer et al., 2004, Eisen et al., 2006, Jou and Nelson, 1998, Jou et al., 1998). It is possible that the reason for such similar affects from either constitutively active or
inactive Rac1 may be due to the inability of this protein to cycle, i.e. turn on or off. The maintenance of junctions, as mentioned previously, is a dynamic process, constantly undergoing reorganisation. This is mediated in part by the switch in Rho GTPase proteins. Therefore, the inability of cells to turn off Rac1 when no longer required, could affect the integrity of the TJ as much as not being able to activate this protein when needed. Indeed a report by Guillemot, L 2008, showed that during the formation of TJs, in a kidney epithelial cell line, there were two peaks in Rac1 activity, early (10-20 minutes) and later (3-8 hours) (Guillemot et al., 2008). This study therefore indicates that constant Rac1 activity is not involved in normal TJ formation (Guillemot et al., 2008). Interestingly in this study, the later peak in Rac1 activity corresponded with a peak in transepithelial resistance (TER), a method that measures the tightness of TJs and as such the state of the permeability barrier, therefore indicating Rac1 is playing a role in formation and integrity of the permeability barrier (Guillemot et al., 2008). However, the use of a Rac1 inhibitor in a lung epithelial cell line resulted in an almost immediate promotion of TER (Lorenowicz et al., 2007). These studies provide evidence that Rac1 is indeed influencing TJs, but the effects in different epithelial cell lines indicate that its function is not simplistic.

Studies on the involvement of Rac1 in the TJ function in keratinocytes seem to support the importance of this protein in the integrity of TJs. During the formation of TJs, it was found that a Rac1-aPKC mediated signalling pathway was necessary for recruitment of JAMs, occludins and claudins in primary and immortalised keratinocytes (Mertens et al., 2005). UVB irradiation of keratinocytes reduced the activity of both Rac1 and aPKC, resulting in dispersed occludin and claudin expression, and indeed reduced TER (Yuki et al., 2011). Kirschner, N 2011, provides both in vivo and in vitro evidence that active Rac1 may play a role in TJ formation (Kirschner et al., 2011).

To date, no studies link Rac1 to the formation or secretion of lamellar bodies. However, for proper secretion, cells must display apical polarisation and cell surface specialisations. As mentioned previously, along with providing a paracellular barrier, TJs also provide a fence function, preventing the mixing of apical and basolateral membrane proteins, thereby contributing to cell polarisation (Kirschner et al., 2010,
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Kirschner and Brandner, 2012, Shin et al., 2006). Therefore any defects caused in the structure and function of TJs by deletion of Rac1 may be reflected in misdirected extrusion of lamellar body contents, for e.g. extrusion of their contents at the lateral border of the cells.
It is therefore reasonable to assume that deletion of Rac1 in keratinocytes could affect the integrity of the permeability barrier.

2.4.1 Materials and Methods

2.4.1.1 Mice

(Generated by the lab of Professor Cord Brakebusch)

To obtain mice with a keratinocyte-restricted deletion of Rac1, transgenic mice expressing Cre recombinase under the control of the K5 promoter (Ramirez et al., 2004) were intercrossed with mice homozygous for a floxed Rac1 allele (Rac1 (fl/fl) K5Cre) (Chrostek et al., 2006). These mice will be referred to as K5-Rac1 deficient mice. Littermates (Rac1 (fl/+)) K5Cre and Rac1 (fl/fl) no Cre) were used as controls. All mice were on a 129Sv/C57Bl6 outbred background. All animal studies were carried out according to Danish rules of animal welfare.
Hosting and sacrifice of mice, harvesting and fixation of tissue samples was carried out in the Animal Facility of the BRIC (Copenhagen University), in accordance with all local ethical and legal requirements. For ultrastructural analysis small samples of back skin from control (n=1) and K5-Rac1 deficient (n=2) mice, aged 30 weeks old, were taken and fixed in TEM fixation solution. Samples were then shipped to Anatomy, NUI Galway for further processing and analysis.

2.4.1.2 Secondary fixation

In order to ultrastructurally analyse the possible leakiness of the TJs of the K5-Rac1 deficient mice as a potential cause for the increase in susceptibility to inflammation, a
number of different post-fixation methods utilizing different combinations of heavy metals and an electron dense tracer were employed.

In a study determining the optimal protocol for the examination of the ultrastructure of skin it was found that the best post-fixation protocol for the preservation of skin morphology was achieved using 1% OsO₄, 0.2% Ruthenium tetroxide (RuO₄) with 0.25% potassium ferricyanide (K₃Fe(CN)₆) (Van den Bergh et al., 1997). This post-fixation method is employed and adapted here.

Electron dense tracers enable the delineation of extracellular spaces, and assist in studying intercellular junctions and also biological permeability barriers. The electron dense tracer employed for this study is Lanthanum nitrate (La(NO₃)₃), a trivalent cation. In order for this heavy metal to be used as a tracer it must be used in a pH range of 7.2 to 8.5 which gives the solution a higher colloidal to ionic ratio. Therefore, the tracer precipitates immediately and cannot cross the membrane. In 1967 Revel and Karnovsky published a technique utilizing this property of La(NO₃)₃ for visualization of intercellular junctions. By adding La(NO₃)₃ at the appropriate pH to all solutions up to dehydration the extracellular space becomes filled with this electron dense material. They also reported that while it is best to add the La(NO₃)₃ to all the solutions, it can just be added to the OsO₄ (Revel and Karnovsky, 1967). A variation on this method was employed to try and essentially outline each of the cells of the stratum granulosum to make it easier to find the tight junctions. This method also aimed to help visualize the leakiness of the tight junctions; if tight junctions are not functioning correctly then the La(NO₃)₃ should be found between the two membranes of the tight junction.

**Post-fixation 1: OsO₄ and RuO₄**

- Rinse in TEM buffer X3
- Post fix with 1% OsO₄ in TEM buffer for 1 hour at 4°C in the dark.
- Rinse in TEM buffer X3
- Secondarily post fix with 0.2% RuO₄ with 0.25% K₃Fe(CN)₆ for 2 X 1 hour at 4°C in the dark.
- Rinse in TEM buffer X3
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Post-fixation 2: OsO₄ and extended RuO₄

- Rinse in TEM buffer X3
- Post fix with 1% OsO₄ in TEM buffer for 1 hour at 4°C in the dark.
- Rinse in TEM buffer X3
- Secondarily post fix with 0.2% RuO₄ with 0.25% K₃Fe(CN)₆ for 2 X 2 hour at 4°C in the dark.
- Rinse in TEM buffer X3

Post-fixation 3: RuO₄

- Rinse in TEM buffer X3
- Post fix with 0.5% RuO₄ with 0.25% K₃Fe(CN)₆ for 2 X 2 hours at 4°C in the dark.
- Rinse in TEM buffer X3

Post-fixation 4: OsO₄, RuO₄ and La(NO₃)₃

- Rinse in TEM buffer containing 2% La(NO₃)₃ X3
- Secondarily post fix with 1% OsO₄, 2% La(NO₃)₃ in TEM buffer in the dark at room temperature.
- Rinse in TEM buffer containing 2% La(NO₃)₃ X3
- Secondarily post fix with 0.2% RuO₄ with 0.25% K₃Fe(CN)₆ for 2 X 1 hour in the dark.
- Rinse in TEM buffer containing 2% La(NO₃)₃ X3

All steps for this post-fixation method were carried out on a rotator.

Post-fixation 5: OsO₄ and La(NO₃)₃

- Place samples in a 50:50 mix of the TEM fixation solution and 4% La(NO₃)₃ to give a final concentration 2% paraformaldehyde, 1% glutaraldehyde and 2% La(NO₃)₃, overnight at room temperature in the dark. This was carried out in order to see if it was possible to get the La(NO₃)₃ to penetrate the tissue by diluting the fixation solution.
- Rinse in TEM buffer containing 2% La(NO₃)₃ X3
• Secondarily post-fix with 1% OsO₄, 2% La(NO₃)₃ in TEM Buffer in the dark at room temperature for 2 hours.
• Rinse in TEM buffer containing 2% La(NO₃)₃ X3

2.4.1.3 Infiltration and embedding

As described previously (See page 59)

2.4.1.4 Sectioning, staining and image capture

Samples were processed, sectioned and imaged for transmission electron microscopy as per standard protocol described previously with the following minor changes:
  - Some semithin sections were left unstained
  - Ultrathin sections were collected on 200 mesh formvar-coated copper grids

RuO₄ is a very highly lipophilic compound and therefore it is an optimal metal for increasing contrast of the cell membranes at the TEM. In addition to this, another advantage of using RuO₄, for the purpose of analyzing the stability of the permeability barrier or more particularly the leakiness of the TJs is that it has high reactivity with the tissue and generally its slow penetration through the stratum corneum, but not the other exposed edges of the samples. The reactivity of the RuO₄ causes artifactual damage to the exposed surfaces of the tissue making it appear to be chemically burned turning brown/black colour and torn. Due to the slow penetration of the RuO₄ into the stratum corneum it should not cause any artifactual damage to the IFE unless there is a defect in the permeability barrier. Therefore during this study the level of penetration/reactivity of the RuO₄ was analysed by looking at both stained and unstained semithin sections.

Stratified epithelia, such as the epidermis, do not show the typical TJ structure as identified in simple epithelia (Langbein et al., 2002). In stratified epithelia they range from a single ‘kissing point’ to an extended close contact junction. Therefore for identification of TJs in the IFE the study by Langbein, L et al 2002 was referenced (Langbein et al., 2002).
2.4.2 Results

2.4.2.1 Analysis of penetration/reactivity of RuO₄

Semithin sections from samples using post-fixation method 1 (OsO₄ and RuO₄) revealed that the edges of the tissue had reacted with the RuO₄ resulting in a black/brown colouration of the tissue in this region in both the control and K5-Rac1 deficient mice (Fig. 2.10 a&b). When the stratum corneum was viewed at higher magnification (Fig. 2.10 c-f), this same type of colouration to the stratum corneum was seen. However, the other layers of the IFE appeared unaffected in both groups.

Figure 2.10 OsO₄ and RuO₄ post-fixation of K5-Rac1 deficient skin. (a&b) show an overview of the sections in which a black colouration can be seen at the edges of the tissue samples (red arrows). Scale bars = 100µm. (c-f) shows higher magnification of toluidine blue stained (c&d) and unstained sections (e&f) where colouration of the stratum corneum is visible (red arrows). Scale bars = 20µm. E = epidermis, D = dermis.
A similar result on the effects of penetration of heavy metals seen for post-fixation method 1, was also found with post-fixation method 2 (OsO$_4$ and extended RuO$_4$). However, in samples post-fixed using method 3 (RuO$_4$) the level of penetration/reactivity of the RuO$_4$ into the tissue from the exposed sides and dermis below, was much greater when compared with the previous two methods of post-fixation. Most the cells of the cornified layer appear to have been artifactual damage as they have become black/brown in colour due to the RuO$_4$ treatment, but the rest of the IFE appears unaffected (Fig. 2.11). No difference in the degree of tissue reactivity could be seen between the control and K5-Rac1 deficient samples.

![Figure 2.11](image)

**Figure 2.11 RuO$_4$ post-fixation of K5-Rac1 deficient skin.** (a&b) show an overview of the sections in which a black colouration can be seen at the edges of the tissue samples (red arrows). Scale bars = 100µm. (c-f) shows higher magnification of toludine blue stained (c&d) and unstained sections (e&f) where colouration of the stratum corneum is visible (red arrows). Scale bars = 20µm. E = epidermis, D = dermis.
Like with the previous post-fixation methods, post-fixation method 4 (OsO₄, RuO₄ and La(NO₃)₃) caused discolouration and damage to the stratum corneum and cut edges of the tissue samples in both groups. Additionally, as seen in Fig. 2.12a, an isolated region of black colouration on either side of a hair follicle in one of the K5-Rac1 deficient samples was found. Ultrastructural analysis showed this region to appear heavily stained with a number of tears in the sections due to the instability of this region (Fig. 2.12b&c).

Images b and c in Fig. 2.12 were taken from the same region with approximately 2µm distance between them showing that the extent of staining in the region decreases as more sections are taken from the block. After a number of sections the heavily stained region was no longer found indicating that it had been cut through. A keratinocyte in the outer layer of the stratum granulosum with no heavy colouration was seen, while keratinocytes underneath did display such colouration (Fig. 2.12b&d). The heavy staining continued laterally in the region of the basement membrane for a short distance (Fig. 2.12e).

No similar region of colouration was found when this sample from this K5-Rac1 deficient mouse was serial sectioned (Fig. 2.13). In addition, serial sectioning of a control sample, another sample from this K5-Rac1 deficient mouse, and also two samples from the other K5-Rac1 deficient mouse did not lead to the discovery of any other such region of colouration.
Figure 2.12 OsO₄, RuO₄ and La(NO₃)₃ post-fixation of K5-Rac1 deficient skin. (a) shows toluidine blue stained semithin section from K5-Rac1 deficient mouse showing region of colouration (black arrow) which is indicative of damage to the epidermis just either side of the hair follicle (hf). Scale bar = 100μm. (b&c) show low magnification ultrastructural images of the region of colouration seen in (a) taken at approximately 2-3μm apart. This region is heavily stained (black arrows) but the extent of staining is greater in (b) when compared with (c). A number of tears (white arrows) in the tissue can be seen due to the instability of the sections. Also in (b) a keratinocyte (blue/black arrow) in the outermost layer of the stratum granulosum within the heavily stained region which has no dark staining. Scale bars = 10μm. (d) shows higher magnification of cell shown in (b) . Scale bar = 2μm. (e) shows an area around the basement membrane (orange/black arrow) to the right of the heavily stained region which is also heavily stained. Scale bar = 500nm. SC = Stratum corneum, K= Keratinocyte, E = Epidermis, D = Dermis.
Figure 2.13 Serial sections from K5-Rac1 deficient sample post-fixed with OsO4, RuO4 and La(NO$_3$)$_3$. Images show corresponding stained (a) and unstained (b) serial sections taken from this block ~5µm apart. Scale bars = 80 microns. No damage to the brownish colouration was found in the epidermis.
2.4.2.2 Ultrastructural Analysis of TJs in the IFE

For orientation purposes and for consistency, desmosomes between outermost keratinocytes (i.e. superficial stratum granulosum just deep to the first layer of corneocytes) were first located and the corresponding tight junctions superficial to these were visualised and studied. On this basis, using post-fixation method 1 (OsO$_4$ and RuO$_4$) TJs were consistently found in this region in both control and K5-Rac1 deficient tissue (Fig. 2.14).

![Figure 2.14 Ultrastructure of TJs using OsO$_4$ and RuO$_4$ post-fixation of K5-Rac1 deficient skin.](image)

(a&b) show low magnification images where TJ (pink arrow) can be seen located above a desmosome (black arrow) at the upper border of the stratum granulosum (SG). Scale bars = 500nm. (c&d) show higher magnification of the desmosome and TJ shown in (a&b). Scale bars = 100nm. K=keratinocyte, SC = stratum corneum.
Longer incubation with RuO$_4$, i.e. post-fixation method 2 (OsO$_4$ and extended RuO$_4$) did not provide any extra enhancement to the level of contrast in the ultrathin sections and therefore, sections with this post-fixation were not extensively analysed for the presence of TJs. Ultrastructural analysis of samples post-fixed with RuO$_4$ only could not be carried out as in the absence of OsO$_4$ no sufficient contrast was achieved.

Using post-fixation methods containing La(NO$_3$)$_3$ no electron dense tracer was observed in the intercellular spaces. Nevertheless, TJs were consistently found located superficial to the desmosome between the outermost keratinocytes of the SG (Fig. 2.15).

**Figure 2.15 Ultrastructure of TJs with La(NO$_3$)$_3$ based post-fixation method of K5-Rac1 deficient skin.** All images show high magnification images of a TJ (pink arrow) located superficial to a desmosome (black arrow) at the upper border of the stratum granulosum (SG). Scale bars = 100nm. K= keratinocyte,
SC = stratum corneum. (a&b) OsO₄, RuO₄ and La(NO₃)₃ post-fixation (c&d) OsO₄ and La(NO₃)₃ post-fixation.

However, in three cases, identification of a TJ superficial to a desmosome was not possible (Fig. 2.16). In two of these cases (Fig. 2.16b&d) it was not possible to see closure of the membrane immediately after the desmosome and it seemed rather as if the membranes were separating. In the third one (Fig 2.16f) rather than the membranes separating, they were running parallel to each other, but still no observable kissing point was found.

Lower magnification images (Fig. 2.16 a, c & e) of the regions where TJs were expected but could not be clearly located, showed that the cells have a similar arrangement when compared with previous images using different post-fixation methods.

**Figure 2.16 Atypical junctional complexes in K5-Rac1 deficient mice (see opposite page).** Images show corresponding low and high magnification of atypical junctional complexes where TJs could not be located above the desmosome (black arrow) at the upper border of the stratum granulosum (SG) in K5-Rac1 deficient mice. At lower magnification (a,c&d) the normal arrangement of the keratinocytes (K) forming the junctional complex in the stratum granulosum (SG) can be seen. The stratum corneum (SC) can be seen overlying the stratum granulosum. Scale bars = 500nm. At higher magnification (b, d & f) no TJ can be seen above the desmosome. The course of the membranes above the desmosome has been outlined (white/black arrows) and it is clear that the membranes don’t come together to form a TJ. Scale bars = 100nm.
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Ultrastructural Analysis of Lamellar bodies

Figure 2.17 Ultrastructure of Lamellar bodies. (a&b) show an overview of the upper epidermal layers where numerous lamellar bodies (orange arrows) are present. Scale bars = 500nm. (c&d) show lamellar bodies with characteristic stacked internal appearance. (e&f) show extrusion of lipids (white/black arrows) from the apical border of keratinocytes of the stratum granulosum (SG). Also evident is the presence of secreted lipids (*). Scale bars for (c-f) = 100nm. (a-d) OsO₄, RuO₄ and La(NO₃)₃ post-fixation. (e&f) OsO₄ and RuO₄ post-fixation. SC = stratum corneum.
Qualitative analysis on the distribution of lamellar bodies in K5-Rac1 deficient mice found that these were consistently found at a level comparable to the control (Fig. 2.17a&b). Indeed while not all lamellar bodies show the typical characteristic of a stacked lamellar internal structure, on occasion such characteristic lamellar bodies were found in both groups (Fig. 2.17c&d). Accordingly, lamellar body secretion of lipids at the apical border of the keratinocytes below the stratum corneum and the presence of secreted lipids between the upper epidermal cells were found in both groups (Fig. 2.17e&f).

2.4.3 Discussion

Previous work carried out on mice bearing a keratinocyte deletion of Rac1 in the skin revealed they have an increased immune reactivity, even under baseline conditions (Pedersen et al., 2012). One potential hypothesis that could account for the reason for this heightened inflammation was that Rac1 deletion could cause a defect in the permeability barrier of the skin. This is supported by studies in keratinocytes that indicate the importance of Rac1 in maintaining the integrity of TJs (Kirschner et al., 2011, Mertens et al., 2005, Yuki et al., 2011). Although to date no direct link between Rac1 and the normal functioning of lamellar bodies has been reported, TJs function in maintaining polarity (Kirschner et al., 2010, Kirschner and Brandner, 2012, Shin et al., 2006). Therefore any defect in TJs may affect the extrusion of lipids from lamellar bodies at the apical side of keratinocytes in the upper layer of the stratum granulosum.

Analysis of the degree of penetration of RuO$_4$ into the tissue in semithin sections indicates that the permeability barrier is not affected by the deletion of Rac1 in the keratinocytes. The degree of penetration of RuO$_4$ was found to be comparable between control samples and K5-Rac1 deficient samples. An isolated presence of higher penetration of RuO$_4$ was found in one of the K5-Rac1 deficient samples. However, extensive serial analysis of this sample and other K5-Rac1 deficient samples, in addition to the presence of an unaffected stratum corneum and keratinocytes above the discoloured region, suggests that this was probably due to some artificial damage to the
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tissue due to the handling of the sample rather than to the leakiness of the permeability barrier.

Following extensive analysis of ultra-thin sections, utilizing different post-fixation methods, it was evident that normal arrangement and morphology of TJs can be found in the K5-Rac1 deficient mice in the IFE. Only three incidents, which accounted for less than 5% of all junctional complexes found and examined, where a desmosome was located at the upper border of the stratum granulosum, a TJ was not observed in K5-Rac1 deficient samples. Although such incidents were not observed in the control samples examined, it must be noted that the number of K5-Rac1 deficient samples examined was higher than that of the control samples. To this end, it is possible that such incidents are a normal occurrence that may be uncovered with further analysis of the controls. However, as the occurrence of these incidents where TJs were not located above the desmosome in the K5-Rac1 deficient samples was so low, it indicates that this is not due to the deletion of Rac1 in the keratinocytes.

The dispensability of Rac1 for maintaining TJs in keratinocytes in vivo is further substantiated by the fact that no evidence of misdirected extrusion of lamellar bodies, which could result from defects in TJ maintaining cell polarity, was observed. Indeed lamellar body extrusion was only seen at the apical border of the keratinocytes of the stratum granulosum in the absence of Rac1. In addition, examination of the lamellar bodies in the upper layers of the IFE found that their distribution in K5-Rac1 deficient mice was comparable to controls.

Taken together, extensive ultrastructural analysis of the permeability barrier of the IFE did not reveal substantial evidence for alterations in its key components due to deficiency in Rac1 in keratinocytes in vivo. Therefore, a defect in the permeability barrier does not account for the increase in the immune reactivity of these mice.

Of interest, as outlined above, a defect in the permeability barrier was only one hypothesis that may account for the increased hypersensitivity in these K5-Rac1 deficient mice. Also proposed was that this may be due to a residual effect from
breakdown of the hair follicles or that the deletion of Rac1 in the keratinocytes may result in aberrant crosstalk between keratinocytes and the immune system. Indeed, it was found by other members of the group that deletion of Rac1 in the keratinocytes resulted in aberrant crosstalk between the keratinocytes and immune response cells thus contributing to skin inflammation. In these Rac1 deficient keratinocytes there was an increased expression of Signal Transducers and Activators of Transcription 1 (STAT1) and interferon gamma (IFNγ) response genes including interleukin-6 and the chemokines Cxcl1 and Cxcl10. This deletion of Rac1 also increased the sensitivity of the cells to IFNγ creating a positive feedback loop further increasing immune reactivity (Pedersen et al., 2012). This therefore supports the ultrastructural analysis here showing that a defect in the barrier does not account for the increased hypersensitivity of these mice.

Interestingly, JAK-STAT pathway is involved in the aberrant regulation of the immune response in atopic dermatitis (Bao et al., 2013). As mentioned previously, defects in the TJJs have been shown to be a contributing factor to the overall barrier dysfunction typical of atopic dermatitis (De Benedetto et al., 2011). Possibly, with increasing age of the K5-Rac1 deficient mice, the heightened inflammation may result in an atopic dermatitis phenotype. Therefore, under these increased stress conditions on the skin it is possible that a mutant barrier phenotype may be revealed. This represents an interesting area of future research.

2.5 Conclusions

There are strong indications for the role of the Cdc42 effector N-WASP, and the Rho GTPase proteins RhoA and Rac1 in maintaining the structural integrity of the IFE. N-WASP has been primarily linked to maintaining AJs and desmosomes, but also indicated in a role in cell-ECM adhesion. The role of RhoA in the normal structure and function of AJs and desmosomes is contradictory. On the other hand, strong evidence suggests its deletion would result in breakdown of the DEJ. Evidence suggests that Rac1 through its regulation of TJJs in keratinocytes, would be important for maintaining the permeability barrier of the IFE. Here, from ultrastructural analysis of mice bearing
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keratinocyte restricted deletion of these proteins in the skin, it is concluded that these proteins are dispensable for maintaining these aspects of the ultrastructural integrity of the IFE
Chapter 3

**Challenging the Skin in Mice Bearing Deletion of Rac1 in Keratinocytes**
Challenging the skin in mice bearing a Rac1 deletion in keratinocytes
3.1 Introduction

In the present chapter, work on the effects of Rac1 deletion in keratinocytes on the DEJ and on the tissue localisation and arrangement of collagen fibrils and of decorin, will be presented.

Studies in mice bearing a keratinocyte restricted deletion of Rac1 in the skin have shown contrasting results on its role in maintaining the integrity of the IFE (Benitah et al., 2005, Castilho et al., 2007, Chrostek et al., 2006, Benitah and Watt, 2007). Severe abnormalities were reported after deletion of Rac1 in adult mice by expression of Cre recombinase fused with a mutant estrogen receptor under the control of the keratin 14 promoter (CreER/K14) (Benitah et al., 2005). In these mice, it was found that approximately two weeks after the induction of the Rac1 deletion by the application of tamoxifen, there was partial and in some places complete loss of epidermal layers resulting from depletion of epidermal stem cells (Benitah et al., 2005). The same group later reported thickening and hyperkeratosis of the IFE, as well as development of skin inflammation and spontaneous wounds when deletion of Rac1 was induced endogenously under the control of the keratin 5 promoter (Cre/K5) (Benitah and Watt, 2007). Contrasting to this, as part of a previous project led by Professor Cord Brakebusch, also utilising Cre/K5 promoter for endogenous Rac1 deletion (i.e. the same model used in this project), only a mild effect on the IFE was found (Chrostek et al., 2006). Here a slight increase in suprabasal cells and an occasional increase in the intercellular spacing between keratinocytes seen at the ultrastructural level was reported (Chrostek et al., 2006). A mild effect on the integrity of IFE was also found in a third study using a related endogenous model of keratinocyte Rac1 deficiency where deletion was under the control of keratin 14 (Cre/K14) (Castilho et al., 2007). Additionally, in a study conducted on transgenic mice expressing the Rac1 dominant inhibitory mutant N17Rac1 in basal keratinocytes driven by keratin-14, no defects on the IFE occurred (Tscharntke et al., 2007). The reason for contrasting results has been discussed by these different authors (Benitah and Watt, 2007, Castilho et al., 2007, Chrostek et al., 2006,
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Tscharntke et al., 2007). Suggestions include different genetic background of the mice, the age of the mice when the Rac1 deletion was induced, the application of tamoxifen to induce the deletion rather than endogenous deletion, or different levels of inhibition of Rac1 (Benitah and Watt, 2007, Castilho et al., 2007, Chrostek et al., 2006, Tscharntke et al., 2007).

Where the integrity of the IFE was severely affected by Rac1 deletion in keratinocytes, the reason was reported to be due to depletion of epidermal stem cells (Benitah et al., 2005). This was associated with a progressive reduction on the expression of integrin α6β4 and the ultrastructurational integrity of HDs (Benitah et al., 2005). In contrast, this was not the case where deletion was driven using Cre/K5 at least in two week old mice (Chrostek et al., 2006). However, Rac1 has been reported to co-localise (Benitah et al., 2005) and co-precipitate (Sehgal et al., 2006) with integrin α6β4 in keratinocytes. This integrin in turn is a main ligand of laminin-332, which, as discussed previously is a major component of the epidermal BM (Breitkreutz et al., 2013, Breitkreutz et al., 2009, Hashmi and Marinkovich, 2011). In \textit{vitro} studies, focusing primarily on the migration of keratinocytes, found that the interaction of integrin α6β4 to laminin-332 regulated Rac1 activity and subsequently the organisation of the laminin-332 matrix (Sehgal et al., 2006, Kligys et al., 2007). Rac1 was also found to co-precipitate with the β1 integrin in keratinocytes (Sehgal et al., 2006). A Rac-GEF, Tiam1, has been shown to be a key molecule in integrin α3β1 induced Rac protein activation for both the production and secretion of laminin-332 in keratinocytes in-vitro (Hamelers et al., 2005). These studies therefore raise the question as to whether Rac1 may be necessary for regulating laminin-332 organisation \textit{in vivo}, and subsequently the overall homeostasis of the epidermal basement membrane.

While \textit{in vitro} works provide indication that Rac1 may play a role in maintaining the integrity of the BM, this effect does not appear to be directly translated when the protein is deleted \textit{in vivo}. In fact, qualitative analysis of the ultrastructure of the BM did not show abnormalities due to Rac1 deletion using Cre/K5 model in two week old mice. Indeed in this model the deposition of laminin-332 and nidogen was normal (Chrostek et al., 2006). In transgenic mice expressing the Rac1 dominant inhibitory mutant N17Rac1 in basal keratinocytes driven by keratin-14 it was also found that there was normal
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deposition of laminin-332 and in addition, normal deposition of collagen type IV (Tscharntke et al., 2007). However, to date no quantitative study of the integrity of epidermal BM in Rac1 deficient skin has been reported. Therefore, it cannot be excluded that the lack of Rac1 might also have additional more subtle effects which could be unravelled by an overall detailed quantitative assessment of the ultrastructural integrity of the DEJ.

The effect of Rac1 deletion on the integrity of the DEJ and on adhesion of keratinocytes at the DEJ is in fact not fully elucidated, at least under the physiological conditions described above. In order to achieve a clearer picture on the biological role of Rac1 in keratinocytes in maintaining the homeostasis of the DEJ, and to unravel potentially more subtle effects, here investigations on its integrity in the absence of Rac1 during aging and in a short term of ICD model. As mentioned above, the model used for this project, where Rac1 deletion is under the control of the Cre/K5 promoter for endogenous deletion showed no major morphological alterations in the IFE in young mice, i.e. two weeks old (Chrostek et al., 2006).

With increasing age the skin undergoes structural and functional changes most notably slower cell renewal, compromised barrier protection, and delayed wound healing and immunological responses (Kottner et al., 2013, Lavker et al., 1987, Wulf et al., 2004). Of particular interest is the effect on the DEJ which becomes flatter due to retraction of rete ridges and serrations making the skin more susceptible to mechanical injury (Lavker et al., 1987, Timar et al., 2000, Wulf et al., 2004). ICD is defined as inflammation of the skin (Prakash and Davis, 2010, Slodownik et al., 2008). Exposure to an irritant results in pathophysiological changes in the skin due to barrier disruption and epidermal damage. The combination of both age and ICD would cause serious stress and weaken the skin. Therefore, under these conditions, the effects on the DEJ of Rac1 deletion in the keratinocytes may manifest more clearly.

Interestingly, in response to irritants keratinocytes play a major role in an immunological response by acting as pro-inflammatory signal transducers. Through the release of cytokines, chemokines and adhesion molecules, keratinocytes recruit and activate immune and mesenchymal cells to repair the tissue (Slodownik et al., 2008,
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Stamatas et al., 2013). Among the major cells of mesenchymal origin that respond to inflammatory signals are fibroblasts, which will then synthesize collagen and promote ECM crosslinking (Barrientos et al., 2008, Gallant-Behm et al., 2011, Werner et al., 2007). Fibroblasts also stimulate the activity of keratinocytes to repair the tissue through secretion of paracrine and growth factors (Werner et al., 2007, Harrison et al., 2006, Stark et al., 2004). Indeed it is well established that crosstalk between keratinocytes and fibroblasts is essential for both normal physiological homeostasis and repair of homeostasis of the skin (Gallant-Behm et al., 2011, Harrison et al., 2006, Wojtowicz et al., 2014, Sorrell and Caplan, 2009, Werner et al., 2007). Of note, reciprocal crosstalk between keratinocytes and fibroblasts is essential for the synthesis and maintenance of the DEJ (Marionnet et al., 2006).

As highlighted in Chapter 2, our group recently showed that deletion of Rac1 in keratinocytes causes heightened inflammation due to aberrant crosstalk with immune cells (Pedersen et al., 2012). Indeed, this is not the first report where genetic deletion of proteins in keratinocytes resulted in an increase in inflammation of the skin (Brakebusch et al., 2000, Demehri et al., 2009). Of interest, in these reports, in addition to effects on the epidermis, the integrity of the dermis was also compromised, primarily due to the development of fibrosis (Brakebusch et al., 2000, Demehri et al., 2009). Taken together, this raises the question as to whether the deletion of Rac1 in the keratinocytes may also affect the integrity of the dermis through aberrant crosstalk between keratinocytes and dermal fibroblasts. Or given that heightened or chronic inflammation is intimately linked to fibrotic diseases of the skin, (Darby and Hewitson, 2007, Shaw et al., 2010, Eming et al., 2007) this raises the possibility that the deletion of Rac1 may affect the function of the fibroblasts indirectly as a response to heightened inflammation.

Dermal fibroblasts function primarily in the production and organisation of the dermal ECM. Therefore it is reasonable to think that if the deletion of Rac1 in the keratinocytes is affecting the function of these fibroblasts, either directly or indirectly, this may result in disturbances in the structural organisation of the dermal ECM. Given the fact that collagen fibrils account for the vast majority of the total ECM in the dermis, the effects of Rac1 deletion in keratinocytes on fibroblasts would be evident in the organisation of the collagen matrix.
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Among the key factors involved in the deposition and organisation of collagen fibrils is decorin (Bosman and Stamenkovic, 2003, Schaefer and Iozzo, 2008, Reed and Iozzo, 2002). Of particular interest here is that decorin has been found to be a mediator of inflammatory signalling (Moreth et al., 2012, Frey et al., 2013). Indeed during an allergic inflammatory response in skin, decorin enhances the stability of IFNγ resulting in increased activation of STAT1 (Bocian et al., 2013). The heightened inflammation due to the absence of Rac1 in the keratinocytes is due to the increased expression of STAT1 in the keratinocytes and an increase in their sensitivity to IFNγ resulting in the promotion of a positive feedback inflammatory loop (Pedersen et al., 2012). Therefore, whether decorin localisation is altered in response to heightened inflammation due to deletion of Rac1 in keratinocytes is of particular interest.

Taken altogether, it is feasible to think that the deletion of Rac1 in keratinocytes may affect the integrity of the DEJ and the dermal collagen matrix, two vital ECM components necessary for the maintenance of the integrity and homeostasis of the skin. Therefore, here qualitative and quantitative analysis is performed to assess these components, primarily in an aged model of ICD.

To this end, quantification on the integrity of the DEJ was first carried out in a pilot study in younger mice. Although an effect was seen due to the deletion of Rac1, namely the occasional occurrence of duplication of the BM, overall no major alterations on the integrity of the DEJ were found. Therefore it was decided to extend the study to older mice. In addition to analysing the integrity of the DEJ in these older mice, qualitative and quantitative analysis on the size distribution of the diameter of collagen fibrils, the percentage of the extracellular space (ECS) occupied by collagen fibrils, the architectural organisation of the collagen matrix, as well as the distribution of decorin was carried out.
3.2 Materials and Methods

3.2.1 Mice

(This work was carried out by the lab of Professor Cord Brakebusch)

To obtain mice with a keratinocyte-restricted deletion of Rac1, transgenic mice expressing Cre recombinase under the control of the K5 promoter (Ramirez et al., 2004) were intercrossed with mice homozygous for a floxed Rac1 allele (Rac1 (fl/fl) K5Cre). These mice will be referred to as K5-Rac1 deficient mice. Littermates (Rac1 (fl/+), K5Cre and Rac1 (fl/fl) no Cre) were used as controls. All mice were on a 129Sv/C57Bl6 outbred background. All animal studies were carried out according to Danish rules of animal welfare.

Hosting, treatment and sacrifice of mice, harvesting and fixation of tissue samples was carried out in the Animal Facility of the BRIC (Copenhagen University), in accordance with all local ethical and legal requirements. Overall, the tissue studied here was ear skin samples from control and K5-Rac1 deficient mice aged between 2-5½-month-old (n=2 control + 2 K5-Rac1 deficient mice) and 11-month-old (n=4+4). These will be referred to as younger and older mice respectively.

For the younger mice, the age was recorded for the 5½-month-old, but the age was not recorded for the other mice in this group. It is known that they would fall in this age range, and that for this Rac1 deletion, the control is age matched.

Irritant contact dermatitis (ICD) was induced in anesthetized mice by treatment with 2% croton oil in a 4:1 acetone/olive oil mixture to both sides of the right ear (These samples will be referred to as Con-ICD and K5-Rac1 deficient-ICD). The left ear was used as a control by treatment with vehicle (4:1 acetone/olive oil mixture) (These samples will be referred to as Con-vehicle and K5-Rac1 deficient-vehicle). Mice were sacrificed after 8 hours.

For light and fluorescence microscopy, ear skin samples from older mice were fixed in 4% paraformaldehyde in phosphate buffer.

For ultrastructural analysis small samples of ear skin from younger and older mice were taken and fixed in TEM fixation solution.
All samples were then shipped to Anatomy, NUI Galway for further processing and analysis.

### 3.2.2 Light & Fluorescence Microscopy

#### 3.2.2.1 Sample Embedding in Paraffin

(Carried out by technical staff in Anatomy, NUI Galway)

Tissue samples from older Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD were embedded in paraffin using the following protocol:

- Dehydrate through graded series of ethanol (70% overnight, 80%, 95% 30 minutes each, and 100% (2 X 1 hour)).
- Place samples in 50:50 mix of 100% ethanol and xylene for 30 minutes.
- Place samples in two changes xylene for 1 hour and 30 minutes respectively.
- Place samples in two changes of paraffin wax at 60°C for 3 hours and overnight respectively.
- Put samples into moulds with melted paraffin and leave to solidify at room temperature.

#### 3.2.2.2 Sectioning

For all histology and immunofluorescence 5µm sections were cut using a Leica RM2125RT. Sections were mounted on twin frost microscope slides for light microscopy and superfrost ultraplus for fluorescence microscopy. Sections were left to dry overnight at 37°C. Before all histology and immunofluorescence staining sections were deparaffinized in xylene (2 X 10 minutes) and rehydrated through a graded series of ethanol (100%, 100%, 95%, 70% and 50% for 2 minutes each).

#### 3.2.2.3 Mayers Haematoxylin and Eosin (H&E)

For general histological overview deparaffinised and rehydrated sections were stained using the following protocol:
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- Remove ethanol by rinsing in tap water for 2mins
- Stain in Mayers Haematoxylin for 6 minutes
- Blue nuclei in running tap water for 4 minutes
- Stain in Eosin for 2 minutes
- Rinse quickly in tap water
- Dehydrate sections through graded series of ethanol (50% & 70% for 10 seconds each, 95%, 100%, 100% for 2 minutes each)
- Clear in xylene (2 X 10 minutes)
- Mount slides in DPX mountant

Sections were imagined using a Leica DM750 upright microscope using Leica Application Suite V4.1.

3.2.2.4 Phosphomolybdic acid – Picosirius Red Staining for Dermal Collagen

For each ear skin sample from older Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD three random sections were stained on different days and imaged.

Staining on deparaffinised and rehydrated sections was carried out as follows:

- Remove ethanol by washing in distilled water (2 X 5 minutes)
- Place sections in 0.2% aqueous phosphomolybdic acid for 1 minute
- Stain in 0.1% Picosirius Red for 90 minutes
- Differentiate sections in 0.5% Acetic Acid (2 X 30 seconds)
- Rinse in distilled water (30 seconds)
- Dehydrate sections through graded series of ethanol (50%, 70%, 95%, 100%, 100% Dip 5 times each)
- Clear in xylene (2 X 5 minutes)
- Mount slides using DPX mountant

Images were captured as Z-stacks on an Andor Olympus Spinning Disk Microscope, using Andor IQ software, with 60x oil immersion objective lens – NA 1.42, at 0.5µm apart. Prior to collecting the Z-stack, exposure time and EM gain were set and recorded.
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to the channels (488 and 564) of illumination. 10 non-overlapping regions of IFE were captured per section. Stacks were imported to ImageJ software (Schneider et al., 2012) for Fractal Dimension and Lacunarity analysis described below.

3.2.2.5 Immunofluorescence for Decorin

For each ear skin sample from older Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD three random sections were stained using an anti Decorin antiserum (LF-113, generously provided by Dr. Larry Fisher, NIH, Bethesda, MD) (Fisher et al., 1995) on different days and imaged. For each section a no primary antibody negative control was included.

Staining on deparaffinised and rehydrated sections was carried out as follows:

- Remove ethanol by washing in 1X Tris-buffered saline (TBS) (3 X 5 minutes)
- Incubate in 1X Protaqs I Antigen Enhancer in millipore water at 60°C for 20 minutes (antigen retrieval)
- Wash in 1X TBS on shaker (3 X 5 minutes)
- Place 50μl of Protease XXIV 200mg/ml on each section and incubate at 37°C for 2 minutes.
- Wash in 1X TBS on shaker (3 X 5 minutes)
- Block by placing 50μl of 5% normal goat serum (NGS) on each section and place in a humid chamber. Incubate at room temperature for 3 hours.
- Draw off NGS. Add 50μl of LF-113 anti-decorin (1:200) diluted in 5% NGS to each section. Incubate in humid chamber overnight at 4°C.
- Wash in 1X TBS on shaker (3 X 5 minutes)
- Add 50μl of secondary antibody (TRITC (594) goat anti rabbit) 1:200 to each section and incubate in humid chamber for 1 hour at room temperature.
- Wash in 1X TBS on shaker (3 X 5 minutes)
- Counterstain sections with Hoechst (1:2000) and Phalloidin 488 (1:200) solution at room temperature for 20 minutes.
- Wash in 1X TBS on shaker (3 X 5 minutes)
Mount section with Fluoromount and store flat in the dark until dry. Seal with clear nail varnish.

Images were captured as Z-stacks on an Andor Olympus Spinning Disk Microscope, using Andor IQ software, with 40x oil immersion objective lens – NA 1.3, at 1µm apart. Prior to collecting the Z-stack, exposure time and EM gain were set and recorded to the channels (405, 488 and 564) of illumination. 10 non-overlapping regions of interfollicular dermis were captured per section. Stacks were imported to ImageJ software (Schneider et al., 2012) for Fractal Dimension and Lacunarity analysis described below.

3.2.2.6 Fractal Dimension (F_D) and Lacunarity (Λ) Analysis

Fractal dimension (F_D) is a measure of complexity of an object, which is calculated by measuring how a pattern changes (increases in detail) with respect to scale (Smith et al., 1996). For example, this method has been used to show changes in collagen matrix complexity in fibrosis (Dioguardi et al., 2006, Moal et al., 2002) and dermal scars (Khorasani et al., 2011). It therefore provides a quantitative descriptor that can be used to determine changes in the morphology of biological structures.

Here F_D was determined using the box counting dimension (DB) method which is calculated using the following formula:

\[ DB = \lim_{\varepsilon \rightarrow 0} \frac{LogN(\varepsilon)}{Log\left(\frac{1}{\varepsilon}\right)} \]

where N(ε) is the number of boxes of various size lengths (ε) that contain an object. This formula returns a value between 1, indicating low complexity, and 2, indicating high complexity of the tissue.

Lacunarity (Λ) is a measure of heterogeneity or structural variance in an image (Smith et al., 1996). It can be used to describe the pattern of fractals, as two fractals may have the same F_D but have different patterns (Smith et al., 1996). It is calculated using the following formula:
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$$\Lambda \varepsilon = (\sigma | \mu)^2$$

where for each box of various size lengths ($\varepsilon$) the mean ($\mu$) and standard deviation ($\sigma$) are calculated. This formula returns a value between 0 and 1, where 0 is completely homogenous and 1 is completely heterogeneous.

Z-stacks were imported into ImageJ (Schneider et al., 2012) and separated into their respective channels. Only those taken in the 594 channel were analysed, i.e. collagen and decorin stained as described above. These Z-stacks were corrected for background using the “background subtraction from a region of interest” function using a scaling factor of 3. A maximum intensity projected image, i.e. where the pixels of maximum intensity (in focus) from each slice in the stack are merged into one image, was created from each Z-stack. For each Z-stack and maximum intensity projected image, three regions of interest (1.1mm$^2$) parallel to the DEJ were analysed using the Frac_Lac plugin. Here, images were auto-converted to binary images and a slow scan sliding box method was used from a minimum box size of 1 pixel to a maximum size of 45% of the region of interest (Khorasani et al., 2011).

3.2.2.7 Statistics

Mean and Standard deviation are shown for groups. For all quantification coefficient of variation (mean/standard deviation), expressed as percentage, was calculated for each group (interindividual variation) and also for each mouse in the group (intraindividual variation). Results were analysed using one way ANOVA and subsequent Tukey’s test where appropriate in Mintab version 16 taking $p = <0.05$ as the level of statistical significance.

Chi squared distribution test was used to determine statistical significance in the size distribution of the diameter of collagen fibrils using the following formula:

$$x^2 = \sum \frac{(o - e)^2}{e}$$

where $o =$ observed distribution and $e =$ expected distribution. After calculating $x^2$ using the CHIDIST function in Microsoft Excel® the $p$ value was determined.
3.2.3 Transmission Electron Microscopy

3.2.3.1 Infiltration and embedding

As described previously (See page 59)

3.2.3.2 Sectioning, staining and imaging

Samples were processed, embedded, sectioned and imaged for transmission electron microscopy according to a standard protocol described previously (See page 49), with a slight modification: sections were stained with uranyl acetate for 10 minutes and lead citrate for 5 minutes in a Leica EM AC20 stainer.

3.2.3.3 Quantitative Analysis of the integrity of the DEJ

This was performed in samples of both younger and older mice using the method as described previously (see page 59). One change was made to this protocol, instead of using Adobe® Photoshop® cs4, to superimpose a square grid on the images, the grid function in ImageJ (Schneider et al., 2012) was used for this purpose.

3.2.3.4 Analysis of size distribution of the diameter of collagen fibrils

Size distribution of the diameter of collagen fibrils was analysed in older Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD samples. For each ear three tissue samples were analysed. From each tissue sample 30 non-overlapping images of the interfollicular dermis were sequentially taken at 20,000X magnification. These 30 images are made up of 10 taken just deep to the DEJ (region 1), 10 just below these (region 2) and the final 10 just below region 2 (region 3) therefore covering approximately 15-20µm depth from the DEJ (Fig.3.1a).
Figure 3.1 Regions of interest for analysis of collagen fibrils. a) Low magnification image of the interfollicular dermis outlining an example of the different regions (region 1 (red box), region 2 (green box) and region 3 (blue box)) that were imaged for analysis of size distribution of the diameter of collagen fibrils. E = epidermis, D = dermis, Scale bar = 2µm. b) Image used for measurement of collagen fibril size outlining the regions of interest used for analysis. Scale bar = 500nm.

Measurements of collagen fibril diameter were performed using ImageJ software (Schneider et al., 2012). For each image, 4 regions of interest of an area of 4µm² were analysed (Fig 3.1b). These areas were kept constant for each image to remove bias. In order to accurately measure the diameter of collagen fibrils images were converted to 8-bit, smoothened, contrast enhanced using saturation of 2.0, manually thresholded to outline cross sectioned collagen fibrils and then any touching particles were separated by running watershed segmentation. Using the analyze particle function the area of collagen fibrils cut in cross section was measured. For this the following parameters for inclusion were set; size 50-7000 in pixel units, circularity between 0.8-1, exclude on edges and fill holes. Show outlines and display results were also selected in order to enable removal of items that were measured other than cross sectioned collagen fibrils from the results.
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Results were collected and analysed using Microsoft Excel®. Diameter of fibrils was calculated by dividing area by \( \pi \). The percentage of collagen fibrils in different diameter ranges was tabulated for each sample.

### 3.2.3.5 Percentage of ECS occupied by Collagen fibrils

In order to calculate the percentage of ECS occupied by collagen fibrils the stereological method of volume fraction (V/v) was applied using the following formula (Howard and Reed, 2010).

\[
V/v = \frac{\text{Volume of Collagen}}{\text{Volume of ECS}}
\]

Using the same images and the same regions of interest as for collagen fibril diameter analysis a stereological square grid was superimposed on the images using the grid function in ImageJ (Schneider et al., 2012). Where the lines of this grid cross (hits) the object is counted (Fig. 3.2). The size of the grid here resulted in 25 hits per region of interest, i.e. 100 hits per image. The number of hits of collagen and the number of hits on ECS (including collagen) was tabulated. (Note: Hits on any other object, for e.g. cells or vessels, were not included) Results were collected and analysed using Microsoft Excel®. Using the formula above V/v was calculated, and this value was converted to percentage. The percentage of ECS occupied by collagen was calculated per sample.

![Figure 3.2 V/v Collagen:ECM counting method.](image)

Stereological grid (red lines) is super imposed on an image, hits on collagen (yellow circles) and hits on ECM (blue circles) are tabulated. Scale bar = 500nm.
3.3 Results

3.3.1 Younger Mice

3.3.1.1 Pilot analysis of the ultrastructural integrity of the DEJ in younger K5-Rac1 deficient model of ICD

Qualitative analysis of the DEJ in younger mice revealed that the keratinocytes appeared closely adhered to the underlying BM in all groups (Fig. 3.3). For the most part the BM appeared ultrastructurally defined with a continuous electron dense layer, namely the LD, underlying an electron lucent layer, namely the LL. There were also isolated regions where an ultrastructurally defined BM was not present in all groups.

Figure 3.3 Ultrastructure of the DEJ in Young Control and K5-Rac1 deficient mice in ICD model. (a,b,c&d) show normally structured DEJ in all groups. Keratinocytes (K) are adhered to an ultrastructurally defined BM (blue arrow). HDs (yellow arrow) are seen studded along the BM. Anchoring fibrils (orange arrow) can be seen extending from the BM into the underlying dermis (D). Scale Bars = 500nm.
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No regions of obvious breakage or discontinuation of the BM was observed due to the deletion of Rac1 or induction of ICD. However, in two of K5-Rac1 deficient samples, namely one K5-Rac1 deficient-vehicle and one K5-Rac1 deficient-ICD sample, occasional duplication of the BM was seen (Fig. 3.4). Contained within these regions of duplication was amorphous material.

Figure 3.4 Duplication of the BM in Younger K5-Rac1 deficient mice. Images show DEJ in K5-Rac1 deficient vehicle treated (a) and croton oil treated (b) where duplication of the BM is evident (white/black arrows). Normally structured BM (blue arrow) and HDs (yellow arrow) were seen in the regions either side of this duplication. K = Keratinocytes, D = Dermis. Scale Bars = 500nm.

Quantitative analysis included the measurement of defined BM, the frequency and percentage of defined DEJ occupied by HDs and thickness of the whole BM, in addition to the LD and LL.

In terms of the percentage of ultrastructurally defined BM at the DEJ no statistically significant difference between Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups (Fig. 3.5).

The frequency and percentage of defined DEJ occupied by HDs also showed a statistically insignificant difference between the all groups (Fig. 3.5).
Figure 3.5 Quantitative analysis of the integrity of the DEJ in young K5-Rac1 deficient model of ICD. (n=2). p value shown was determined by one way ANOVA comparing all groups.
In terms of the thickness of the defined BM at the DEJ there does appear to be a slight thickening of the whole BM in the K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups although this is not statistically significant (Fig. 3.6). When the individual layers were analysed for thickness, it appeared that there was a slight thickening of the LL in the K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups, but this also does not show a statistically significant difference (Fig. 3.6). The thickness of the LD is consistent in all groups and does not display a statistically significant difference (Fig. 3.6).

Therefore the only effect on the DEJ seen here due to the absence of Rac1 in the keratinocytes was a rare occurrence of duplication of the BM.
Figure 3.6 Thickness of the DEJ in young K5-Rac1 deficient model of ICD. (n=2). p value shown was determined by one way ANOVA comparing all groups.
3.3.2 Older Mice

3.3.2.1 Histological Analysis of the skin in older K5-Rac1 deficient model of ICD

Histological investigation of ear skin samples from older Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD revealed no obvious structural difference between the epidermal layers in the different groups (Fig. 3.7). The epidermis appeared as a cohesive layer, with no evidence of blistering, in any groups. No evidence of alterations in the thickness of the epidermis was observed either due to the Rac1 deficiency or ICD. The structural architecture of the dermis was comparable in all groups.

![Figure 3.7 General histological overview of older K5-Rac1 deficient model of ICD. Image show H&E stained ear skin from 11 month mice showing no obvious morphological difference between groups. E= Epidermis, D= Dermis. Scale bars = 200µm.](image-url)
3.3.2.2 Analysis of the integrity of the DEJ in older K5-Rac1 deficient model of ICD

**Qualitative analysis** of the DEJ in older Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD samples revealed keratinocytes closely adhered to the underlying BM (Fig. 3.8). The BM appeared ultrastructurally defined in the majority of the regions analysed with isolated regions of blurred BM seen in all groups.

![Figure 3.8](image)

**Figure 3.8 Ultrastructure of the DEJ in older Control and K5-Rac1 deficient mice in ICD model.** (a,b,c&d) show normally structured DEJ in all groups. Keratinocytes (K) are adhered to an ultrastructurally defined BM (blue arrow). HDs (yellow arrow) are seen studded along the BM. D = Dermis. Scale Bars = 500nm.
Figure 3.9 Duplication of the BM in older K5-Rac1 deficient mice. Images show DEJ in K5-Rac1 deficient vehicle treated (a, c, e) and croton oil treated (b, d, f) where duplication of the BM is evident (white/black arrows). Parts of cells can be seen within these duplications (green arrows (a, c, d, e)). Normally structured BM (blue arrow) and HDs (yellow arrow) were seen in the regions either side of this duplication. K = Keratinocytes, D = Dermis. Scale Bars = 500nm.
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No regions of obvious breakage or discontinuation of the BM were observed due to the deletion of Rac1 or the induction of ICD. Like with the younger K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD mice there was the sporadic presence of duplication of the BM (Fig. 3.9). However, this was more frequent than that seen in the younger individuals, in that it was found in seven of the K5-Rac1 deficient-vehicle samples and eight of the K5-Rac1 deficient-ICD samples. The number of incidents of this duplication per sample was low, the most incidents occurring in one region analysed for quantification of the integrity of the DEJ was five, but on average three incidents per region was observed. For the most part, these duplications contained amorphous material, but in roughly about one third of the incidents, cellular material was evident (Fig 3.9 a, c, d, e). No such incidents of the duplication of the BM were observed in either the Con-vehicle or Con-ICD samples.

As for the younger mice quantitative analysis included the measurement of defined BM, the frequency and percentage of defined DEJ occupied by HDs and thickness of the whole BM, in addition to the LD and LL. Quantification of the presence of ultrastructurally defined BM at the DEJ revealed a statistically significant difference between the groups (Fig. 3.10). Using Tukey’s test it was determined that the statistically significant difference is due to the Con-ICD and K5-Rac1 deficient-ICD groups, where a higher level of defined BM is seen in the Con-ICD group compared to the K5-Rac1 deficient-ICD group. No statistically significant difference was seen for the frequency of HDs and percentage of defined DEJ occupied by HDs between the all groups (Fig. 3.10).
Figure 3.10 Quantitative analysis of the integrity of the DEJ in Older K5-Rac1 deficient model of ICD. For graphs where a statistically significant difference ($p = <0.05$) was found between the groups, letters A and B are used to show where the difference between the groups occurs. To this end groups that do not share a letter are significantly different from each other. ($n=4$). $p$ value shown was determined by one way ANOVA comparing all groups.
Figure 3.11 Arithmetic Mean Thickness of the DEJ in Older K5-Rac1 deficient model of ICD. For graphs where a statistically significant difference (p = <0.05) was found between the groups, letters A and B are used to show where the difference between the groups occurs. To this end groups that do not share a letter are significantly different from each other. (n=4). p value shown was determined by one way ANOVA comparing all groups.
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Quantification of the thickness of the defined BM, and also the LL and LD, in Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD revealed a statistically significant difference. For the thickness of the whole BM, the Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups showed a statistically significant increase when compared to the Con-vehicle group (Fig. 3.11).

When the LL, was measured a statistically significant increase in thickness was found in both the K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups compared to the Con-vehicle (Fig. 3.9). For the LD a statistically significant increase in thickness of the LD in the Con-ICD and K5-Rac1 deficient-ICD groups was seen (Fig. 3.11).

Interindividual and intraindividual variation for all parameters of the integrity of the DEJ showed low variability between individuals and within individuals (Table 3.1).

<table>
<thead>
<tr>
<th></th>
<th>Control Vehicle</th>
<th>Control ICD</th>
<th>K5-Rac1 deficient Vehicle</th>
<th>K5-Rac1 deficient ICD</th>
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<td></td>
<td></td>
<td></td>
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<td>10%</td>
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<td>10%</td>
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<td>7%</td>
<td>8%</td>
<td>11%</td>
</tr>
<tr>
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<td>6%</td>
<td>7%</td>
<td>13%</td>
</tr>
<tr>
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<td>11%</td>
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<tr>
<td><strong>Intraindividual Variation</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
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</tr>
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</tr>
<tr>
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<td>13%</td>
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<td>13%</td>
</tr>
<tr>
<td>BM Thickness</td>
<td>10%</td>
<td>14%</td>
<td>8%</td>
<td>10%</td>
</tr>
<tr>
<td>LD Thickness</td>
<td>11%</td>
<td>10%</td>
<td>7%</td>
<td>10%</td>
</tr>
<tr>
<td>LL Thickness</td>
<td>7%</td>
<td>8%</td>
<td>9%</td>
<td>11%</td>
</tr>
</tbody>
</table>

Table 3.1 Co-efficient of Variation for the integrity of the DEJ
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**In summary**, from the qualitative analysis of the DEJ, sporadic duplication of the BM in both younger and older K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD mice was found. The occurrence of this increased with the age of the mice. Quantification of the integrity of the DEJ in the older mice revealed a statistically significant difference in the percentage of the DEJ occupied by a defined BM between the Con-ICD and the K5-Rac1 deficient-ICD groups.

In addition, in the older mice an increase in the thickness of the whole BM in all groups compared to the Con-vehicle was found. In the K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD and increase in the thickness of the LL compared to the Con-vehicle was found. In terms of the thickness of the LD a statistically significant increase for the Con-ICD and K5-Rac1 deficient-ICD compared to the Con-vehicle was seen.
3.3.2.3 Analysis of the Collagen architecture in the Dermal Extracellular Matrix in older K5-Rac1 deficient model of ICD

Qualitatively the collagen architecture of the dermis appeared to have a similar morphology in all groups (Fig. 3.12). Bundles are generally seen running parallel to the epidermis with occasional bundles running perpendicularly.

Figure 3.12 Dermal Collagen in Older Control and K5-Rac1 deficient mice in ICD model. Maximum intensity projected images of Phosphomolybdic acid – Picosirius Red Staining of collagen (red). This method also highlights the cytoplasm of cells (green). E = epidermis, D = dermis. Scale bars = 10µm.
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Quantification of the complexity and heterogeneity of the collagen network by $F_D$ and $\Lambda$ analysis in Z-stacks did not reveal a statistically significant difference (Fig. 3.13). However, when the same regions were analysed in maximum intensity projected images (i.e. where the pixels of maximum intensity (in focus) from each slice in the stack are merged into one image) a statistically significant difference was found for both parameters (Fig. 3.13). For $F_D$ this is due a statistically significant difference between the K5-Rac1 deficient-ICD group compared to the Con-ICD and the K5-Rac1 deficient-vehicle groups. For $\Lambda$ this is due to an increase in heterogeneity in the K5-Rac1 deficient-ICD group compared to all other groups.
Figure 3.13 $F_D$ and $\Lambda$ of collagen matrix in Older Control and K5-Rac1 deficient mice in ICD model. Graphs show results of $F_D$ and $\Lambda$ of Z-stacks and maximum intensity projected images. For graphs where a statistically significant difference ($p < 0.05$) was found between the groups, letters A and B are used to show where the difference between the groups occurs. To this end groups that do not share a letter are significantly different from each other. ($n=4$). $p$ value shown was determined by one way ANOVA comparing all groups.
3.3.2.4 Analysis of the Ultrastructure of the Dermal Extracellular Matrix in older K5-Rac1 deficient model of ICD

Qualitatively the collagen fibrils in these regions did not appear to have any pathological abnormalities in response to the deletion of Rac1 in the keratinocytes or the induction of ICD (Fig. 3.14). Collagen fibrils in all groups appeared to have a circular profile when cut in cross section.

Quantification of the size distribution of the diameter of collagen fibrils revealed that in the Con-vehicle there is a tendency toward smaller collagen fibrils compared to all other groups when images were analysed all together i.e. not broken down into regions (Fig. 3.15a). Chi- squared test found that there is a statistically significant difference in the size distribution of the diameter of collagen fibrils when the Con-vehicle was compared to the Con-ICD ($p=0.000$), K5-Rac1 deficient-vehicle ($p=0.006$) and K5-Rac1 deficient-ICD ($p=0.034$). The size distribution of the diameter of collagen fibrils in the Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups all follow a similar pattern (Fig. 3.15a). Indeed when these groups were compared with each other using Chi-squared test no statistically significant difference in their size distribution of the diameter of collagen fibrils was found.

The size distribution of the diameter of collagen fibrils in region 1, i.e. just below the DEJ, did not reveal a statistically significant difference between any of the groups (Fig. 3.15b).

In region 2 (Fig. 3.15b) the size distribution of the diameter of collagen fibrils was found to be statistically significant when the Con-vehicle was compared to the Con-ICD ($p=0.000$), K5-Rac1 deficient-vehicle ($p=0.000$) and K5-Rac1 deficient-ICD ($p=0.005$). For region 3 (Fig 3.15b), results followed the same pattern as region 2, showing $p=0.000$, $p=0.000$ and $p=0.000$ for the Con-ICD, K5-Rac1 deficient-vehicle, and K5-Rac1 deficient-ICD groups respectively, when compared to the Con-vehicle.

In both region 2 and region 3 the size distribution of the diameter of collagen fibrils in the Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups all follow
as similar pattern (Fig. 3.15b) and indeed no statistically significant difference was found between these groups.
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Figure 3.14 Ultrastructure of Extracellular Matrix in Older Control and K5-Rac1 deficient mice in ICD model. (opposite page) Non-overlapping images of the dermis divided into region 1, 2 and 3 depending on the depth for the DEJ, where region 1 was taken just below the DEJ where the predominant feature seen is fibrillar collagen. Scale bars = 500nm.

Figure 3.15 Size Distribution of the Diameter of Collagen Fibrils in Older K5-Rac1 deficient model of ICD. (a) Shows a heat map representing the size distribution of the diameter of collagen fibrils for all images, i.e. not broken down into regions. (n=4). (b) Shows heat maps representing the size distribution of the diameter of collagen fibrils for separate regions based on their distance from the DEJ. Distributions that do not share a letter are statistically different.
Figure 3.16 V/v Collagen:Extracellular Space for (a) all images i.e. not broken down into regions and (b) for region 1 (just deep to the DEJ), region 2 (just below region 1) and region 3 (just below region 2). (n=4). For graphs where a statistically significant difference (p = <0.05) was found between the groups, letters A and B are used to show where the difference between the groups occurs. To this end groups that do not share a letter are significantly different from each other. . p value shown was determined by one way ANOVA comparing all groups.
The percentage of the ECS occupied by collagen was found not to be significantly different in all the groups when this parameter was examined for the entire area (Fig. 3.16a). When images were broken down in different regions based on their distance from the DEJ, a significant difference was found only for region 3, i.e., the deepest region (Fig. 3.16b). This was due to a decrease in the percentage of ECS occupied by collagen in the croton oil treated control (Fig. 3.16b). This was not significantly different from the K5-Rac1 deficient-ICD which had a slight decrease in the percentage of ECS occupied by collagen. However, this was not significantly reduced compared to the Con-vehicle and K5-Rac1 deficient-ICD (Fig. 3.16b).

Interindividual variation for the percentage of ECS occupied by collagen showed similar low values for all groups (Table 3.2). In terms of intraindividual variation, an increase is seen for Con-ICD in region 3. All other values are similar between groups (Table 3.2).

![Table 3.2 Co-efficient of Variation for V/v of Collagen:ECS](image)

In summary, ultrastructural analysis of the dermal ECM revealed a tendency toward thicker collagen fibrils in the Con-ICD, as well as K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD compared to the Con-vehicle. Also, a decrease in the percentage of ECS occupied by collagen fibrils in the Con-ICD and K5-Rac1 deficient-ICD groups was found. However, this was only statistically significant for the Con-ICD compared to the Con-vehicle.
3.3.2.5 Analysis of Decorin in the Dermal Extracellular Matrix in older K5-Rac1 deficient model of ICD

Immunofluorescent staining for decorin revealed a similar pattern of staining in the dermis of all groups (Fig. 3.17). This result is substantiated by quantification of the complexity and heterogeneity of staining by means of $F_D$ and $\Lambda$ analysis. For these parameters, no statistically significant difference was found in either Z-stacks or when the same regions were analysed in maximum intensity projected images (Fig. 3.18).

Figure 3.17 Analysis of the distribution of Decorin in Older Control and K5-Rac1 deficient mice in ICD model. Immunofluorescence staining for decorin (red) showed similar distribution in all groups. Sections were counterstained with phalloidin (green) and hoechst (blue). E = Epidermis, D = Dermis. Scale bars = 10µm.
Figure 3.18 $F_0$ and $\Lambda$ of Decorin in Older Control and K5-Rac1 deficient mice in ICD model. Graphs show results of $F_0$ and $\Lambda$ of Z-stacks and maximum intensity projected images. (n=4). $p$ value shown was determined by one way ANOVA comparing all groups.
3.3.3 Summary of Results

Histological analysis in the older mice did not reveal any obvious structural difference in the epidermis between any of the groups. There was no indication of blistering due to induction of ICD or the deletion of Rac1. One effect found here in both K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups was sporadic duplication of the BM, a feature that became more frequent with age.

Quantitative analysis on the integrity of the DEJ was found to only be affected in the older mice. In these older mice there was a statistically significant difference in the percentage of the DEJ occupied by a defined BM between the Con-ICD and the K5-Rac1 deficient-ICD groups. In addition, an increase in the thickness of the whole BM in all groups compared to the Con-vehicle was found. In the K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD and increase in the thickness of the LL compared to the Con-vehicle was found. In terms of the thickness of the LD a statistically significant increase for the Con-ICD and K5-Rac1 deficient-ICD compared to the Con-vehicle was seen.

While no obvious morphological alteration on the collagen architecture was qualitatively visible at the light microscopic level, quantification analysis of this revealed a significant difference between K5-Rac1 deficient-ICD and the Con-ICD and K5-Rac1 deficient-vehicle groups revealing a reduction in collagen matrix structural complexity in the K5-Rac1 deficient-ICD group as measured by fractal analysis. Also, an increase in lacunarity, i.e. heterogeneity, was found in the K5-Rac1 deficient-ICD group compared to all of the other groups.

Quantitative analysis of the fibrillar collagen matrix at the ultrastructural level revealed a tendency toward smaller collagen fibrils in the Con-vehicle, compared to all other groups. Also a decrease in the percentage of ECS occupied by collagen fibrils in the Con-ICD and K5-Rac1 deficient-ICD groups was seen, although this was only statistically significant for the Con-ICD compared to the Con-vehicle.

No difference was found in the decorin staining between the different groups when measured by fractal and lacunarity analysis.
3.4 Discussion

Deletion of Rac1 in the keratinocytes of the skin has been found to cause different levels of severity with regard to maintaining the integrity of the IFE in different models. The effects from endogenous deletion in the embryo ranges from mild (Castilho et al., 2007, Chrostek et al., 2006, Tscharntke et al., 2007) to severe (Benitah and Watt, 2007). When deletion of Rac1 in keratinocytes of the skin is induced in adult mice, it was also reported to have severe effects on the IFE (Benitah et al., 2005).

The severe effects, namely the partial and in some places complete loss of epidermal layers, where deletion of Rac1 was induced in adult mice, was reported to be an effect of depletion of epidermal stem cells (Benitah et al., 2005). Indeed in the K5-Rac1 deficient model used for this project, although mild effects on the IFE were previously reported (Chrostek et al., 2006), an increase in differentiation genes was found (Pedersen et al., 2012). Therefore, the deletion of Rac1 may induce epidermal stem cells to detach from the BM and undergo terminal differentiation. However, in vivo, contrasting results were found for the expression of integrin α6β4 and the integrity of HDs due to deletion of Rac1, where it was found to affect them (Benitah et al., 2005) or have no effect (Chrostek et al., 2006).

Indeed, contrasting results on the role of Rac1 in maintaining the epidermal BM are also reported. In vitro work, provides indication that Rac1 activity is necessary for maintaining the DEJ (Kligys et al., 2007, Sehgal et al., 2006). However, such an effect has not been found in vivo to date (Chrostek et al., 2006, Tscharntke et al., 2007), including in previous work carried out in the K5-Rac1 deficient model used for this project, when mice were two weeks old (Chrostek et al., 2006). In addition, while a detailed analysis of the DEJ was not carried out in K5-Rac1 deficient back skin sections used for analysis of the tight junctions (See Chapter 2), it can be said that no obvious effects on the DEJ were observed.

Therefore, in order to try and further elucidate the function of Rac1 in the keratinocytes on the DEJ, here additional stress in the form of age and ICD are applied to the skin. As discussed above, increasing age results in structural and functional changes to the skin (Kottner et al., 2013, Lavker et al., 1987, Wulf et al., 2004) and ICD causes
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pathophysiological changes due to barrier disruption and epidermal damage (Prakash and Davis, 2010, Slodownik et al., 2008). Therefore, such stress on the skin may manifest the effects of Rac1 deletion more clearly.

Ultrastructural examination of the DEJ revealed incidents of duplication of the BM in both younger and older K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD samples. As this was not seen in the any of the control samples, either young or older, vehicle or ICD samples, it can be considered here to be an effect caused by the deletion of Rac1 in the keratinocytes of the skin. This is substantiated by the fact that the occurrences of duplication were observed to a similar level in both vehicle and ICD K5-Rac1 deficient samples.

Although the occurrence of duplication increases with the age of the mice, it still is a sporadic occurrence and indeed does not affect the overall percentage of defined BM at the DEJ for both the K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups compared to the Con-vehicle. However, a higher level of defined BM was found in the Con-ICD, and this was statistically significantly different from the K5-Rac1 deficient-ICD group. What the underlying cause for such a difference in the ICD groups is unclear, possibly this may be a result of a different reaction to the treatment of the keratinocytes at the DEJ in the absence of Rac1.

Duplication of the BM has been reported to be a normal occurrence due simply to reformation of the DEJ as basal keratinocytes migrate into the space left after detachment of stem cells as they begin the process of terminal differentiation (Briggaman and Wheeler, 1975). As previously discussed, there is in vitro and in vivo evidence that in the absence of Rac1 keratinocytes show increased differentiation (Benitah et al., 2005, Chai et al., 2010, Chrostek et al., 2006, Pedersen et al., 2012). Therefore this may account for the duplication of the BM seen here. However, if this is the case, such incidents should also be seen in the control samples, albeit to a lesser extent, as keratinocyte terminal differentiation is a normal physiological process. This however was not the case. It is possible that the keratinocyte mediated reformation of the DEJ is affected due to a reduced ability to organise the laminin-332 matrix. Indeed in vitro evidence suggests that interaction of α6β4 and α3β1 integrin with laminin-332
regulates Rac1 activity and subsequently the organisation of this matrix (Hamelers et al., 2005, Kligys et al., 2007, Sehgal et al., 2006).

The incidents of duplication of the BM became much more frequent with age, as it was seen to a much larger extent in the older mice. The younger mice used in this study would be considered to be mature adults, and the older mice would be considered to be middle aged (http://research.jax.org/faculty/harrison/ger1vLifespan1.html). Indeed, duplication of the BM has been reported to be an age related feature in normal human skin, suggested to possibly compensate for reduced adhesion of the epidermis to the dermis due to retraction of rete ridges (Lavker et al., 1987). Photoaging of the skin has also been reported to cause duplication of the BM (Amano, 2009) and indeed such occurrences due to age have also been reported for other tissues (Alvarado et al., 1983, Kilo et al., 1972, Khalil et al., 1996). It could be postulated that the deletion of Rac1 in the keratinocytes of the skin may result in premature age effects on the BM. Although, if this was the case, it would be expected that such duplication, at least to minor extent, would be seen in the older control samples. However, as stated above, these mice are middle aged and therefore may not have developed such effects on the DEJ yet.

Interestingly, in a study showing age related duplication of the BM in the corneal epithelium, it was reported that duplication only occurred in young adults where there was repeated traumatic abrasion, edema, ulceration or chronic inflammation (Alvarado et al., 1983) which would support the idea that such an effect is pathologic. Indeed duplication of the BM is extensively associated with pathological conditions affecting the skin, including inflammatory skin conditions (Ashton, 2004, Emanuel et al., 2006, Fleischmajer et al., 2000, Hamada, 2002, Rozas Munoz et al., 2011, Shahidullah et al., 1995, Mirancea et al., 2007).

Duplication at the DEJ that is a hallmark of Kindler syndrome (Ashton, 2004). This is a congenital skin blistering condition caused by a mutation in the gene encoding Kindlin-1, an integrin binding protein that regulates their function and mediates their adhesion to the actin cytoskeleton (Karakose et al., 2010, Ye et al., 2011, Ye et al., 2014). Interestingly, duplication of the BM was reported in regions of micro blistering in mice bearing a keratinocyte restricted deficiency of the α3β1 integrin in the skin (Margadant et al., 2009). This would support the idea that duplication of the BM may be the
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consequence of reduced keratinocyte adhesion at the DEJ. In an independent study utilising Cre/K5 promoter for Rac1 deletion in keratinocytes, spontaneous wounding was reported although the underlying cause was not reported (Benitah and Watt, 2007). In the present study, the ultrastructural integrity of the HDs was not affected by the deletion of Rac1 in keratinocytes or ICD in younger or older mice. In addition no statistically significant difference was found in the frequency of HDs or the percentage of the DEJ occupied by HDs in both age groups. In addition there was no evidence of microblistering at the DEJ.

If there is reduced keratinocyte adhesion at the DEJ this would be most evident at the LL. Quantification of the thickness of the BM found that in 11-month-old mice there was a statistically significant increase in the control ICD model and both K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD model compared to the Con-vehicle. In terms of the LL, in 11-month-old mice the thickness in the K5-Rac1 deficient-vehicle treated was statistically significantly increased compared to the vehicle treated control, indicating reduced adhesion at the DEJ. However, there was no evidence of blistering at the light microscopic level or indeed at the ultrastructural level in this group. In the Con-ICD and K5-Rac1 deficient-ICD groups an increase in thickness was also observed compared to the Con-vehicle, although this was only significant for the K5-Rac1 deficient-vehicle samples. This result would indicate that induction of ICD is causing an increase in the thickness of the LL, but that this is more marked where Rac1 has been deleted in the keratinocytes. Indeed, although not statistically significant, there does seem to be a trend towards a thicker LL in the young mice also. The fact that this is also true for the K5-Rac1 deficient-vehicle samples, suggests that this could be due to the already existing heightened inflammation in these mice, and that ICD is not having an additional effect on the LL. Indeed, even though the vehicle treated ear samples are from the same mice as the ICD samples, the inflammatory response to the different regions is statistically significantly different (Pedersen et al., 2012). The vehicle treatment did induce a statistically significant increased inflammatory response in the ears of K5-Rac1 deficient mice compared to the Con-vehicle, however, this was still statistically significantly less than that seen for the ICD model in both the control and K5-Rac1 deficient samples (Pedersen et al., 2012).
A significant increase in thickness of the LD was also found in both control and K5-Rac1 deficient models of ICD compared to the Con-vehicle. An increase in the thickness of the K5-Rac1 deficient-vehicle was also found but this was not significantly different to the Con-vehicle. This would indicate that ICD is causing thickening of the LD. Thickening of the BM is associated with numerous diseases where inflammation is a factor of the disease (Daldon and Lage, 2011, Jeffery, 2001, Joussen et al., 2004, Kim, 2008, Kudo et al., 2013, Soy et al., 2013) including the skin (Regauer et al., 2005). However, these incidents, these are long term conditions. Here, thickening of the LD occurred in eight hours, and also the effect was only found in the older mice. This would indicate that the aged LD is predisposed to thickening in response to ICD. Therefore here it appears that this effect seems to be due to age and ICD and not an effect of Rac1 deletion.

In summary, with regard to potential alterations of the DEJ, it appears that the main effect due to the deletion of Rac1 in keratinocytes in the skin is the presence of sporadic duplication of the BM. This is more common in older mice, and, it raises the questions as to whether Rac1 may be relevant in skin aging.

What the underlying cause for such an effect is unclear at present. Although, due to the increase in thickness of the LL seen in the K5-Rac1 deficient mice, it may appear that there may be reduced adhesion of keratinocytes to the BM. However, no effect on the HDs was observed, or indeed microblistering between the HDs. This would suggest that there is no loss of HDs, α3β1 integrin or indeed other adhesive components at the basal side of keratinocytes. It could be postulated that there may be some alterations in the molecular structure of these junctions or possibly some redox imbalance (See further in Introduction for Chapter 4), possibly resulting in reduced adhesive power. This warrants further investigation. Considering the fact that duplication of the BM was found in association with Kindler syndrome (Ashton, 2004) and with microblisters in α3 KO skin (Margadant et al., 2009), it could be possible that the duplication found here could be, at the most, a very initial indicator of reduced adhesion at the DEJ.

Another important factor for the synthesis and maintenance of the integrity of the DEJ is reciprocal crosstalk between keratinocytes and fibroblasts (Marionnet et al., 2006).
Therefore, the duplication of the BM may actually be an effect of alterations in this crosstalk. If this is the case, it would be expected that the function of fibroblasts would be affected, and therefore, it is reasonable that a defect in fibroblasts may also result in alterations in the ECM production and organisation. In addition to this, the heightened inflammation in K5-Rac1 deficient mice due to the aberrant crosstalk between keratinocytes and the immune system (Pedersen et al., 2012) may have further effects with repercussions on the integrity of the dermal ECM.

In the present study the architecture of dermal collagen was analysed to determine if the deletion of Rac1 in keratinocytes of the skin caused defects in the crosstalk with fibroblasts thereby affecting their function.

To this end the collagen matrix was qualitatively and quantitatively analysed at both the microscopic and ultrastructural level. Qualitative microscopic analysis following staining of the dermal collagen network revealed no clear evidence of the formation of fibrosis, or indeed morphological alterations in the collagen network in response to the deletion of Rac1 or the induction of ICD. Also at the ultrastructural level no indications of any abnormalities due the deletion of Rac1 or the induction if ICD was evident.

In order to quantify the architectural organisation of the dermal collagen matrix fractal and lacunarity analysis was applied. In addition to this, the collagen matrix was analysed at the ultrastructural level for the size distribution of the diameter of collagen fibrils and the percentage of ECS occupied by collagen. Through all these methods of analysing the collagen matrix, no clear pattern of alterations due to the deletion of Rac1 or the induction of ICD emerged across all results. However, this analysis did reveal some perturbations in the dermal collagen fibrils, as discussed below.

Fractal and lacunarity analysis has been used previously to determine changes in collagen matrix organisation (Charadram et al., 2012, Chow et al., 2014, Dioguardi et al., 1999, Dioguardi et al., 2006, Frisch et al., 2012, Rocha et al., 2006), including the skin (Herreros et al., 2007, Khorasani et al., 2011, Wu et al., 2014). Here, a statistically significant decrease in the complexity of the collagen network in the K5-Rac1 deficient-ICD group, when compared to the Con-ICD and K5-Rac1 deficient-vehicle groups, but not the Con-vehicle group, was found. An interpretation to this result in the view of its
biological significance is difficult to determine, but there are alterations. For the lacunarity a statistically significant difference due to an increase in heterogeneity of the collagen matrix in the K5-Rac1 deficient-ICD group compared to all other groups was found. Given that lacunarity is used to describe patterns of fractals, taken FD and Λ together it does appear that the combination of the deletion of Rac1 in the keratinocytes and ICD is having an effect on the architectural organisation of the dermal collagen. The effects on the collagen matrix observed here was only found in the maximum intensity projected images, and not when this method was applied to Z-stack images. Why this was not picked up from analysis on the Z-stacks may be due to the fact that the max intensity projected image is a clearer image as it combines the pixels with maximum intensity, i.e. in focus pixels, from each slice and merges them into one image. It may also be worth performing this analysis at higher magnification. In a study analysing the collagen architecture in dermal scars who also used Sirius red staining and confocal imaging, images used were of higher magnification than that used here (Khorasani et al., 2011). This may help elucidate, or pick up subtle differences in the dermal collagen matrix.

Analysis of the size distribution of the diameter of collagen fibrils at the ultrastructural level revealed a tendency toward larger collagen fibrils in Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups when all images of the dermis from these groups, within a range of 15-20µm from the DEJ were compared to the Con-vehicle. When these images were broken down into regions based on distance from the DEJ, this statistically significant result was also found for region 2 and region 3, but not region 1 i.e. just below the DEJ. The pattern of distribution was found to be similar in the Con-vehicle, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD groups. As previously mentioned deletion of Rac1 in keratinocytes in the skin results in a heightened inflammation in the skin due to increased expression of STAT1 and increased sensitivity of keratinocytes to IFNγ (Pedersen et al., 2012). ICD is defined as inflammation of the skin (Prakash and Davis, 2010, Slodownik et al., 2008). Therefore, taken together, it would appear that a higher inflammatory state is influencing collagen fibril diameter. One key point which must be highlighted, is that this effect on the size distribution of the diameter of collagen fibrils occurred in a relatively short time period of eight hours.
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Therefore, given that notoriously long and slow turnover of collagen fibrils, it is conceivable that the effects observed here may occur on the already existing collagen matrix rather than through production of new collagen fibrils. There are two predominant factors that influence collagen fibril diameter, namely fibril associated collagens with interrupted triple-helices (FACIT) collagens and small leucine rich proteoglycans (SLRPs). In terms of FACIT collagens, Collagen XIV in particular is known, through studies in tendon and lung tissue, to regulate the diameter of collagen fibrils (Banos et al., 2008, Tzortzaki et al., 2003, Young et al., 2000, Zhang et al., 2005). However, to date this has not been shown in skin, and no link between collagen XIV and inflammation has been made. SLRPs on the other hand, have been shown to regulate collagen fibril diameter in skin (Chen and Birk, 2013, Kalamajski and Oldberg, 2010) and are also associated with inflammatory responses (Iozzo and Schaefer, 2010, Moreth et al., 2012, Schaefer and Schaefer, 2010).

Decorin, one of the most prominent proteoglycans in the skin (Bosman and Stamenkovic, 2003, Reed and Iozzo, 2002) is a member of the SLRP family. This SLRP is of particular interest here due to the recent finding that during an allergic inflammatory response, decorin enhances the stability of IFNγ resulting in increased expression of STAT1 (Bocian et al., 2013). In addition to this, decorin binds to collagen fibrils and constrains the rate of fibrillogenesis resulting in more uniform thinner fibrils (Iwasaki et al., 2008, Seidler et al., 2005). Indeed decorin KO mice show an increase in collagen fibril diameter in the dermis, in conjunction with a more irregular appearance of fibrils (Danielson et al., 1997). In the present study, while there is a tendency towards larger collagen fibrils, the fibrils themselves did not display any structural abnormalities as they have a circular outline in cross section. Also, qualitative analysis of immunofluorescent staining for decorin did not reveal any changes in the pattern or intensity of staining in the dermis. This was substantiated quantitatively by fractal analysis, or more importantly analysis of lacunarity. Indeed, lacunarity would reflect differences in staining pattern, and this did not reveal a statistically significant difference between the groups. It may be possible that if it is decorin that is responsible for the increase in diameter of collagen fibrils, the inflammatory response did not alter the localisation of this proteoglycan, but affected its function. Therefore, rather than
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influencing fibrillogenesis, in this case, decorin may become more involved in
mediating inflammation. Indeed it is well established that decorin plays a major role in
inflammation (Frey et al., 2013, Iozzo and Schaefer, 2010, Moreth et al., 2012).

What is of particular interest here, is that although the vehicle treatment does induce an
inflammatory response in mice bearing keratinocyte restricted deletion of Rac1 in the
skin, this is still much less than that seen for the croton oil treatment in the control and
K5-Rac1 deficient mice (Pedersen et al., 2012). Indeed the croton oil treatment on the
K5-Rac1 deficient mice elicited a 3-fold increased response compared to the same
treatment in the control (Pedersen et al., 2012). Therefore, the level of inflammation
does not seem to be a contributing factor, just that inflammation is present. Seen as the
same effect is observed in all three groups, where higher inflammation is present, i.e.
Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD, it would appear that
this is an additional effect on the skin due to the heightened inflammation when Rac1 is
deleted in the keratinocytes. Therefore, here, this effect may be a consequence of
perturbations in indirect crosstalk with the fibroblasts.

Analysis of the percentage of ECS occupied by collagen revealed a statistically
significant decrease in region-3 (i.e. deepest region examined) in the Con-ICD group
compared to the Con-vehicle and K5-Rac1 deficient-vehicle groups. The most obvious
reason for the decrease in the percentage of ECS occupied by collagen would be due to
swelling of the tissue in response to croton oil treatment. However, it has been
previously shown that the K5-Rac1 deficient model of ICD showed the highest
inflammatory response in comparison to the other groups (Pedersen et al., 2012).
Therefore, if this is a direct effect of swelling of the tissue it would be expected that this
would be most evident in the K5-Rac1 deficient-ICD group. Given that this statistically
significant difference was only seen in region 3 of the Con-ICD model, i.e., the region
furthest from the DEJ, where the collagen matrix would be more variable, such an effect
is considered to be of minor significance. Indeed the degree of intraindividual variation
for this region was found to be higher in the other 3 groups.

Taken altogether, this effect on the structural architecture of the dermal collagen fibril
matrix observed here was that there was a tendency toward larger collagen fibrils, most
likely in response to inflammation in the skin. This effect was not evident at the light microscopic level, although a decrease in complexity of the collagen matrix accompanied by an increase in heterogeneity was found for the K5-Rac1 deficient-ICD group. As stated previously, these effects, in addition to the increase in thickness in the LD for the Con-ICD and K5-Rac1 deficient-ICD groups, occurred in a short period of time. What may be of particular interest in the terms of the deletion of Rac1 in the keratinocytes is whether following the resolution of inflammation, these structures return to their normal state. If this was not the case, it may indicate the beginnings of the formation of fibrosis in the tissue. Therefore, it is possible that the overall integrity of the skin in the absence of Rac1 is more sensitive to repeated assault of the tissue.

3.5 Conclusions

The clearest evident effect on the DEJ seen here due to the deletion of Rac1 in keratinocytes of the skin was sporadic duplication of the BM. The underlying cause for such an effect remains to be fully elucidated. In terms of the effect of Rac1 deletion on the integrity of the dermal collagen matrix, it would appear that alterations in the size distribution of the diameter of collagen fibrils correspond to conditions typically characterised by an inflammatory response, i.e. deletion of Rac1 and ICD. Whether such an effect is the first step that may eventually lead to fibrosis needs to be elucidated and represent an interesting follow up study. This study has revealed previously unknown effects on the DEJ and the ECM due to the deletion of Rac1 in keratinocytes typically manifest in response to stress in the form of age progression and ICD.
Chapter 4

Rac1 and NADPH Oxidase 1 complex in the Interfollicular Epidermis
Rac1 and Nox1 in the IFE in vivo

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4.1 Introduction

In this chapter work on the immunofluorescent detection of Nox1 in the IFE is presented. The main aim of this study is to determine whether the deletion of Rac1 in keratinocytes affects the distribution and the intensity level of Nox1 immunofluorescence in the IFE in vivo. In addition, whether these parameters are affected in a short term model of irritant contact dermatitis (ICD) in the IFE of control and K5-Rac1 deficient mice is examined.

Maintaining normal homeostasis of the epidermis requires regulated cellular signalling for proper cell adhesion, proliferation, differentiation, migration, gene expression, immunity, and wound healing. Reactive oxygen species (ROS), such as superoxide or hydrogen peroxide, which were originally thought to be just toxic by-products of oxygen metabolism, have emerged in recent years as important mediators in several signalling pathways that regulate such processes (Bedard and Krause, 2007, Miki and Funato, 2012). Their involvement in such signalling pathways is mediated by their ability to induce post-translational oxidative modifications of sulphur containing amino acid side chains of proteins (Roos and Messens, 2011). Among the proteins that can be redox regulated through this mechanism, as introduced in chapter 1, are members of the Rho GTPase family (Mitchell et al., 2013, Stanley et al., 2014).

However, due to the very nature of ROS, they cannot impart target selectivity or specificity, and are therefore not typical signalling molecules. In order to overcome this problem, the cell must have some mechanisms in place to prevent off target effects. One of the key mechanisms that enable targeted redox regulation of proteins is through dedicated enzymatic systems, namely NADPH oxidase (Nox) complexes, which provide regulated ROS production (Bedard and Krause, 2007, Miki and Funato, 2012).

The Rac subfamily proteins (Rac1, Rac2 and Rac3) are among the central factors that directly control the activity of certain Nox complexes, primarily Nox1 and Nox2 (Hordijk, 2006, Stanley et al., 2012). Interestingly, while Rac proteins have been identified for their involvement in the activation of Nox complexes (Hordijk, 2006), it has been demonstrated that the activity of Rac1 can be redox regulated (Heo and
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Campbell, 2005). In addition, Rac2 and Rac3 have been shown to contain the motif that in Rac1 allows redox regulation and, therefore, the activity of these proteins also have the potential to be regulated through this mechanism (Heo and Campbell, 2005). Therefore, an interesting situation arises where Rac proteins can be both regulators and targets of Nox complex activity (Stanley et al., 2014).

Nox2 (originally named gp91phox), the first member of the Nox protein family to be identified, serves as a prototype for the remaining members (Bedard and Krause, 2007, Lambeth, 2004). This protein is highly expressed in phagocytic cells and although it can be activated by Rac1, it shows preference for Rac2 (Bedard and Krause, 2007, Gu et al., 2001).

Nox1, on the other hand, is expressed in many non-phagocytic cells, showing high constitutive expression in colon epithelial cells where it is proposed to function in innate immune defence and cell proliferation (Rokutan et al., 2008, Rokutan et al., 2006, Bedard and Krause, 2007). It is accepted that Rac1 is the Rac subfamily protein that is necessary for Nox1 activation, (Hordijk, 2006, Leto et al., 2009, Miyano and Sumimoto, 2007).

In addition to Rac1, Nox1 activity is also reliant on other proteins (Fig. 4.1), which was primarily elucidated through in vitro work in endothelial cells (Hordijk, 2006, Ray and Shah, 2005, Sundaresan et al., 1996, Werner, 2004). Nox1 requires p22phox for membrane stabilisation, resulting in the formation of a heterodimer flavocytochrome b_{558} (Parkos et al., 1988, Suh et al., 1999). Its activation is dependent on the cytosolic regulators, first identified in colon epithelial cells, Nox activator 1 (Noxa1) and Nox organiser 1 (Noxo1) (Banfi et al., 2003, Takeya et al., 2003, Geiszt et al., 2003). These proteins are homologues for the Nox2 (the Nox family prototype) cytosolic regulators p67phox and p47phox respectively. While Nox1 is more highly active in the presence of Noxa1 and Noxo1, it can also interact with p67phox and p47phox (Banfi et al., 2003, Geiszt et al., 2003, Takeya et al., 2003).

Classically, active Nox complexes are known to function at the cell membrane and produce superoxide extracellularly, however, it is now known that these complexes can
also associate with, among others, caveolae/lipid rafts, endosomes, the nucleus and the actin cytoskeleton (Leto et al., 2009, Ushio-Fukai, 2009, Stanley et al., 2014).

Figure 4.1 Model of activation of the Nox1 complex. When inactive the Nox1 and p22phox subunits form the membrane bound flavocytochrome b558. The Noxo1 and Noxa1 form a cytosolic regulatory complex. Rac1 resides in the cytosol in an inactive GDP-bound state, and is sequestered by Guanine nucleotide dissociation inhibitor (GDI) protein. Upon activation, the cytosolic regulatory complex translocates to the membrane and binds to the flavocytochrome b558. Rac1 translocates separately following conversion into its active GTP-bound state and binds to the Noxa1 subunit. This completes the formation of the Nox1 complex, which has now been activated enabling the transfer of electrons from NADPH to O$_2$ resulting in the formation of superoxide (O$_2^-$). CM = cell membrane, EX = extracellular space, CY = cytoplasm

As mentioned previously, the activity of Nox1 is reliant on active Rac1, an association that was first uncovered from studies in a kidney cell line; HEK293H cells, a cervical...
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cancer cell line; HeLa cells, and in an ovarian epithelial cell line; CHO-K1 cells (Cheng et al., 2006, Miyano et al., 2006, Ueyama et al., 2006). While to date, the endogenous interaction of these proteins in whole cells has not been demonstrated, it is accepted that Rac1 is necessary for Nox1 activation in non-phagocytic cells (Hordijk, 2006, Leto et al., 2009, Miyano and Sumimoto, 2007). However, despite evidence of the existence of functioning Nox complexes in keratinocytes in vitro, a clear definitive functional correlation between Rac1 and Nox1 in this cell type has not been demonstrated (Stanley et al., 2012).

Nox complexes in keratinocytes are associated with oxidative stress in response to ultraviolet irradiation (He et al., 2005, Henri et al., 2012, Ryu et al., 2010, Valencia and Kochevar, 2006, Valencia and Kochevar, 2008, Wu et al., 2008) tumour formation (Rezvani et al., 2011a, Rezvani et al., 2011b, Chamulitrat, 2010) and apoptosis (Tseng et al., 2012). Of particular interest is that it was reported that Nox complex inhibition blocked ROS production in keratinocytes in vitro response to an irritant, benzalkonium chloride (Kim et al., 2012). In vitro work showed that an increase in ROS production after treatment with various growth factors and hormones could be prevented through inhibition of Nox complexes (Goldman et al., 1998, Goldman et al., 1999, Nakai et al., 2008). Much of the above evidence for functioning Nox complexes comes from the use of unspecific general Nox complex inhibitors. Therefore, while these studies do provide an indication that the effects are mediated by Nox complexes, they do not provide specification as to which complex is involved.

Nox1 protein expression has been demonstrated through western blotting of cultured keratinocytes (Chamulitrat et al., 2004, Wu et al., 2008). Indeed silencing of Nox1 in a human keratinocyte cell line prevented increased ROS production following bacterial exposure (Grange et al., 2009) and knockdown of Nox1 prevented HaCaT cell migration (Nam et al., 2010).

However, to date the expression of Nox1 in keratinocytes in vivo has not been shown.

There are some indications that the function of Rac1 in keratinocytes may be mediated through Nox dependent ROS production. For example, constitutively active Rac1 in
HaCaT cells were shown to enhance neuregulin induced Nox mediated ROS production necessary for the activation of SSH-IL-cofilin pathway of cell migration (Kim et al., 2011). Overexpression of active Rac1 in HaCaT cells also increased VEGF expression in a Nox-ROS dependent manner (Sen et al., 2002). Indeed, as part of this study, application of constitutively active Rac1 to cutaneous wounds resulted in increased VEGF expression and improved wound healing (Sen et al., 2002). However, most interesting is a study in Tiam1-deficient keratinocytes which provides a possible direct link of Rac activity, Nox mediated ROS production and ERK phosphorylation for keratinocyte cell survival (Rygiel et al., 2008). Mice bearing a keratinocyte restricted deletion of Rac1 in the skin show defects in ERK signalling under hyperproliferative conditions, namely wound healing (Tscharntke et al., 2007) and tumour formation (Wang et al., 2010).

In addition to the functional link between Nox1 and Rac1 due to mutual regulatory activity, another interesting aspect that may functionally connect these proteins in keratinocytes is given by the following observation made in the present study. As shown in the previous chapter, sporadic duplication of the BM was one of the effects found due to the deletion of Rac1 in keratinocytes of the skin. As discussed in Chapter 3, this effect on the BM has been previously found in association with reduced adhesion due to defects in integrins at the DEJ namely in Kindler syndrome (Ashton, 2004) and with microblisters in the skin of mice bearing deletion of α3β1 in keratinocytes (Margadant et al., 2009). As a possible explanation for the BM duplication found here in the K5-Rac1 deficient skin, it was postulated that this could be a very initial indicator of reduced adhesion at the DEJ (see discussion of Chapter 3). It is well known that α6β4 and α3β1 integrins are the main components involved in adhesion of keratinocytes at the DEJ (Carter et al., 1990, Hopkinson et al., 2014, Zhang and Labouesse, 2010). It would therefore, be expected that in loss of adhesion at the DEJ, integrin function would be compromised, at least in part.

In the last number of years, integrins have been shown to be under the influence of redox regulation (Eble and de Rezende, 2014), and, therefore, a redox imbalance may have repercussion on integrin function at the DEJ in the absence of Rac1. However, no
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definitive statement can be made as to whether this is the case here. In addition, the sporadic duplication seen here and the absence of blistering, would suggest that at most a very modest reduction of adhesion in the absence of Rac1. Nevertheless, investigating Nox1 in this system would add insight on a potential redox imbalance in absence of Rac1 in keratinocytes and a consequent integrin dysfunction.

While interactions and functional correlations between Rac1 and Nox1 have been studied in different systems, and are likely to also exist in keratinocytes in vivo, whether the deletion of Rac1 may have an effect on the level of mRNA and protein is unknown.

Firstly, loss of Rac1 might alter the turnover of Nox1, since Nox1 lacks Rac1 as a complex partner. Secondly, in terms of the control of expression of Nox1 in epithelial cells, the Nox1 gene contains two regions with a GATA sequence (Brewer et al., 2006), and also a region containing a γ-activated sequence (Kuwano et al., 2006). It was shown that the GATA sequence was involved in the constitutive expression of Nox1 in a colon epithelial cell line (Brewer et al., 2006). On the other hand, the γ-activated sequence, through binding STAT1, was found to be involved in increased Nox1 expression in a large intestinal epithelial cell line in response to IFNγ (Kuwano et al., 2006). It is known that deletion of Rac1 in keratinocytes results in increased expression of STAT1 as well as increased sensitivity to IFNγ (Pedersen et al., 2012). This may provide indication for a link between Rac1 function and control of Nox1 production. Therefore, it is reasonable to think that the deletion of Rac1 in keratinocytes may have repercussions on the expression of Nox1 protein. Of interest, the study by Kuwano et al., 2006, showed that mRNA expression of Nox1 increased after 4 hours treatment with IFNγ, and continued to rise for 24 hours. Subsequently, Nox1 protein expression was significantly increased after 8 hours IFNγ treatment and was still elevated at 24 hours. This corresponded with an increase in Nox1 activity and superoxide production (Kuwano et al., 2006).

Finally, in addition to the Nox1 gene containing two regions with a GATA sequence (Brewer et al., 2006), and a region containing a γ-activated sequence (Kuwano et al., 2006) it also contains regions for binding interferon regulatory factor, AP-1, NFκB, CREB, (Kuwano et al., 2006), HNF-1α and Cdx1 (Valente et al., 2008). Of interest AP-1
Rac1 and Nox1 in the IFE in vivo

(Schonthaler et al., 2011) NFκB (Lawrence, 2009) CREB (Wen et al., 2010) are well known transcription factors involved in inflammatory responses. Indeed transcription of Nox1 protein has been found in response to inflammatory stimuli (Lambeth et al., 2007). Taking this in account, given the established role of Nox complexes as mediators of inflammation (Pelletier et al., 2012, Segal et al., 2012), and considering in vitro evidence that Nox complex inhibition reduced ROS production in keratinocytes in response to an irritant (Kim et al., 2012), the localisation and expression of Nox1 is also examined in a short term models of ICD.

Given the above considerations, the deletion of Rac1 may not only affect the activity of Nox1, but also its protein expression. This study aims therefore to show the in vivo expression and localisation of Nox1 protein in keratinocytes through immunofluorescence in the IFE and to determine whether this is affected due to the heightened inflammation typical of K5-Rac1 deficient skin and/or ICD.

4.2 Materials and Methods

4.2.1 Analysis of Nox1 Expression in the Interfollicular Epidermis of K5-Rac1 deficient mice

4.2.1.1 Mice

Samples used for this analysis were taken from paraffin embedded tissue from 11-month-old Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD as described in chapter 3 (see page 100).

4.2.1.2 Immunofluorescence for Nox1

For each 11 month Con-vehicle, Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD three sections were stained using anti-nox1 rabbit polyclonal antibody, followed by TRITC (594) goat anti rabbit on different days. For all sections, a staining
negative control was included in the corresponding batch where the primary antibody was omitted. The staining procedure was carried out according to the following protocol:

- Deparaffinise sections in xylene 2 X 10 minutes
- Rehydrate sections through a graded series of ethanol (100%, 100%, 95%, 70% and 50% for 2 minutes each)
- Remove ethanol by washing in 1X Tris-buffered saline (TBS) (3 X 5 minutes)
- Incubate in 1X Protaqs I Antigen Enhancer in millipore water at 60°C for 20 minutes (antigen retrieval)
- Wash in 1X TBS on shaker (3 X 5 minutes)
- Place 50μl of Protease XXIV 200mg/ml on each section and incubate at 37°C for 2 minutes.
- Wash in 1X TBS on shaker (3 X 5 minutes)
- Block by placing 50μl of 5% normal goat serum (NGS) on each section and place in a humid chamber. Incubate at room temperature for 3 hours.
- Draw off NGS. Add 50μl of Anti-nox1 rabbit polyclonal antibody (1:200) diluted in 5% NGS to each section. (5% NGS only was placed on negative control sections). Incubate in humid chamber overnight at 4°C.
- Wash in 1X TBS on shaker (3 X 5 minutes)
- Add 50μl of secondary antibody (TRITC (594) goat anti rabbit) 1:200 to each section and incubate in humid chamber for 1 hour at room temperature.
- Wash in 1X TBS on shaker (3 X 5 minutes)
- Counterstain sections for nuclei with Hoechst (1:2000) and F-actin with Phalloidin 488 (1:200) solution at room temperature for 20 minutes.
- Wash in 1X TBS on shaker (3 X 5 minutes)
- Place a coverslip on the section and mount with Fluoromount. Store flat in the dark until dry. Seal with clear nail varnish.
4.2.1.3 Imaging for immunofluorescence for Nox1

Images were captured as Z-stacks on an Andor Olympus Spinning Disk Microscope, using Andor IQ software, with 60x oil immersion objective lens – NA 1.4, at 0.5µm apart. Prior to collecting the Z-stack, exposure time and electron multiplied gain were set and recorded to the channels (405, 488 and 564) of illumination. For each section, 10 non-overlapping regions of IFE were captured.

4.2.1.4 Measurement of Nox1 fluorescence in sections

Z-stacks were imported into ImageJ (Schneider et al., 2012) and separated into their respective channels. Nox1 stained Z-stacks were corrected for background using the “background subtraction from a region of interest” function using a scaling factor of 3. This removes any low level background signal to normalise the data across all treatments. A maximum projected intensity image was created for the green channel i.e. phalloidin stained. This was used in order to draw around the epidermis, excluding the stratum corneum, to create a region of interest for measurement in Nox1 stained Z-stacks. For these regions of interest integrated density in Z-stacks for the red channel i.e. Nox1 stained was measured. In addition the area of the region of interest was measured. Results were collected and analysed using Microsoft Excel®. Integrated density for each sample was determined by correcting for area i.e. dividing total integrated density by total area measured per section.

4.2.1.5 Statistics

Mean and Standard deviation are shown for groups. For all quantification coefficient of variation (standard deviation/mean), expressed as percentage, was calculated for each group (interindivdual variation) and also for each mouse in the group (intraindividual variation). Results were analysed using one way ANOVA in Mintab version 16 taking p = <0.05 as the level of statistical significance.
4.2 Results

4.2.2 Analysis of Nox1 immunofluorescence in IFE K5-Rac1 deficient model of ICD

Qualitative analysis of the Nox1 immunofluorescent staining, in Con-vehicle sections, revealed consistent staining throughout the IFE (Fig. 4.2). This staining appeared to be quite punctate. Also evident in these samples, was in many cells in the IFE there is a clear line of staining surrounding the nuclei. Also evident in these samples was Nox1 staining in cells of the dermis.

This pattern of Nox1 staining in the Con-vehicle staining was comparable to the staining in Con-ICD, K5-Rac1 deficient-vehicle and K5-Rac1 deficient-ICD sections (Fig. 4.2).

Figure 4.2 Analysis of the distribution of Nox1 in Control and K5-Rac1 deficient mice in ICD model. (Opposite page) Immunofluorescence staining for Nox1 (red) showed similar distribution in all groups. An evident clear line of staining was obvious in many cells surrounding the nuclei (white arrows). Sections were counterstained for nuclei Hoechst (blue). E = Epidermis, D = Dermis. Scale bars = 10µm.

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Quantitative analysis of the intensity of Nox1 fluorescence staining did not reveal any statistically significant difference in the IFE between the groups (Fig. 4.3) even when the groups were individually compared with each other.

Interindividual variation for Nox1 fluorescence intensity showed a similar low level in all groups (Table 4.1). On the other hand, a high level of intraindividual variation was seen in all groups. This was slightly raised in the Con-ICD and K5-Rac1 deficient-ICD groups (Table 4.1).

![Nox1 Fluorescence Intensity in the Interfollicular Epidermis](image)

Figure 4.3 Analysis of Nox1 Fluorescence Intensity in Older Control and K5-Rac1 deficient mice in ICD model. $p$ value shown was determined by one way ANOVA comparing all groups.

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<td>Interindividual Variation</td>
<td>13%</td>
<td>11%</td>
<td>11%</td>
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<tr>
<td>Intraindividual Variation</td>
<td>37%</td>
<td>47%</td>
<td>31%</td>
<td>49%</td>
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Table 4.1 Co-efficient of Variation of Nox1 Fluorescence Intensity in the IFE.
4.2.3 Summary of Results

This study showed that keratinocytes do express Nox1 protein in vivo. No alterations in the distribution or a statistically significant difference in the staining intensity of Nox1 in the IFE was found in the presence of deletion of Rac1 in the keratinocytes and/or ICD. There was a slight increase in the intraindividual variation of Nox1 fluorescence intensity in the Con-ICD and K5-Rac1 deficient-ICD groups.

4.3 Discussion

Nox complex mediated ROS production has in recent years emerged as key factor for redox regulated of cellular signalling (Chen et al., 2009, Jiang et al., 2011, Ushio-Fukai, 2009). Rac proteins are among the central components for the activation of Nox1 and Nox2 complexes. A major focus of this on-going project is to explore and visualise to what extent reciprocal interaction between Rac1 and Nox1 may be of functional relevance in keratinocytes. As a first step towards this end, the intensity level of Nox1 staining in the IFE, and the influence of Rac1 on this intensity are investigated. Therefore, Nox1 localisation and the intensity level of staining are first examined here through immunofluorescence in the IFE in control and K5-Rac1 deficient mice. In addition, due to the established role of Nox complexes in inflammation, and the fact that mice bearing a keratinocyte restricted deletion of Rac1 in the skin are more prone to inflammation (Pedersen et al., 2012), these parameters are also assessed in a K5-Rac1 deficient short term model of ICD.

The present study shows for the first time that Nox1 is normally present in the IFE in vivo.

Nox complexes are known to function at the cell membrane, but can also associate with caveolae/lipid rafts, endosomes, the nucleus and the actin cytoskeleton (Leto et al., 2009, Ushio-Fukai, 2009, Stanley et al., 2014). With regard to Nox1, it has been
Rac1 and Nox1 in the IFE in vivo

reported to show weak cytoplasmic and strong perinuclear staining in a transformed gingival keratinocyte cell line (Chamulitrat et al., 2003). Staining in vascular smooth muscle cells was found associated with endoplasmic reticulum in one study (Janiszewski et al., 2005) and punctate staining along associated with caveolae along the plasma membrane in another (Hilenski et al., 2004). It has also been reported to be associated with endosomes (Lassegue, 2007, Miller et al., 2007, Oakley et al., 2009).

In the present study, the pattern of staining of Nox1 in the IFE, revealed a punctate staining with a clear line of staining surrounding the nuclei. At the light microscopic level, it is difficult to define the exact subcellular location of the staining pattern seen here. However, the punctate nature of the staining, which seemed to be also present in the cytoplasm, in addition to the clear line of staining surrounding the nuclei, provides indication that Nox1 seen here may be associated with distinct cellular structures. This could indeed be the plasma membrane of which Nox1 is known to associate (Bedard and Krause, 2007, Leto et al., 2009), and also additional intracellular structures containing a membrane (Hilenski et al., 2004, Janiszewski et al., 2005, Lassegue, 2007, Miller et al., 2007, Oakley et al., 2009). This could also suggest Nox1 mediated ROS production for the mediation of intracellular signalling. Despite the fact that the present investigation cannot specifically demonstrate the exact subcellular location of Nox1, it does clearly show Nox1 protein expression in the IFE in vivo for the first time. Therefore, an interesting future follow–up study would be the analysis on the subcellular distribution of Nox1 in keratinocytes.

One other additional feature of Nox1 staining in the skin seen here is worth mentioning. In addition to the staining in the IFE, Nox1 staining was also evident in dermal cells in this tissue. While it cannot be determined here what cell type these cells are, given that fibroblasts are the primary cell type in the dermis, it is highly likely that these are fibroblasts. Nox2 and Nox4 have been shown to be expressed in dermal fibroblasts (Chenevier-Gobeaux et al., 2006, Loughlin and Artlett, 2010). Therefore, the staining seen here in cells of the dermis may indicate that dermal fibroblasts also express Nox1 in vivo.
Interestingly, while here a high degree of Nox1 staining was found in the skin, in constitutive Nox1 KO mice, it is reported that they do not display a spontaneous phenotype (Gavazzi et al., 2006). This may obviously suggest that the skin is unaffected in these mice. While this does not rule out that the deletion of this protein is not having subtle effects on the skin, it may strongly speak for the fact that Nox1 is not essential for normal functioning of the epidermis. Therefore, while the biological significance of Nox1 in the epidermis in vivo, still remains to be clarified, the present work clearly demonstrates for the first time that Nox1 is normally expressed in keratinocytes in vivo.

Additionally, the present study also shows for the first time that the protein amount of Nox1 is not under the control of Rac1. In fact, the pattern of Nox1 staining seen in the Con-vehicle samples was not visibly altered in response to the deletion of Rac1. Also, no statistically significant difference in the level of Nox1 fluorescence intensity was found in these samples. Due to the heightened expression of STAT1 in keratinocytes lacking Rac1 (Pedersen et al., 2012) and given that transcription of Nox1 was shown to be driven through the JAK/STAT1 in a large intestinal epithelial cell line (Kuwano et al., 2006), it was hypothesized that the deletion of Rac1 in keratinocytes may result in the increased expression of Nox1. The results here provide indication that this may not be case in keratinocytes in vivo, however, this would need to be confirmed by western blotting.

As mentioned previously, Rac1 restricted deletion in keratinocytes results in increased expression of STAT1, which is a downstream effector of IFNγ. This makes K5-Rac1 deficient keratinocytes much more sensitive to inflammation (Pedersen et al., 2012). Despite this, in addition to the fact that Nox complexes are involved in the mediation of inflammation (Pelletier et al., 2012, Segal et al., 2012), results here suggest that any potential involvement of Nox1 in an IFNγ mediated inflammatory response in keratinocytes would not involve regulation of its expression. This also appears to the case in response to ICD as no difference in the level of Nox1 immunofluorescence intensity and no alteration in the pattern in response to the induction of ICD in either control or K5-Rac1 deficient groups was found.
However, one result which may suggest some alteration in the pattern of Nox1 staining in response to ICD is that there was a slight increase in intraindividual variation in the control and K5-Rac1 deficient groups.

Taken altogether, despite the increased inflammation typical of the skin in K5-Rac1 deficient mice or ICD, the localisation and level immunofluorescence intensity of Nox1 protein in keratinocytes in vivo remains unaffected.

Although it is generally accepted that Rac1 is an activator of Nox1, this study now shows that the control mechanism by which Rac1 controls Nox1 may not involve Nox1 expression. Therefore, it appears that the entire control of Nox1 through Rac1 occurs on a functional level, at least in keratinocytes in vivo.

It must be noted that this study cannot provide indication for the functional activation of Nox1. Therefore, it still remains to be determined whether the deletion of Rac1 affects the activity of Nox1 in keratinocytes in vivo. Measuring the activity of Nox complexes employs probes that can detect ROS generation (Maghzal et al., 2012, Nauseef, 2014). Currently, while ROS detection has been reported in in vivo (Kielland et al., 2009) and in ex vivo (Brandes and Janiszewski, 2005, Maghzal et al., 2012) studies, such methods are currently not specific or sensitive enough for detection to determine the sources of ROS production (Brandes and Janiszewski, 2005, Maghzal et al., 2012). The use of ROS detection methods in vitro, combined with Nox complex inhibitors, do provide some indication as to the source of ROS, but cannot provide definitive proof. Indeed, such methods for detecting Nox activity were developed during the study of Nox2 mediated ROS generation in phagocytic cells, where due to the high levels of ROS produced through Nox2, it is easy to determine that this is the source of ROS. Detection of Nox mediated ROS production in non-phagocytic cells is more complicated, as non-phagocytic Nox complexes produce much less ROS. This makes it difficult to specify Nox mediated ROS production from cellular sources of ROS in such cell types (Maghzal et al., 2012, Nauseef, 2014).
Indeed, as part of this on-going project it is aimed to set up an in vitro model to examine the effects of an interaction of Rac1 and Nox1. This is the next step in this investigation and some preliminary work has been done.

Ideally, such a model should be established in primary keratinocytes. However, primary keratinocytes only last a few passages in culture, and, K5-Rac1 deficient primary keratinocytes are even more challenging from this point of view (Pedersen et al., 2012, Chrostek et al., 2006). Therefore, in order to establish a protocol it was decided to use a stable epithelial cell line which is commonly used in our laboratory. To this end, Madin-Darby Bovine Kidney (MDBK) cells were utilised, to study changes in ROS production after treatment with one of the most widely used inhibitor of Nox complexes, namely Diphenyleneiodonium chloride (DPI). Preliminary staining for ROS in these cells using a commonly used probe for ROS detection, (namely H2DCFda), did not reveal any changes in the level of ROS in presence even after DPI treatment. However, the major problem with the use of this inhibitor was that after 3 hours the cells stopped moving and later detached from the culture dish. Given that the use of current methods for detecting ROS production are not able to distinguish between the different cellular sources of ROS (Maghzal et al., 2012, Nauseef, 2014) and the response of the cells to DPI found here, it was decided to seek an alternative mechanism to study the effects of the functional interaction of Rac1 and Nox1. To this end, preliminary co-localisation experiments for active Rac1 and Nox1 in MDBK cells were carried out, trying also to inhibit not only Nox complexes but also Rac1.

Co-localisation analysis of two fluorescently labelled proteins is a method that can be used to infer interaction (Dunn et al., 2011, Zinchuk et al., 2007). Given that it is well accepted that Rac1 is part of the Nox1 complex, and is necessary for its activation (Hordijk, 2006, Leto et al., 2009), their co-localisation would also suggest Nox1 activation.

The outcomes from these experiments were unsatisfactory for a number of reasons. Other than the above mentioned poor response of the cells to DPI, other issues were encountered. Firstly, no observable effect from the use of the Rac1 inhibitor was found when used at the recommended concentration or indeed much higher concentrations. Secondly, no visible change in the staining for active Rac1 in the cells after treatment
with the Rac1 inhibitor (NSC23766) was noted. Thirdly, staining cells with the active Rac1 antibody revealed cytoplasmic staining with little staining at the plasma membrane, which, independent of any additional potential localisation, was to be expected for Rac1 in its active state.

This preliminary work highlighted many limitations with the use of pharmacological inhibitors and with the anti-active Rac1 antibody, which in this system, needs further testing. Therefore, before moving to a primary keratinocyte model, it may be more promising to examine the effects of the functional interaction of Rac1 and Nox1 through other means, such as for example the use of genetic manipulation studies on stable cell lines utilising RNA interference technology.

### 4.4 Conclusions

This study demonstrates that keratinocytes of the IFE do express Nox1 in vivo. The localisation and the level immunofluorescence intensity of Nox1 is not affected by the deletion of Rac1 or the induction of ICD, or indeed the combination of Rac1 deletion and ICD. Although it cannot be ruled out that Rac1 may not take part in the functional regulation of Nox1 activity in keratinocytes in vivo, it can definitely be stated that it does not take part in the regulation of its localisation and expression.
Summary of Thesis

Work presented in this thesis elucidated important questions pertaining to the role of the Cdc42 effector N-WASP, RhoA and Rac1 in maintaining the structural integrity of the IFE. A critical aspect of the structural integrity of the interfollicular epidermis (IFE) is the ability of keratinocytes to form a stable cohesive sheet which is fundamentally achieved through cell-cell and cell-matrix junctions. While many techniques can provide indirect evidence for structural defects in the components that contribute to this integrity, e.g. basement membranes, desmosomes etc., the contribution of ultrastructural analysis is fundamental to visualise tissue features which are critical for the fulfilment of functional requirements. This, therefore, points at the fact that a thorough integration of research methods, including ultrastructural histological evaluation, which is traditionally seen as an “old” method of investigation, is still indispensable in modern science to obtain the full answer to cell biological questions.

Through the investigations presented in Chapter 2, it was possible to definitely demonstrate that the structural integrity of the IFE is not affected by the lack of RhoA and N-WASP, and, that the increased susceptibility to inflammation observed in Rac1-deficient skin is not due to a barrier disruption.

Such analysis was also utilised in Chapter 3 to study the debated role of Rac1 in maintaining the overall integrity of the IFE. This revealed mainly a mild effect on the DEJ, mainly visible as age progresses and after a detailed examination of the DEJ. These included sporadic duplication of the BM and increased thickness of the LL. These results, taken together, and, integrated into the current cell biological knowledge may be an indication of very early defects of epidermal adhesion at the DEJ, and, also raise the question of a possible involvement of Rac1 in the context of skin aging. Furthermore, in the same chapter, ultrastructural analysis revealed that there was an alteration in the size distribution of collagen fibrils corresponding to conditions typically characterised by an inflammatory response, i.e. Rac1 deletion and ICD. Therefore, this study has revealed
additional effects of Rac1 deletion in the skin and highlighted unknown aspects of crosstalk between keratinocytes and the underlying connective tissue environment.

The work presented in Chapter 4 showed for the first time that keratinocytes in the IFE \textit{in vivo} do express Nox1 under basal conditions, and, that this expression is not visually or quantitatively altered in the absence of Rac1 and in inflammatory conditions in general. This highlights the fact that the activity of Nox1 (which, also in keratinocytes \textit{in vivo}, may likely be implicated in ROS production both in basal and inflammatory conditions) does not undergo a regulation through enhancement of its expression. This includes also the fact that any potential regulatory role of Rac1 on Nox1 activity in keratinocytes \textit{in vivo} is only carried out at the functional level and not through control of its expression. Further development of research tools facilitating specific detection of Nox1 derived ROS \textit{in vivo} or establishing an appropriate and very specific \textit{in vitro} model may complement the present investigation. This may also help to shed light on the specific role of Rac1 in the activation of Nox1 and on functional control of specific ROS production in skin keratinocytes.
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Appendix 1: List of Materials Reagents and supplier

**Reagents**

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**Materials**

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