Effect of Flightless protein on skin architecture, cellular responses and Epidermolysis Bullosa

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February 2011

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ABSTRACT

Wound healing is an area of largely unmet medical need with patients often relying on wound management practice rather than specific therapies. Recent research in our laboratory has identified a cytoskeletal protein Flightless (Flii) as a negative regulator of wound healing. This highly conserved protein is important in development and has a unique structure allowing it to act as a multifunctional protein. Flii expression increases in response to wounding, inhibiting cellular migration and proliferation while its deficiency improves wound healing. The aim of this study was to investigate the effect of differential Flii expression on skin architecture, cellular responses during wound healing, adhesion-mediated cell signaling and skin blistering associated with the genetic skin disorder Epidermolysis Bullosa (EB).

Chapter 3 of this thesis describes the effect of differential Flii expression on skin architecture and formation of hemidesmosomes which anchor the skin layers. Using primary fibroblasts and keratinocytes with varying Flii expression this study investigated the effect of Flii expression on cellular adhesion, spreading and migration on different extracellular matrix substrates. The results presented in Chapter 3 also describe the effect of Flii neutralising antibodies on primary keratinocyte proliferation illustrating improved proliferation in response to decreased Flii expression.

In Chapter 4 an incisional wound healing model was used to investigate the effect of differential Flii expression on different components of hemidesmosomes. Flightless was shown to regulate hemidesmosome formation through its effects on integrin-mediated cellular adhesion and migration. Using immunoprecipitation studies, Flii association with structural and signaling proteins present at the dermal-epidermal junction was investigated. Flii was found to form a cytoskeletal complex with talin, vinculin and paxillin suggesting its role in downstream signaling. The association of Flii with paxillin was further investigated in Chapter 5 where the effect of Flii over-expression on fibroblast adhesion and formation of adhesion structures was examined. Flii over-expression inhibited paxillin activation and the turnover of adhesion structures through down regulation of signaling proteins involved in cell adhesion signaling pathways.

Chapter 6 of this thesis summarises the effect of Flii in skin blistering by utilizing both human samples and two mouse models of Epidermolysis Bullosa. Flii expression is significantly increased in response to skin blistering and its effects on integrin mediated cellular adhesion, migration and type VII collagen expression make Flii a negative contributor to blister formation. Decreasing Flii expression genetically or using neutralizing antibodies reduces skin blistering, improves cellular adhesion and decreases TGF-β mediated collagen contraction.

In summary, Flii adversely affects skin strength and blister formation. Using a multidimensional approach of both in vitro and in vivo methodologies, human tissue and animal models this thesis reveals several novel findings and contributes to better

understanding the involvement of Flii in both maintaining skin homeostasis and regulating wound repair. Flii is a novel target for development of mechanistic based therapy for improved wound healing. Findings presented in this thesis may open doors to significant changes in clinical practice and contribute to better therapeutic design by which would healing of blisters in patients with Epidermolysis Bullosa might be improved.

DECLARATION

"This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to **Zlatko Kopecki** and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text"

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Zlatko Kopecki

ACKNOWLEDGMENTS

Prof Allison Cowin

Thanks to my inspiring supervisor, Prof Allison Cowin who has provided me with enormous support, enthusiasm and encouragement throughout my PhD journey. I was privileged to work in your laboratory and have enjoyed the research challenges we faced. Thank you for your advice, knowledge, guidance, patience and reassurance all of which have been invaluable.

*

A/Prof Barry Powell

Thank you for being my second supervisor. I truly appreciate all your support, advice, guidance, attention to detail and help with my PhD project and the thesis.

*

Women's and Children's Health Research Institute

To all the members of the Wound Healing Laboratory group I offer particular thanks for your advice and assistance, support and friendship throughout my PhD journey. Thanks to the entire Women's and Children's Health Research Institute staff, especially students for a fun and enjoyable time during my PhD.

*

A/Prof Ruth Arkell

Thank you for providing the Flightless transgenic mice used in this project and for your assistance and advice during manuscript preparation.

*

Prof Detlef Zillikens and Prof Ralph Ludwig

Thank you for assistance with the development of the EBA mouse model and for providing the ColVII antibody used in this project.

Prof Leena Bruckner-Tuderman and Prof Christina Hass

Thank you for having me in your laboratory, for your advice and for providing the ColVII hypomorphic mice and Kindler patient samples used in this project.

*

Prof Geraldine O'Neil

Thank you for your advice in protocol development and for your assistance with manuscript preparation.

*

Prof Dedee Murrell

Thank you for having me in your laboratory, your advice and for providing me with the human EB samples used in this project.

*

Department of Paediatrics, University of Adelaide

To all staff of the Department of Paediatrics, University of Adelaide I offer sincere thanks for encouragement, administrative support and advice throughout my PhD.

*

NHMRC, Channel 7 Children's Research Foundation and DEBRA-SA

Thank you for your support and funding over the years.

*

Jasminka, Josip and Vedrana Kopecki

I would like to express everlasting gratitude to my family, my parents Josip and Jasminka who have raised me into a person I am today, and always offered encouragement, advice and unconditional support and love. Thanks to my sister Vedrana who has always been there for help and advice and has offered endless support and love throughout the years. Thank you all for helping me fulfil my dreams and pursue happiness, I love you all.

PUBLICATIONS ARRISING FROM WORK IN THIS THESIS

Kopecki Z, Cowin AJ. Flightless I: an actin-remodelling protein and an important negative regulator of wound repair. *Int J Biochem Cell Biol*. 2008;40(8):1415-9. **IF 4.804 RIF 39/156.**

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NATIONAL AND INTERNATIONAL SCIENTIFIC MEETINGS AND AWARDS ARISING FROM WORK PRESENTED IN THIS THESIS

Kopecki Z, Arkell R, Powell B.C and Cowin, A.J (2008) Flightless I regulates hemidesmosome formation and integrin mediated cellular adhesion and migration during wound repair. **1st Australasian Wound and Tissue Repair Society Conference, oral presentation – Darwin 2008.**

Kopecki Z, Adams D, Arkell R, Powell B.C and Cowin, A.J. Flightless I affects hemidesmosome formation and integrin mediated cellular adhesion and migration during wound repair. **Australian Wound Management Association Conference – Darwin 2008.**

Kopecki Z, Adams D, Arkell R, Powell B.C and Cowin, A.J. Flightless I affects hemidesmosome formation and integrin mediated cellular adhesion and migration during wound repair. **Australian Society for Medical Research, oral presentation – South Australia Annual Conference 2008.**

Kopecki Z, Adams D, Arkell R, Powell B.C and Cowin, A.J. Flightless I regulates hemidesmosome formation and integrin mediated cellular adhesion and migration during wound repair. **Combio Conference - Canberra 2008.**

Kopecki Z. Helping the butterfly children: Role of Flightless in Epidermolysis Bullosa. **Invited Young Investigator presentation – Women's and Children's Health Research Institute Annual Public Meeting 2009.**

Kopecki Z, Adams D, Arkell R, Powell B.C and Cowin, A.J. Role of Flightless protein in skin blistering disease Epidermolysis Bullosa **Australian Society for Medical Research, oral presentation – South Australia Annual Conference 2009.**

Kopecki Z, Adams D, Arkell R, Powell B.C and Cowin, A.J. Flightless I, a negative regulator of wound healing, affects cellular adhesion and blister formation in Epidermolysis Bullosa – Abstract presentation, **Wound Healing Society and European Tissue Repair Society conference, Limoge, France 2009.**

Kopecki Z, Adams D, Arkell R, Powell B.C and Cowin, A.J. Flightless I, a negative regulator of wound healing, affects cellular adhesion and blister formation in Epidermolysis Bullosa – **Plenary Talk – European Society for Dermatological Research Annual Meeting, Budapest, Hungary 2009.**

National Health and Medical Research Council (NHMRC) Research Grant "Function of Flightless I in skin blistering disorder Epidermolysis Bullosa" AJ Cowin and DF Murrell. (Grant number # 626802) 2010 -2013. Assisted with grant proposal.

Channel 7 Children's Foundation "Function of Flightless in the skin blistering disorder Epidermolysis Bullosa" Cowin AJ. 2008-2010. Assisted with grant proposal.

Young Investigator Award Finalist – Excellence in Science and Communication 2010 Children, Youth and Women's Health Service.

Tissue Therapies Conference Travel Award for attendance and abstract presentation at the 1st Australasian Wound and Tissue Repair Society Conference – Darwin 2008.

Dystrophic Epidermolysis Bullosa Research Association (DEBRA) – South Australia Research Grant 2009-2010.

European Society for Dermatological Research Travel Fellowship 2009.

Channel Nine Young Achiever Awards – Semi Finalist – University of Adelaide Faculty of Sciences Science & Technology Award category 2009.

Brenda Nelson Travel Fellowship, AFUW-SA 2009.

Faculty of Health Sciences Travel Fellowship University of Adelaide 2009.

Research Abroad Scholarship, Department of Paediatrics University of Adelaide 2009.

Australian Society for Medical Research – Ross Wishart Memorial Award Semi-Finalist 2009.

Dystrophic Epidermolysis Bullosa Research Association –SA President 2009-2010.

LIST OF ABREVIATIONS

A	absorbance
α	alpha
AD	autosomal dominant
AF	collagen VII anchoring fibrils
AP	anchoring plaque
APS	ammonium persulphate
AR	autosomal recessive
Arp 2/3	actin related protein
AF	anchoring fibrils
α-SMA	Alpha Smooth Muscle Actin
AP-1	activating protein-1
APS	ammonium persulfate
B	blister
β	beta
β-tubulin	beta tubulin
BP	bullous phemphigoid
bp	base pair
BCA	bicinchoninic acid
BSA	bovine serum albumin
°C	degrees Celsius
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cm	centimetre

CaMK-II	calcium/calmodium dependent protein kinase type II
c-DNA	complementary deoxyribonucleic acid
CISK	cytoline-inderpendent survival kinase
CO_2	carbon dioxide
ColVII	type VII Collagen protein
COL7A1	human type VII alpha-1 collagen gene
COL17A1	human type XVII alpha-1 collagen gene
d	dermis
dATP	deoxtadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEB	Dystrophic Epidermolysis Bullosa
DDEB	Dominant Dystrophic Epidermolysis Bullosa
DEJ	dermal-epidermal junction
dGTP	deoxyguanosine triphosphate
dTTP	deoxythyamine triphosphate
d0	day 0 post-wounding
d3	day 3 post-wounding
d7	day 7 post-wounding
d14	day 14 post-wounding
d21	day 21 post-wounding
DAPI	4',6 – Diamidino-2-phenylindole dihydrochloride
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's media
DNA	deoxyribonucleic acid
e	epidermis
EB	Epidermolysis Bullosa

EBS	Epidermolysis Bullosa Simplex
ECL	enhanced chemical luminescence
ECM	extracellular matrix
EDTA	ethylediaminetetraacetic acid
EGF	epidermal growth factor
EM	emission wavelength
EX	excitation wavelength
FA	focal adhesion
FAK	focal adhesion kinase
FC	focal complex
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
Flii	Flightless I protein
Flii	Flightless I gene
$Flii^{+/-}$	Flightless heterozygous
$Flii^{Tg/+}$	Flightless transgenic (contains $Flii^{+/+}$; $Flii^{Tg/+}$)
$Flii^{Tg/Tg}$	Flightless transgenic (contains $Flii^{+/+}$; $Flii^{Tg/Tg}$)
fli-1	Flightless I homolog
FLAP-1	Flightless associated protein-1
FLAP-2	Flightless associated protein-2
FnAb	Flightless neutralising antibodies
Fp	filopodium
FRT	Flp recombinase target
x g	times the force of gravity
γ	gamma

g	grams
GFP	
HaCaTs	human keratinocytes
HD	hemidesmosome
H&E	Haematoxylin and Eosin
HFF	human foreskin fibroblasts
hr	hour
HRP	horse radish peroxidise
IgG	immunoglobulin-G
IL-1	interlucin-1
IP	inner plaque
ITG	integrin gene
JEB	Junctional Epidermolysis Bullosa
K5	Keratin-5
K15	Keratin-15
KCl	potassium chloride
kDa	kilo Daltons
KIND1	kindler gene
L	litre
LAMA	laminin gene
LD	lamina densa
LL	lamina lucida
LLGL1	Lethal Giant Larve homolog
LM	lamella
LRR	leucine rich repeat
M	molar

MAPK	mitogen activated protein kinase
$MgCl_2$	magnesium chloride
min	minutes
ml	millilitre
mm^2	millimetre square
mM	milimolar
MMP	matrix metalloproteinases
MMP7	matrilysin
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NHS	normal horse serum
NF-κB	Nuclear Factor Kb
nm	wavelength
NR	nuclear receptor
nt	nuceotide
OP	outher plaque
PAGE	polyacrylamide
PBS	phosphate buffered saline
PCR	Polymerase Chain Raction
PCNA	proliferating cell nuclear antigen
PCT	progenitor cell trageted
PDGF	platelet derived growth factor
PGKNeo	phosphoglycerate kinase promoter–driven neomycin phosphotransferase expression
PIP_2	phosphatidylinositol 4,5-biphosphate
PLEC1	plectin 1 gene

PM	plasma membrane
rFlii	recombinant Flii protein
RAI1	retinoic acid induced 1
RDEB	Recessive Dystrophic Epidermolysis Bullosa
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT-qPCR	real time-quantitative polymerase chain reaction
sbdp	hemidesmosomal sub-basal plate
SCC	Squamous Cell Carcinoma
SDS	sodium dodecylsulphate polyacrylamide gel electrophoresis
sec	seconds
SEM	standard error of mean
SF	stress fiber
SMCR7	Smith Magenis Syndrome chromosome region candidate homolog
SMS	Smith Magenis Syndrome
TEMED	N,N,N’,N’-tetramethylethylenediamine
TIR	Toll/IL-1 receptor domain
TLR	Toll-like receptor
TGF-β	transforming growth factor-beta
TGF-β1	transforming growth factor-beta one
TGF-β2	transforming growth factor-beta two
TGF-β3	transforming growth factor-beta three
TNF-α	tumor necrosis factor-alpha
TOPA3A	Topoisomerase DNA III alpha
Tris	tri(hydroxymethyl)methylamine

TRS	target retrieval solution
µg	microgram
µl	microlitre
µm	micrometer
μm^2	micrometer square
µM	micro molar
WT	wild-type
WST-1	2-(4-iodophenyl)-3-(4-nitrophenly)-2H-tetrazolium
x	times
%	percent
+/+	homozygous
+/-	heterozygous
<	smaller than

Chapter 1

LITERATURE REVIEW

Parts of this chapter have been published in following journals:

Kopecki Z, Cowin AJ. Flightless I: an actin-remodelling protein and an important negative regulator of wound repair. *Int J Biochem Cell Biol.* 2008;40(8):1415-9. **IF 4.804 RIF 39/156.**

Kopecki Z, Murrell DF, Cowin AJ. Raising the roof on epidermolysis bullosa (EB): a focus on new therapies. *Wound Practice and Research*. 2009;17(2):76-82.

1.1 Overview

The "normal" non-pathogenic wound healing process relies on a fine balance between cellular proliferation, adhesion and migration resulting in tightly controlled equilibrium between tissue regeneration and fibrosis. The coordination of multicellular responses to tissue injury and control of the dynamic mechanisms involved in wound healing, both of which are complex and difficult to understand, underlie the outcome of wound repair. The outcome of this normal non-pathogenic response to tissue injury involves re-establishment of the skin barrier function, restoration of the skin anatomical structure and physiological function however it also inevitably results in scar formation.

Unfortunately wound healing and scar formation is an area of largely unmet medical need. Various factors and genetic conditions can affect our ability to heal which results in a variety of wound healing disorders with different wound pathologies. Current available medical treatments or clinical methods rely greatly on wound management rather than specific treatment or prevention of different underlying wound pathologies. Wound healing disorders can be loosely categorized into four main types including chronic wounds, diabetic ulcers, burn related hypertrophic scar formation and skin blistering genetic disorders. Each of these conditions is associated with different therapeutic problems and is becoming of increasing clinical importance due to increasing morbidity, mortality and rising health system costs for the community. Healing by fibrosis instead of regeneration places a huge burden on the public health system and is estimated to cost in order of tens of billions of dollars annually (Gurtner *et al*., 2008, Sen, 2009). Dysfunctional healing, physical and psychological effects of these conditions result in a lifelong disability and place a great burden not only on individuals and their families but also on a wider community, highlighting the need for further research and development of the novel

mechanistic based therapeutic interventions which improve wound healing and reduce scar formation. This introduction will detail the fundamental processes of wound healing with particular focus on integrin receptors, actin cytoskeleton and proteins involved in mediating cell changes during wound healing including Flightless (Flii), paxillin and talin. Moreover, it will describe the wound repair in a group of genetic skin blistering diseases called Epidermolysis Bullosa (EB) where mutations in proteins present at the dermal-epidermal junction result in recurrent blister formation.

1.2 Skin Biology

Mammalian skin consists of two layers, a keratinised stratified epithelium consisting of keratinocytes and an underlying thick layer of collagen-rich, dermal tissue containing fibroblasts (Martin, 1997). From the obvious role in providing a protection barrier against infection skin also plays important roles in regulating homeostasis by controlling blood flow, temperature and sweat production and contains a network of sensory nerve endings allowing us to sense the external environment (Alonso and Fuchs, 2003, Koster, 2009).

The epidermis, the outermost component of the skin consists of a population of keratinocytes which are under continual differentiation, division and movement outward towards the surface of the skin. The epidermal layer is the primary barrier that protects the body from dehydration, mechanical trauma and insults and is further divided into five layers depending on the stages of cell differentiation, namely: *Stratum basale*, *Stratum spinosum*, *Stratum granulosum*, *Stratum granulosum* and *Stratum Corneum* (Alonso and Fuchs, 2003, Koster, 2009) (Fig 1.1). Cells of the Stratum basale are undifferentiated keratinocytes which have an amazing ability to self renew. More differentiated cells in the

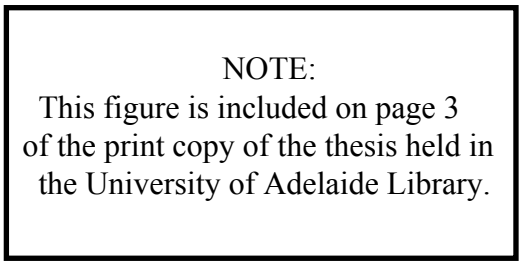


Figure 1.1: Morphology of the skin layers. Diagrammatic representation of the skin epithelial morphology illustrating different layers and cells differentiation throughout the epidermis. Figure adapted from (Alonso and Fuchs, 2003).

epidermis are found in the *Stratum spinosum* where cells have a structural function and produce keratin proteins namely, Keratin 1 and Keratin 10. Cells differentiate further and move into the next layer called *Stratum granulosum* where cells are characterized by the production of the keratohyalin granules made up of histidine-rich proteins and keratin (Kanitakis, 2002). The most stratified layer of the skin, *Stratum corneum*, is characterized by large keratinocytes called corneocytes, which have undergone terminal differentiation, and are replaced from differentiating cells moving from the layers below (Alonso and Fuchs, 2003).

Separating the two layers of the skin, at the dermal-epidermal junction is the basement membrane, a mixture of extracellular matrix proteins secreted by the epidermal and dermal cells, composed together to provide a connection between the two skin layers (Fig 1.2) (McMillan *et al*., 2003). On the epidermal side of the basement membrane are perpendicularly arranged basal keratinocytes which anchor to the basement membrane by the special adhesion structures called hemidesmosomes and form tight connections with the neighbouring cells, forming attachment plaques called desmosomes. Ultrastructurally, these basal keratinocytes contain the electron dense cytoplasmic intermediate filaments (tonofibrils) consisting of keratin polypeptides (Kanitakis, 2002, Litjens *et al*., 2006). The hemidesmosomes are specialized anchoring complexes of proteins providing the stable adhesion of basal epithelial cells to the basement membrane. Type I hemidesmosomes, found in skin, consist mainly of integrin α6β4, plectin, tertraspanin CD151 and bullous pemphigoid (BP) antigens 180 and 230. Human patients with mutations to any of the hemidesmosome components develop the skin blistering disease called EB, and severity of the disease is dependent on the type and location of the mutations and subsequent mRNA and protein levels (Margadant *et al*., 2008, McMillan *et al*., 1998, Tsuruta *et al*., 2003).

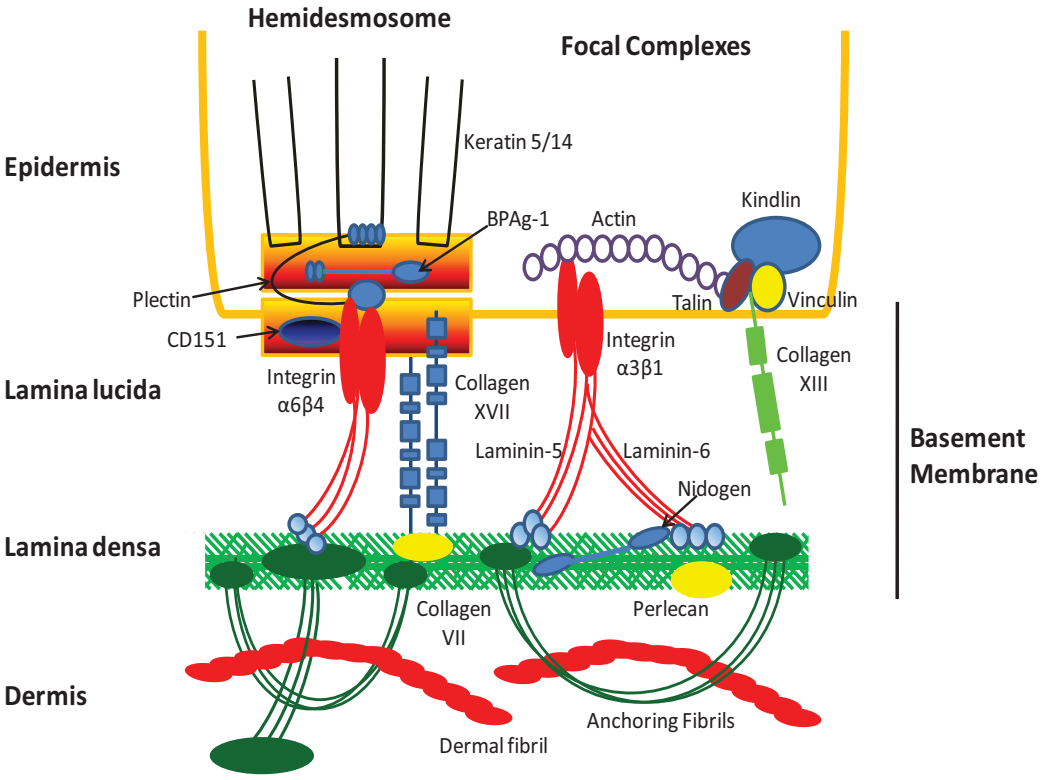


Figure 1.2: Proteins of the basement membrane. Schematic diagram of some of the proteins present at the dermal-epidermal junction providing a strong adhesion of the skin layers.

The basement membrane zone provides the adhesion interface, permeability barrier and controls the cell organisation. Structural components of the basement membrane zone include: keratin tonofilaments, outer and inner plaques of hemidesmosomes, plasma membrane, lamina lucida, lamina densa and type VII collagen (ColVII) anchoring fibrils which span into the papillary dermis (Bruckner-Tuderman *et al*., 1999, Masunaga, 2006).

Beneath the basement membrane is the second layer of the skin called the dermis. Dermis is regulated by the synthesis and degradation of its protein components, fibrous molecules and extracellular matrix (Kanitakis, 2002). The superficial region or papillary dermis is made up of collagen fibres arranged in loose bundles and thin elastic fibres. The deeper region or reticular dermis is made up of coarser collagen bundles, a thicker elastic network, dermal appendages and vascular and nerve plexuses (Sorrell and Caplan, 2004). The majority of the dermal fibres are composed of interstitial collagen type I and III, providing mechanical stability and strength to the skin. Other collagens found in the dermis include collagen IV, a component of the basement membrane and blood vessels, and ColVII, a component of the dermal epidermal junction anchoring fibrils (Kanitakis, 2002). The main fundamental cells of the dermis are fibroblasts. These spindle shape calls not only provide the fibres for dermal architecture but also interact with other cells and growth factors and participate in both inflammatory and wound repair functions. Fibroblast-keratinocyte interactions and cross talk through different signaling pathways is essential for effective skin function while differentiated fibroblasts, called myofibroblasts, provide tensile strength required for closure of the wound margins (Tomasek *et al*., 2002). Figure 1.3 illustrates the morphology of the skin and the basement membrane zone.

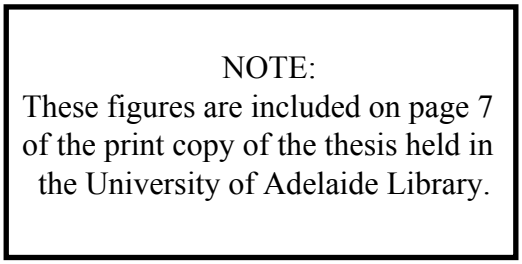


Figure 1.3: Morphology of the skin and the basement membrane zone. A Light microscopy of the skin illustrating an epidermis (E) with different layers of keratinocyte cells and the dermis (D) consisting of collagen fibres, fibroblasts and inflammatory cells in response to tissue injury. **B** Electron microscope image examining the structure of basement membrane and illustrating hemidesmosome adhesion structures (HD), lamina lucida (LL) and lamina densa (LD) and the ColVII anchoring fibrils (AF). N indicates the nucleus of the basal keratinocyte. Scale Bar = 0.2μm. **C** Higher magnification of the hemidesmosome (HD) illustrating outer plaque (OP) and inner plaque (IP), plasma membrane of the basal keratinocyte (PM), hemidesmosome subbasal dense plate (sbdp) in addition to lamina lucida (LL), lamina densa (LD) and anchoring fibrils (AF). Scale Bar = 0.1μm. Figure from (Masunaga, 2006).

1.3 Wound Healing

Wound healing is a highly dynamic biological process that results in restoration of tissue integrity and involves interactions between growth factors, different blood and tissue cell types and extracellular matrix components (Desmouliere *et al*., 2005). The study presented in this thesis will examine and outline the process of wound healing which results in a healed skin and a mature scar at the site of injury. Better understanding of the mechanisms involved in wound healing will allow us to improve our knowledge and practice in treatment of different pathological wounds, ranging from chronic wounds and ulcers to burn contractures and blistering wounds. Research to date has led to our understanding of the processes involved in wound healing which take place in three interrelated and complex phases, namely inflammation, granulation and remodelling. While these phases of wound healing overlap and influence each other they will be described below separately to simplify the review of the wound healing process (Fig 1.4 and Fig 1.5).

1.3.1 Inflammatory Phase

The initial inflammatory stage of wound healing takes place straight after injury and involves the action of platelets that act to restore the balance of haemostasis and prevent further blood loss by forming a blood clot. Activated platelets bind to collagen fibres of connective tissue and aggregate forming a fibrin plug and release more growth factors, cytokines, fibrinogen fragments and other proinflamatory mediators (Grose and Werner, 2004). The process of diapedesis takes place where binding of leucocytes to the integrin receptors result in their internalization through endothelial gaps into the extracellular space. Transient hypoxia in the wound increases keratinocyte migration, early angiogenesis, cell proliferation, clonal expansion of fibroblasts, and transcription and

NOTE:
These 3 figures are included on page 9 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.4: Different phases of wound healing. Diagrammatic representation of the three main phases of wound healing**.** Figures adapted from (Gurtner *et al*., 2008).

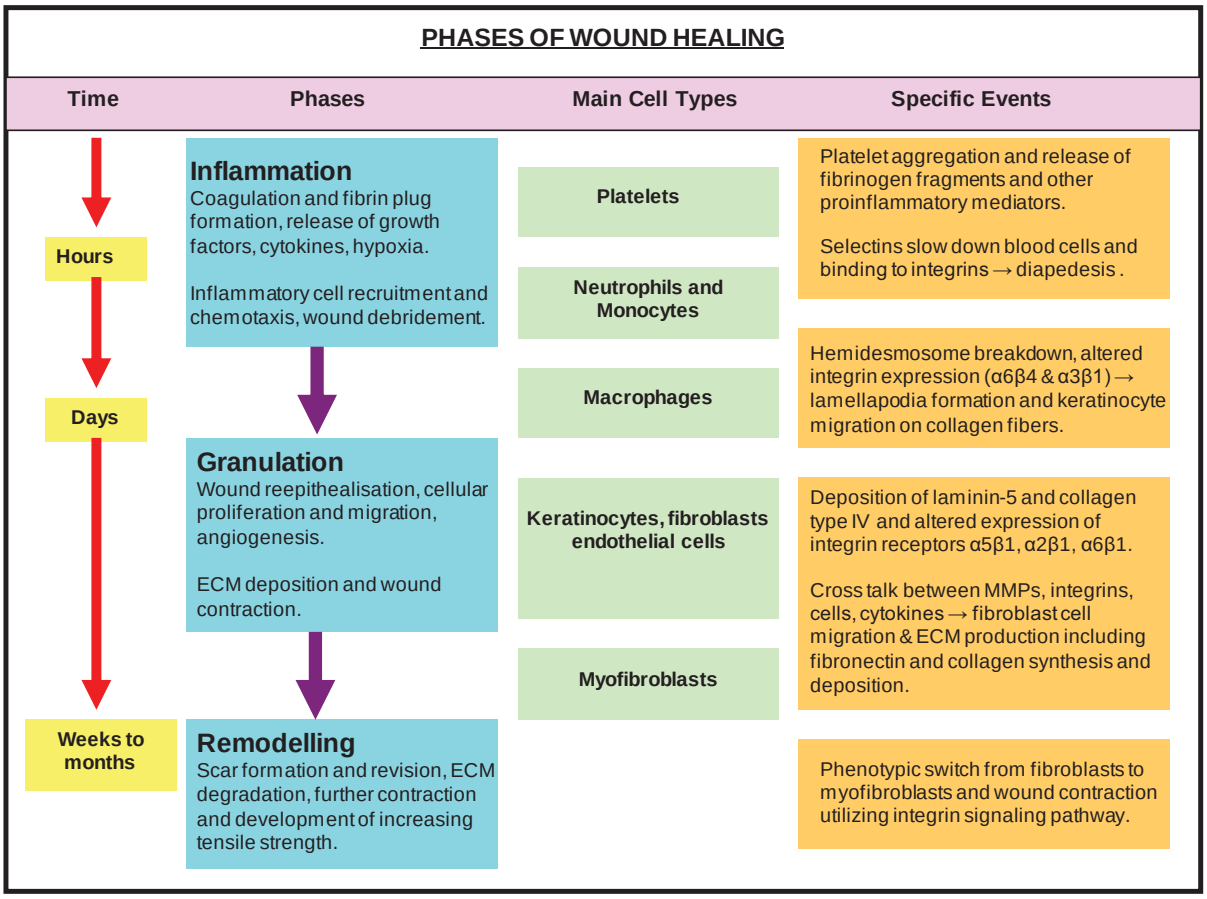


Figure 1.5: Brief description of different phases of wound healing. Illustration of the length of different phases of wound healing with main cell types involved and selected specific events. Figure adapted from (Falanga, 2005).

synthesis of various growth factors and cytokines (Falanga, 2005). Neutrophils recruit more macrophages, endothelial and other inflammatory cells to the site which help remove dermis and attract more fibroblasts which then begin to synthesize collagen, marking the start of the secondary granulation phase in the wound healing process (Amadeu *et al*., 2003).

1.3.2 Granulation Phase

During the granulation phase of wound repair a number of key events take place, including extracellular matrix protein production and keratinocyte and fibroblast cell migration and proliferation (Falanga, 2005). Wound re-epithelialization is a complex process requiring deposition of provisional basement membrane rich in laminin-5 and collagen type IV. Integrin receptors are essential in this phase of wound repair as cell migration across the basement membrane involves specific integrin expression. Cell adhesion molecules of the integrin family consist of 18 alpha (α) and 8 beta (β) subunits which form 24 known αβ-heterodimers depending on cell type and function (Hehlgans *et al*., 2007). These heterodimeric transmembrane receptors consisting of one α and one beta β subunit have large extracellular, short transmembrane and intracellular domains which act to bind the extracellular matrix to the actin cytoskeletal structures and hence mediate cell-cell and cell-matrix communication, adhesion, cell proliferation, differentiation, migration and apoptosis (Grose *et al*., 2002). Integrin expression is altered when the epidermis is damaged, diseased or wounded and specific integrins are known to positively or negatively contribute to the tissue pathogenesis and play an important role in wound repair during cell migration proliferation and adhesion (Watt, 2002).

1.3.2.1 Keratinocyte Migration

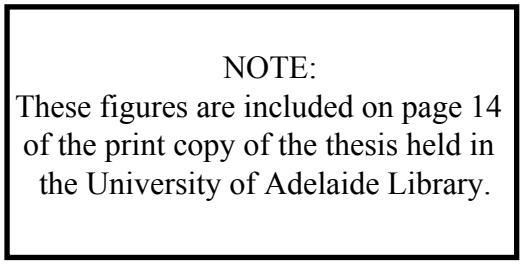
In the unwounded matrix the basal keratinocytes are joined to neighbouring keratinocytes by desmosomes and to the basement membrane by hemidesmosomes. Desmosomes and hemidesmosomes are the major cell surface attachment sites for actin intermediate filaments at cell-cell and cell-substrate contacts, respectively (Gonzalez *et al*., 2001, Green and Jones, 1996). Hemidesmosomes are specialized integrin mediated adhesion junctions composed of α6β4 integrin heterodimers which use laminin-5 anchoring filaments to attach an epithelium to the underlying basal lamina (Dowling *et al*., 1996, Jones *et al*., 1998, Borradori and Sonnenberg, 1999). The importance of hemidesmosomes is demonstrated in a blistering skin disease called Junctional Epidermolysis Bullosa (JEB) which is linked to the mutations in genes encoding the protein components of the hemidesmosomes (Santoro and Gaudino, 2005). Upon wounding keratinocytes present at the wound margins change into the migrating phenotype and undergo subcellular modification including: hemidesmosome disassembly, retraction of intracellular tonofilaments and keratin filaments, dissolution of most desmosomes and formation of peripheral cytoplasmic actin filaments (lamellapodia) and focal complexes (Kirfel and Herzog, 2004, Santoro and Gaudino, 2005).

The disassembly of hemidesmosomes requires cross-talk between growth factors, matrix metalloproteinases, integrins and structural proteins (Werner *et al*., 2007). A shift from stable and resting assembly of laminin-5 with α6β4 integrin takes place due to phosphorylation of the integrin heterodimer and binding to the ‘unbound’ α3β1 integrin occurs. This promotes hemidesmosome disassembly and relocation of α6β4 integrin to the lamellipodia while α3β1 integrin focal complexes regulate actin-myosin driven processes required for cell spreading and migration (Santoro and Gaudino, 2005, Manohar *et al*.,

2004). In this process α3β1 integrin plays an important role in keratinocyte migration acting as a multifunctional receptor regulating adhesion, signalling and proteolysis (Yanez-Mo *et al*., 2001). Consequently, following wounding the integrin expression profile on wound margin keratinocytes changes and results in induction of specific integrins which recognize the proteins of dermal matrix: fibronectin receptor α5β1, collagen receptor α2β1 and laminin receptors α3β1, α6β1 and α6β4 (Falanga, 2005, Grose and Werner, 2004). The first row of keratinocytes migrate on collagen while depositing laminin-5 which serves as a ligand for α3β1 integrin and induces further cell migration (Yanez-Mo *et al*., 2001) while laminin-5 cleavage by plasmin induces hemidesmosome formation and cell anchorage to the extracellular matrix in an integrin dependent fashion (Laplante *et al*., 2001). Figure 1.6 illustrates a) binding of α and β subunits forming different integrin heterodimers and b) change and translocation of α3β1 and α6β4 integrin expression profile upon wounding of wound margin keratinocytes, resulting in hemidesmosome disassembly and cellular migration.

Integrins are also known to associate with several different transmembrane proteins including tetraspanin CD151 which forms a stable complex with α3β1 integrin and regulates its function and cell migration. The tetraspanins are a family of membrane proteins characterized by four transmembrane domains and are known to modulate processes such as signal transduction, cell adhesion and motility (Santoro and Gaudino, 2005, Cowin *et al*., 2006, Sterk *et al*., 2002). Studies have shown that in migrating cells both tetraspanins and integrin α3β1 relocalize to motility related structures including mainly filopodia and membrane ruffles at the cell leading edge and are involved in dynamic adhesions to the substrate different from focal adhesions (Yanez-Mo *et al*., 2001, Penas *et al*., 2000). Furthermore, recent findings indicate that wound repair is defective in

A



B

Figure 1.6: Integrin receptors and wound healing. A Binding of α and β subunits and formation of different integrin heterodimers. **B** Wounding induces a change and translocation of α3β1 and α6β4 integrin expression on wound margin keratinocytes resulting in hemidesmosome disassembly and cellular migration. Figure adapted from (Manohar *et al*., 2004).

mice lacking the tetraspanin CD151 which has been shown to form complexes with all three laminin binding integrin heterodimers (α6β4, α3β1 and α6β1) and is capable of modifying the integrin mediated cell migration in vitro (Cowin *et al*., 2006). Tetraspanins have little effect on integrin – ligand binding however they have been shown to regulate post-ligand binding events, including integrin mediated adhesion strengthening, cell spreading and motility (Goldfinger *et al*., 1999, Wright *et al*., 2004, Hemler, 2003, Hemler, 2005).

1.3.2.2 Keratinocyte Proliferation

Besides keratinocyte migration, cellular proliferation is an essential part of the re-reepithelisation of the wound and an important step in wound repair. Re-epithelialization involves the movement of epithelial cells from the edge of the unwounded tissue using fibrin and fibronectin as foundations in their migration. Keratinocytes start to migrate in order to cover the wound matrix however a keratinocyte proliferative boost is required for adequate wound repair especially in larger wounds where migration alone is insufficient to cover the deficit (Falanga, 2005). In order to reconstitute the full thickness of the skin and tissue integrity, cell proliferation is sustained by various growth factors, integrin signalling and matrix metalloproteinases. Recent studies have indicated that integrins regulate the balance between cell proliferation and differentiation and significantly contribute to the skin inflammation (Watt, 2002).

1.3.2.3 Fibroblast proliferation and myofibroblastic differentiation

In response to injury fibroblasts become activated by a complex array of inflammatory and pro-fibrotic mediators (Zeisberg *et al*., 2000). Fibroblast cells are

capable of expressing numerous growth factor receptors, integrin receptors (α1, α4, α5 and β5), adhesion molecules and non-integrin matrix receptors. The cell mediated adhesion and re-organisation of the granulation tissue in skin wounds is believed to be an integrin-mediated process (Kelynack *et al*., 2000, Norman and Fine, 1999). Continual collagen synthesis and attraction of fibroblasts leads to the development of granulation tissue where collagen fibres act as the template for new tissue growth and formation of a myofibroblastic population of cells (Desmouliere *et al*., 2005).

The most abundant proteins of the extracellular matrix are members of the collagen family. Most prominent myofibroblast extracellular matrix products are collagens type I, III, IV, V and VII (Wang *et al*., 2006b, Hinz, 2007). Collagen type I and III belong to the fibril-forming collagens and are vital matrix contraction, remodelling and formation of scar tissue. During wound healing, collagen type III is firstly produced by active fibroblasts in the granulation phase and is substituted for collagen type I in the later phases of wound repair (Liu *et al*., 2001). Collagen type IV belongs to the basement membrane collagens and is also involved in wound reepithelisation and formation of new basement membrane over the wound matrix. ColVII is important in forming anchoring fibrils in the epidermal-dermal junction and is involved in group of blistering diseases called EB (Gelse *et al*., 2003). Excessive deposition of type I collagen is highly characteristic of numerous fibrotic disorders, including scleroderma, and most likely takes place due to high transcriptional activation of collagen genes in response to various cytokines, such as Transforming Growth Factor-β1 (TGF-β1) (Ghosh, 2002, Piccinini *et al*., 1999, Ihn *et al*., 1996, Ihn *et al*., 1997).

Various cytokines and growth factors are involved in the process of fibroblastic differentiation into myofibroblasts, including TGF-β, Platelet Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), Fibroblastic Growth Factor (FGF), Tumour Necrosis Factor Alpha (TNF-α) and Interferon-γ. TGF-β is known to be an important cytokine involved in different aspects of wound healing, including differentiation of fibroblasts to myofibroblasts, regulation of re-epithelialization, adhesion, synthesis of extracellular matrix proteins, promotion of connective tissue renewal and scar formation (Cowin *et al*., 2001a, Amadeu *et al*., 2003, Cowin *et al*., 2001b). Specific integrin signals affect the composition of connective tissue by altering the expression of extracellular matrix proteins, matrix degrading enzymes and their inhibitors, hence modulating the outcome of wound repair (Kelynack *et al*., 2000). Blocking of α5 and/or β1 integrins *in vitro* prevents the TGF-β1 induced myofibroblast differentiation that is characterized by increased Alpha-Smooth Muscle Actin (α-SMA) expression and enhanced collagen gel contraction in human fibroblasts derived from skin, mouth and kidney (Lygoe *et al*., 2004, Tomasek *et al*., 2002).

1.3.3 Remodelling Phase

The remodelling phase is the third phase of the wound healing process which involves removal of fibronectin and hyaluronic acid and further changes in the reorganization of the tissue matrix. Wound contraction is controlled by myofibroblasts and, as the granulation tissue becomes depleted of fluids and blood vessels, continual increases of tissue strength are observed (Amadeu *et al*., 2003). Collagen binding integrins (α1β1 and α2β1) have also been identified to be involved in wound contraction and collagen matrix remodelling. α1β1 integrin receptor has been identified as a primary integrin receptor utilized by contracting myofibroblasts *in vivo* where it mediates collagen

dependent adhesion/migration and collagen matrix contraction, while α2β1 integrin receptor is expressed by myofibroblasts largely in culture during collagen gel contraction and only weakly *in vivo* where it aids the α1β1 mediated collagen matrix contraction (Kagami *et al*., 1999, Racine-Samson *et al*., 1997).

Wound repair and healing through dermal fibrosis constitute an essential response to tissue injury, restoring the normal skin function, tissue integrity and the balance of homeostasis. However, in some cases, the inflammatory process becomes prolonged and fibrosis results in excessive scar formation (Amadeu *et al*., 2003). Scar formation, a result of the cutaneous wound healing through the fibro-proliferative response and influence of various fibrotic agents causing excessive fibrosis, occurs in both adult humans and higher vertebrate animals and is the last step in the remodelling phase of wound healing (Liu *et al*., 2001). Numerous changes take place in the remodelling phase of wound healing and lead to replacement of granulation tissue by scar formation, including increase in deposition of type I collagen, wound organization and contraction and decrease in vessel numbers and myofibroblasts by process of apoptosis (Desmouliere *et al*., 2005).

One of the important roles in the transition from the remodelling phase to scar formation in wounded tissue is played by proteolytic enzymes, matrix metalloproteinases (MMP) which degrade the extracellular matrix components. The outcome of this stage of wound healing depends on a fine balance between extracellular matrix synthesis and degradation. Newly synthesized matrix is progressively remodelled by various proteases secreted by fibroblasts and macrophages and wound matrix contraction results in an increased density of the new scar tissue (Hinz, 2007, Santoro and Gaudino, 2005). Metalloproteinases include three main groups of enzymes: collagenases which cleave

fibrillar collagen type I, II and III, gelatinases which hydrolyse collagen and fibrin, stromelysins which catabolize extracellular matrix components, including fibronectin, laminin and unstructured collagen. The secretion of this proteins is regulated by various cytokines, growth factors and tissue stress (Amadeu *et al*., 2003). Certain MMPs have the ability to bind integrin receptors, consequently providing a unique mechanism for localized matrix degradation (Kirfel and Herzog, 2004). The actions of MMPs are quickly followed by secretion of new matrix components which stabilize the collagen network and increase the extracellular matrix. These processes are often repeated until the scar tissue is formed and the wound is stabilized (Amadeu *et al*., 2003). The end point of the adult wound healing is often defined both by the functional ability of the skin and the appearance of the scar tissue, which can range from tiny scar tissue visible as fine lines post surgery to extensive skin scarring restricting skin function and patient's mobility (Durani *et al*., 2009). Under certain conditions, including burn injury and certain subtypes of EB blistering skin disease, the scar tissue continues to form, resulting in hypertrophic scar contractures and severe pseudosyndactyly with total "skin cocooning" that impairs mobility and requires continuous surgical release of contractures (Azizkhan *et al*., 2006, Fine *et al*., 2005, Glicenstein *et al*., 2000, Siepe *et al*., 2002). Examples of different scar tissue and surgical release of contractures are shown in Figure 1.7.

1.4 Actin Cytoskeleton

The cytoskeleton is an integral component of all eukaryotic cells composed of actin microfilaments, microtubules, intermediate filaments and stress fibres. The cytoskeleton is under continual remodelling, assembly and severing in response to various cell requirements to generate the mechanical force for cellular contraction, adhesion and motility. An important property of the actin cytoskeleton is its ability to produce cell

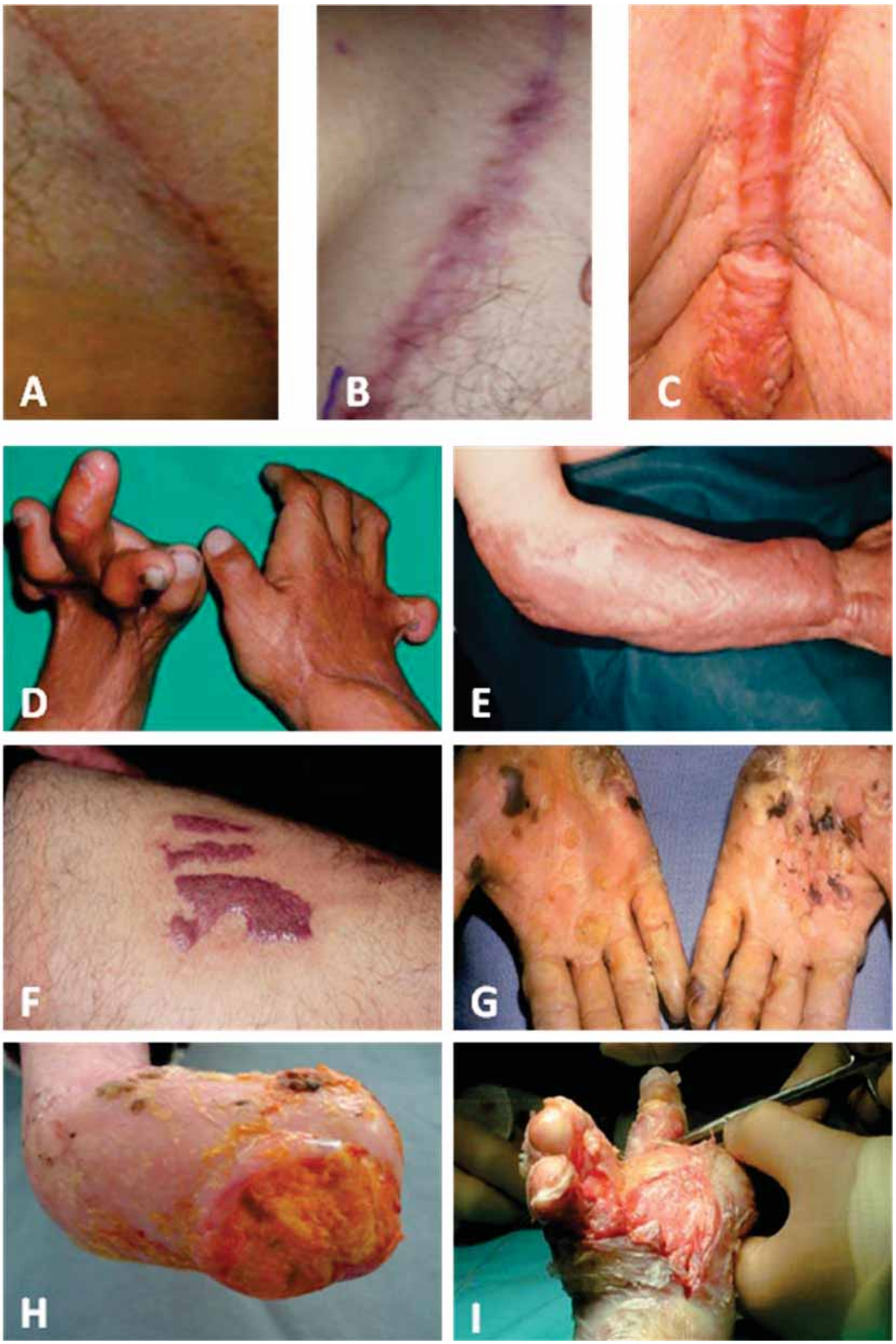


Figure 1.7: Examples of different types of scar tissue following normal and pathological adult wound healing and surgical release of contractures. A-B Fine linear scars post normal wound healing after elective surgery. **C** Hypertrophic scar post open heart surgery. **D-E** Hand and forearm scar contracture post burn injury. **F** Scar 11 months post burn injury. **G-H** Hand scars and pseudosyndactyly due to EB skin blistering. **I** Surgical release of hand contractures due to EB skin blistering. Figures adapted from (Spanholtz *et al*., 2009, Kerrigan and Homa, 2009, Kerrigan and Homa, 2010, Dammak *et al*., 2009, Pillay, 2008, van Zuijlen et al., 2002).

movement in the absence of motor proteins, however this unique ability is dependent on the presence of actin binding/remodelling proteins (dos Remedios *et al*., 2003). Recent studies have shown that the actin cytoskeleton is also involved in modulation of cell signalling, growth, differentiation and gene expression, while components of the actin cytoskeleton work in synergy to provide stronger cell stability during stress (Cairns *et al*., 2004, Esue *et al*., 2006). A number of different structural and dynamic aspects of cell behaviour are dependent on the actin cytoskeleton, including cell morphology, polarity, adhesion complex formation, vesicle trafficking and phagocytosis, cytokinesis and movement (Hall and Nobes, 2000). Actin microfilaments are the smallest components of the actin cytoskeleton network and play a role in cellular motility, structure and division (Lambrechts *et al*., 2004). Two types of actin microfilaments have been categorized; individual non-polymerized globular actin subunits termed G-actin which assemble into long filamentous fibres termed F-Actin (Lambrechts *et al*., 2004). Microtubules and intermediate filaments are larger structures of the cytoskeleton (Lambrechts *et al*., 2004).

Microtubules are composed of α and β tubulin dimers which function in both cellular movement and division. Intermediate filaments are involved in the formation of adhesion complexes namely hemidesmosomes, desmosomes and focal adhesions and directly interact with proteins of the extracellular matrix (Bazile *et al*., 2009). Key roles of the intermediate filaments include signal transduction, cytoskeletal cross talk between the organelles in the cytoplasm and organisation of the cytoplasm (Goldman *et al*., 2008). The structural function of the keratin intermediate filaments was evident when point mutations in keratin 15 (K15) and keratin 5 (K5) genes were identified to cause Epidermolysis Bullosa Simplex (EBS) where extensive skin blistering takes place due to cytolysis of the basal layer of cells (Nguyen *et al*., 2007).

Stress fibres are also a component of the actin cytoskeleton network allowing a cell to modulate its responses to tissue injury. Mammalian cells contain three types of stress fibres: ventral stress fibres attached to focal adhesions at both ends, dorsal stress fibres attached to focal adhesions at one end, and transverse arcs which are the acto-myosin bundles that do not attach to focal adhesions directly (Naumanen *et al*., 2008). The major role of stress fibres is to maintain a balance between contraction and adhesion. This balance results in the stable actin bundles which maintain a constant length under tension, especially in ventral stress fibres attached to the extracellular matrix on both sides (Goffin *et al*., 2006). Numerous proteins are localized to the site of stress fibres, however their function in stress fibre assembly and maintenance is unclear. α-Actinin is one of the proteins recruited to the sites of stress fibre assembly where it bundles and cross-links the actin filaments and connects other adaptor proteins to the stress fibres. RhoA, a member of the Rho family of small GTPases vital in actin cytoskeleton organisation, is most directly linked to stress fibre formation. RhoA acts through downstream effectors including the mDia1/DRF1 formin complex which results in the increase in parallel actin stress fibre formation throughout the cell (Naumanen *et al*., 2008). The GTP bound form of RhoA also activates the Rho-associated kinase which promotes stress fibre formation by inhibiting actin filament depolymerisation and inducing contractibility, however the precise mechanisms of stress fibre assembly are still largely unknown (Hotulainen and Lappalainen, 2006).

1.4.1 Actin dynamics during wound repair

The actin cytoskeleton is involved in many fundamental wound healing processes including cellular proliferation, migration and adhesion. In response to a variety of stimuli the actin cytoskeleton is quickly remodelled in order to generate necessary mechanical

force required for changes in cell contraction, adhesion and motility. The main changes in the actin cytoskeleton during the wound healing process include lamellipodia remodelling during keratinocyte migration and wound reepithelialisation, infiltration of inflammatory cells and migration of fibroblasts required for the deposition and remodelling of the extracellular matrix and dermal wound contraction (Cowin *et al*., 2003, Jacinto *et al*., 2001). Studies by Cowin *et al*. (2003; 2007) have illustrated that wounding has a differential effect on proteins associated with actin dynamics both in fetal and adult skin wound repair. Interestingly, wounding also has an effect on the expression of filamentous F-actin. While "scar free" fetal wounds have predominantly epidermal expression of F-actin, the "scar forming" adult wounds have predominantly dermal F-actin expression and this developmental switch in actin expression might be important in fetal wound contraction and "scar-free" wound repair (Cowin, 2005). Upon wounding, the motile cells at the wound edge are induced by various extracellular matrix signals including growth factors and extra cellular matrix substrates to migrate across the wound matrix and form new and stable adhesion sites (Lambrechts *et al*., 2004). Understanding the processes involved in actin dynamics and cell matrix adhesion are vital in understanding the process of wound repair.

1.4.2 Actin cytoskeleton and cell matrix adhesion

Changes in cell shape, adhesion and migration properties are all regulated by the continuous remodelling of the actin cytoskeleton. In order to migrate in response to the extracellular signals, the cells first assemble actin at the cell front driving the extension of membrane protrusions called lamellipodia and filopodia (Partridge and Marcantonio, 2006). At the leading edge of the cell, adhesions are formed with the extracellular matrix, hence anchoring the protrusions to move the cell body. The combination of the acto-

myosin contractibility and disassembly of the adhesion structures at the rear of the cell allows the cell body to move forward (Le Clainche and Carlier, 2008).

Lamellipodia, filopodia and membrane ruffles are components of the actin cytoskeleton involved in both cell motility and cell matrix adhesions (Small *et al*., 2002). Lamellipodia consist of a network of branched actin filaments that produce the force for cell protrusions at the leading edge. The assembly of the actin based projections is regulated by the GTPases of the Rho family which link the surface receptors to the organisation of the actin cytoskeleton. While Rho GTPase is instrumental in formation of stress fibre and focal adhesion formation, Rac1 and Cdc42 signal formation of the lamellipodia and filopodia respectively (Small *et al*., 2002, Schwartz and Shattil, 2000, Hall, 1998). The activated Actin related protein (Arp2/3) nucleates the new filaments from the sides of pre-existing filaments and induces the formation of a branched filament network at the leading edge of the cell (Insall and Machesky, 2009). Elongation of the new filaments is further regulated by different capping proteins which maintain the short filaments at the protruding region of the cell (Hotulainen and Lappalainen, 2006, Le Clainche and Carlier, 2008). Different components of the actin cytoskeleton of the moving cell and adhesion sites formed as a result of cell migration and the role of Rho GTPases in actin organisation are shown in Figure 1.8.

Filapodia are thin cellular processes consisting of the long parallel actin filaments arranged into tight bundles (Fig 1.8). Elongation of filopodial bundles is primarily regulated by the Cdc42 GTPase and the Ena\VASP family of proteins and formins which function to uncap the barbed ends (Hotulainen and Lappalainen, 2006). Membrane ruffling is characterized by the dynamic movement of the membrane protrusions, consisting of

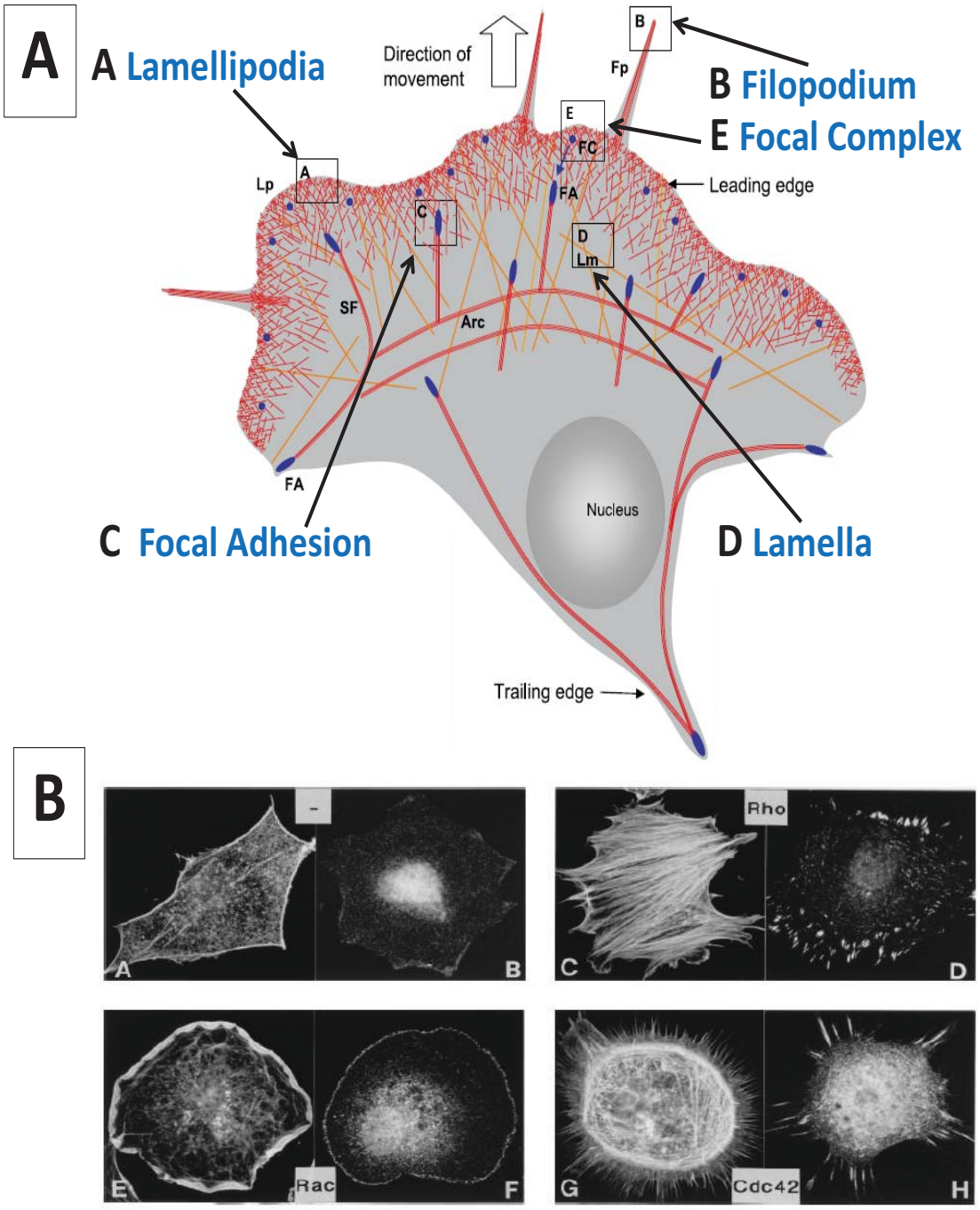


Figure 1.8: Actin Cytoskeleton of the moving cell. A Arrangement of the actin cytoskeleton in a moving cell – A Lamellipodia, B Filopodium, C Focal Adhesion, D Lamella, E Focal Complex **B** Formation of different actin cytoskeleton components in response to GTPase signaling in fibroblasts. Actin filaments visualised with phalloidin staining in A, C, E and G and adhesion complexes visualised with an anti-vinculin antibody in B, D, F and H. Quiescent fibroblasts in A and B show few organised actin filaments or adhesion complexes. In response to Rho stress fibre formation (C) and adhesion complex formation (D) is evident. Microinjection of Rac induces lamellipodia (E) and associated focal adhesion complexes while microinjection of Cdc42 induces filopodia formation (G) and associated adhesion complexes (H). Figure adapted from (Hall, 1998, Le Clainche and Carlier, 2008).

lamellipodia and filopodia, in response to the extracellular signals. Away from the leading edge of the cell at the site of slow actin turnover, lamella are formed and are characterized by proteins involved in the movement of stress fibres namely tropomyosin and myosin II (Le Clainche and Carlier, 2008).

Cell motility along the extracellular matrix substrate depends on membrane protrusions at the leading edge, traction of the cell body and retraction of the cell tail. In these processes primary cellular adhesion structures called focal complexes act as a "molecular clutch" driving the interaction between the actin cytoskeleton and the extracellular matrix substrate. When the clutch is disengaged there is no connection between the cytoskeleton and no protrusions take place, as actin treadmilling is converted into retrograde flow. However, engagement of the clutch results in a strong connection between the cytoskeleton and the substrate leading to conversion of the acto-myosin tension into traction of the cell body, protrusions at the leading edge of the cell and retraction of the cell tail (Le Clainche and Carlier, 2008). Engagement of the "molecular clutch" is determined by molecular interaction between integrin-substrate, integrin-actin binding proteins, and actin filament-actin binding proteins interactions. Tension generated by the acto-myosin contraction induces maturation of the adhesion sites and recruitment of structural and signaling molecules involved in actin dynamics, allowing these adhesion sites to also function as mechanosensors (Le Clainche and Carlier, 2008, Lambrechts *et al*., 2004).

Initial integrin mediated cell-matrix adhesions, termed focal complexes, develop underneath lamellipodia and are driven by actin polymerization. These are highly dynamic structures that exist for a limited time. A proportion of the stable focal complexes develop

into elongated focal adhesions which are associated with contractile stress fibres (Bershadsky *et al*., 2006). A vital function of focal adhesions is the anchoring of polymerized actin filament stress fibres into bundles which provide contractile force required for effective translocation of a cell body during cellular migration (Bach *et al*., 2009). Adhesions which are too weak or two strong result in slower cell migration, hence a fine balance between formation of new adhesions and retraction of the rear of the cell needs to be achieved for efficient cellular migration. This is highly dependent on signaling between a number of proteins recruited to the adhesion sites including paxillin, talin, vinculin, FAK, Src kinase (Lambrechts *et al*., 2004, Petit and Thiery, 2000). Fibrillar adhesions which arise from mature focal adhesions are located more centrally in cells and are associated with fibronectin fibrils and actin cables not stress fibres. The elongation of these adhesion structures is dependent on deformability of the extracellular matrix substrate (Le Clainche and Carlier, 2008).

1.4.2.1 Structural and signaling proteins of cell adhesion sites

Actin binding proteins involved in regulating actin organization and polymerization are essential mediators of membrane protrusions and cell migration. These proteins include both structural and adaptor proteins involved in adhesion signaling pathways, namely vinculin, talin, paxillin, α-actinin and Src kinase. The structural protein vinculin plays a role in stabilisation of the adhesion structure during maturation of focal complexes into focal adhesions (Le Clainche and Carlier, 2008). Talin acts as a “hyper-activator” of integrin receptors where it links the cytoplasmic tail of integrin receptors to the actin cytoskeleton and also increases the affinity of the extracellular domain of integrin to the extracellular matrix ligand. Talin is essential for integrin function and talin knockout results in abnormal cellular migration and early embryonic lethality (Le Clainche and

Carlier, 2008). Cytoplasmic talin is activated in response to phosphatidylinositol 4,5-biphosphate (PIP_2) binding which also terminates the auto-inhibition of talin through the talin head-rod binding. Once activated, the talin sub-domain interacts with the β integrin tails, forms the talin specific loop structure and disrupts the connection between the cytoplasmic tails linking the integrin receptors and the actin cytoskeleton (Moser *et al*., 2009). This model of integrin activation by talin is one example of how cytoplasmic proteins can regulate activation state of integrin receptors and alter cellular adhesion and migration. Fig 1.9 illustrates the schematic model of talin activation of integrin receptor subunits, linking the extracellular matrix, through integrin receptors, to the actin cytoskeleton.

Paxillin protein has a vital role in cellular adhesion and migration. It acts both as the structural and adaptor protein, allowing recruitment and binding of number of different proteins as well as transducing different signals into cytoskeletal responses and changes in gene expression (Cowin *et al*., 2003). Focal adhesion proteins like paxillin provide a point of signal convergence, a platform for protein kinases such as Src which are stimulated by various growth factors like TGF-β, to interact with other signaling proteins, promoting downstream adhesion signaling pathways (Goetz, 2009). Studies have indicated that Src phosphorylation of paxillin tyrosine Y118 creates an ERK binding site on paxillin. This results in ERK recruitment to focal adhesions, binding of Raf and MEK to ERK, phosphorylation of paxillin and increased interactions with FAK, stimulating cellular spreading and motility, and suggests that paxillin association with ERK activation is vital in focal adhesion turnover (Brown and Turner, 2004, Schaller, 2001, Webb *et al*., 2004).

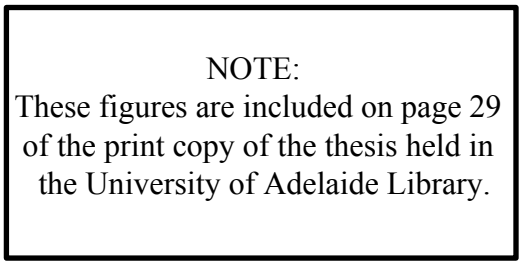


Figure 1.9: Talin activation of integrin receptor subunits. A PIP_2 binding to cytoplasmic talin activates the talin protein by ending the auto-inhibitory interaction with the rod domain. **B** Talin sub-domain engages with the membrane proximal NPxY motif in the β integrin cytoplasmic tail. **C** Talin specific loop structure forms with binding to the MP helical region of the integrin cytoplasmic chain, disrupting the connection between α/β subunit integrin cytoplasmic tails. Pulling forces at the β integrin tail reorient the transmembrane domains, disrupting the packing of the α/β transmembrane domains. Figure adapted from (Moser *et al*., 2009).

Better understanding of the complex adhesion signaling pathways and association of adaptor proteins like paxillin with different actin binding proteins involved in actin dynamics is required for improved understanding of cell adhesion and migration during wound healing and development of novel approaches for improved wound healing outcomes.

1.5 Gelsolin Family of Actin Remodelling Proteins

Actin remodelling and cytoskeletal rearrangement occurs during a variety of cellular functions and involves an array of actin binding and remodelling proteins including ADF/cofilin family, profilin family, gelsolin superfamily, thymosins, capping proteins and the Arp2/3 complex (dos Remedios *et al.*, 2003). However, the members of the gelsolin superfamily of actin remodelling proteins have also been identified as instrumental in reorganizing the actin cytoskeleton (Kwiatkowski, 1999). The actin remodelling protein Flii is a relatively newly discovered member of the gelsolin family of actin-severing proteins which now includes gelsolin, villin, adseverin, capG, advillin, supervillin and Flii (Archer *et al*., 2005). These higher eukaryotic actin-binding proteins are believed to function in the cytoplasm of cells where they control actin organisation by severing pre-existing filaments, capping the fast growing filament ends and nucleating or bundling actin filaments to enable filament reassembly into new cytoskeletal structures (Kwiatkowski, 1999, Archer *et al*., 2005, Goshima *et al*., 1999, Liu and Yin, 1998). These remodelling proteins remain attached to the 'barbed' ends of broken severed actin filament, preventing annealing of the broken filaments or addition of new actin monomers. Subsequently, the broken actin filaments are uncapped by interactions with phosphoinositides which results in rapid actin assembly and allows cells to reorientate the

cytoskeleton and mediate the changes required for adhesion, motility and contraction (Sun *et al*., 1999).

The gelsolin family of actin remodelling proteins has three to six homologous gelsolin-like structural domains known as G1-G6 segmental domains, three actin binding regions and a number of calcium independent monomer and filament binding domains. Villin, supervillin and Flii have evolved to contain additional domains allowing them to have specific multiple roles and interact with a variety of proteins. Villin contains an additional actin binding domain, termed villin head piece; supervillin contains an N-terminus domain capable of protein-protein interactions and nuclear localization while Flii contains a N-terminus Leucine Rich Repeat (LRR) Domain also capable of multiple protein-protein interactions (Fig 1.10). In contrast, CapG only contains three gelsolin-like structural domains however it still retains full actin severing and capping ability and plays a role in cell migration (Le Clainche and Carlier, 2008, Witke *et al*., 2001). There is a high homology in structure of different members of the gelsolin family however the differences in structure observed suggests that these proteins have undergone evolutionary changes, gaining specific and multiple functional properties beyond actin remodelling (Campbell *et al*., 1997, Archer *et al*., 2005).

Gelsolin is the most abundant member of the actin remodelling family that is involved in regulating the dynamics of the filamentous actin by binding, severing and capping actin filaments (Sun *et al*., 1999). High gelsolin levels have also been associated with stress fibre formation and gelsolin was found to play a role in promoting stress fibre formation and actin stabilisation (Arora *et al*., 1999). Gelsolin is also a secreted protein where its role in plasma is to “clean up” actin filaments that have been released into

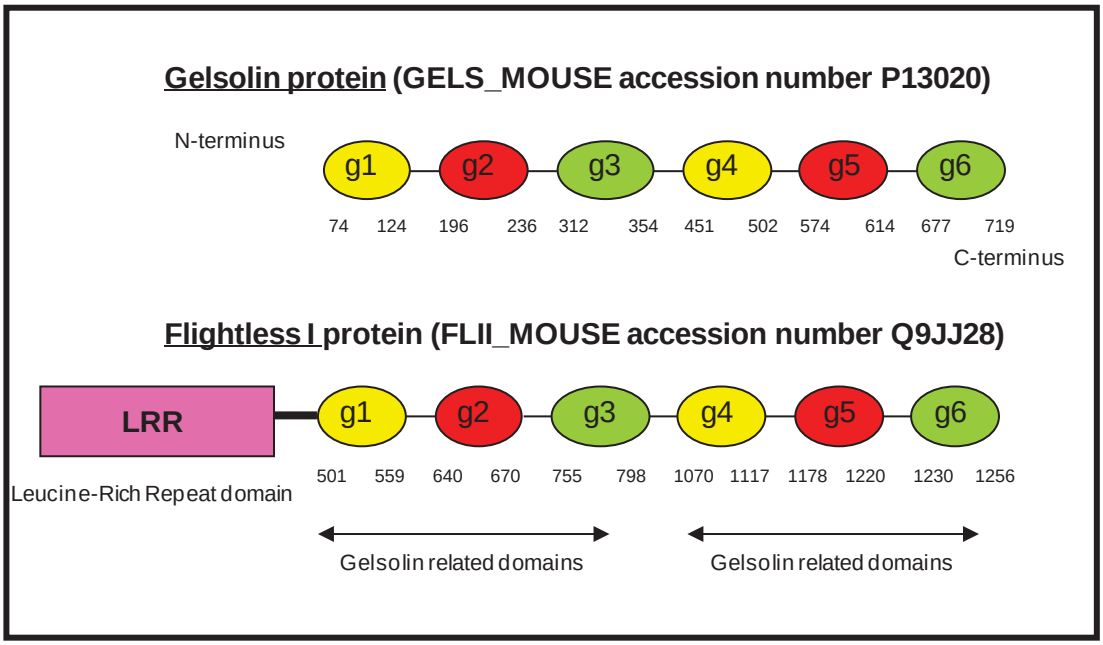


Figure 1.10: Schematic diagram of structure and domain homology between FliI and gelsolin. LRR denotes Leucine Rich Repeat Domain, g1-6 denotes the six gelsolin segmental repeats, numbers denote the amino acid residues. Figure adapted from (Kopecki and Cowin, 2008).

circulation during burn injury and cell necrosis (Kwong *et al*., 2003, Rothenbach *et al*., 2004, Sun *et al*., 1999). However, its roles extend beyond actin dynamics as gelsolin has been implicated to play a role in hereditary gelsolin amyloidosis, Alzheimer's disease, tumour suppression and cellular apoptosis (Azuma *et al*., 1998, Qiao *et al*., 2005, Sagawa *et al*., 2003, Tanskanen *et al*., 2009).

In addition to their role as actin remodelling proteins the members of the gelsolin superfamily have specific roles in a variety of cellular processes including cell motility, apoptosis and gene expression (Silacci *et al*., 2004). Several members including Flii, supervillin and gelsolin have roles in regulating gene transcription and act as nuclear receptor co-activators (Archer *et al*., 2005, Archer *et al*., 2004). Current studies suggest that manipulation of the members of the gelsolin family may be important in wound repair, identifying these proteins as potential new targets to help improve wound healing and reduce scar formation (Cowin *et al*., 2007). Studies examining the effect of gelsolin on wound repair indicate that increases in cellular gelsolin levels in mouse fibroblasts enhance cellular migration and result in increased rate of wound closure (Cunningham *et al*., 1991). Moreover, gelsolin knockout mice studies have shown that absence of gelsolin in skin fibroblasts results in a variety of actin-related defects, including decreased motility and delayed wound closure (Witke *et al*., 1995). However, recent studies revealed a novel role for Flii in wound repair, identifying this protein as a negative regulator of wound healing (Adams *et al*., 2009, Cowin *et al*., 2007).

1.6 Flightless I (Flii) protein

The actin remodelling protein Flii was originally characterised as a gene mutation in *Drosophila melanogaster* that resulted in an irregular actin organisation and defective flight muscles (Campbell *et al*., 1993). This led to the discovery of the human homologue of the Flii gene in 1997 (Campbell *et al*., 1997). The gelsolin superfamily is a conserved family of proteins present in both mammalian and non-mammalian organisms and the Flii protein is the most evolutionary conserved member of this family (Claudianos and Campbell, 1995). The *Drosophila melanogaster* Flii gene is located in the subdivision 19F on the X chromosome and encodes a 1256 amino acid protein with a predicted weight of 143,672 Da which is characterised by the LRR domain and gelsolin-like domains (Davy *et al*., 2000).

The human Flii gene spans 14kb of genomic DNA and although it contains more introns than human gelsolin or human villin it is considerably smaller (Campbell *et al*., 1997). Human Flii locus has been mapped on human chromosome 17p11.2, a region deleted in Smith-Magenis syndrome (SMS), a genetic disease causing developmental and behavioural abnormalities (Elsea and Girirajan, 2008, Liu and Yin, 1998, Campbell *et al*., 2002). Although there are number of different genes located at this particular chromosomal region deleted in SMS patients, researchers have recently shown that the loss of one particular gene retinoic acid induced 1 (RAI1) is responsible for most of the characteristic features associated with SMS while other genes deleted within chromosome 17, including Flii, may contribute to the variability and severity of the condition (Girirajan *et al*., 2006). Further research investigating the function of Flii may lead to better understanding of this syndrome whilst manipulation of Flii levels in vivo may lead to new therapeutic

interventions in wound and burn injuries hence having significant impact on paediatric, child and adult health.

1.6.1 Flightless I structure and expression

The C-terminal end of the Flii protein sequence has significant homology to the gelsolin family of proteins where two large duplicated domains have been identified, each characterised by three repeated sequences of between 125 and 150 amino acids denoting the six gelsolin-like (g1-6) segmental repeats (Claudianos and Campbell, 1995). Figure 1.10 illustrates the structure and domain homology between Flii and gelsolin. The N-terminal end of the Flii mRNA sequence translates to a ~400 residue domain consisting of 16 tandem repeats of a 23 amino acid LRR motif. Proteins containing the LRR motif are known to utilize this domain for various protein-protein or protein-lipid interactions and this may explain the Flii association with a variety of different signaling and structural proteins (Kobe and Kajava, 2001). Existence of the Flii LRR domain suggests that gene insertion events have taken place during evolution of the Flii protein which represents the first case where gelsolin-like and LRR protein domain are joined in the same molecule (Kopecki and Cowin, 2008).

The unique structure of the Flii protein featuring both the actin-binding gelsolin related domain and LRR domain has given rise to speculations that it is involved in augmenting the versatility of actin filament structures and the dynamics behind the actin cytoskeleton re-arrangement by being the intermediate protein linking the cytoskeleton to different signaling proteins (Davy *et al*., 2000). Association of the LRR domain of Flii with Ras has been suggested by kinetic analysis based upon competitive inhibition of Ras-

dependent adenylyl cyclase activity (Goshima *et al*., 1999, Campbell *et al*., 1997, Davy *et al*., 2000). Ras proteins are involved in regulation of actin cytoskeleton where their association with Raf-1 results in activation of the mitogen-activated protein kinase (MAPK) pathway, however Flii interaction with this pathway remains to be elucidated (Goshima *et al*., 1999).

The actin cytoskeleton is an integral component of all cells and its re-organisation, regulation and interaction with actin-remodelling proteins plays an important role in cell motility, contraction and adhesion (Davy *et al*., 2000). Consequently, the action of actin binding proteins like Flii may greatly alter the outcome of the tightly orchestrated processes of wound healing (Cowin *et al*., 2007). Pathways of Flii synthesis and degradation are also still to be investigated, however studies by Davy et al., (2000) have shown that Flii is expressed in different migratory structures, including filopodia, neurites and growth cones and it specifically co-localizes with cytoskeletal structures connected with a migratory phenotype, including actin-rich regions in parasympathetic neurons harvested from chicks, in mouse blastocysts and cellularization structures in Drosophila melanogaster (Davy *et al*., 2000). This implies a role for Flii at different sites of dynamic actin filament reorganisation suggesting that Flii may play an important role in cellular responses where active actin remodelling is required, including adhesion and migration. Despite the close sequence homology of this molecule with other members of the gelsolin family, the actin binding and severing activity of Flii has shown to target different actin based structures in the cell (Davy *et al*., 2000). Studies on Swiss 3T3 fibroblasts have shown that Flii expression is mainly nuclear; however a translocation to the cytoplasm is evident in the presence of serum. Cytoplasmic to nuclear translocation of Flii is not

surprising as this also occurs with other members of the gelsolin family namely supervillin, gelsolin and CapG (Davy *et al*., 2001).

Flii protein localizes to actin based structures both *in vitro* and *in vivo* in mouse fibroblasts (Davy *et al*., 2000) and has the ability to bind and sever filamentous actin in vitro (Azuma *et al*., 1998), indicating that the gelsolin-like domain is functionally active. Unlike gelsolin, Flii possesses F-actin binding and severing activities independent of Ca^{2+} concentration suggesting a role for Flii in dynamic assembly and disassembly of actin filaments (Goshima *et al*., 1999). Co-localization of Flii with different molecules involved in regulating cytoskeletal re-organization has also been observed, including GTP-binding proteins, Ras, Cdc42, and RhoA, implicating the involvement of these molecules in a common functional pathway. Association of Flii with Ras proteins suggests possible involvement of Flii in downstream MAPK signaling which may be one of the signaling pathways by which Flii regulates cell responses during wound healing, however this is yet to be further investigated. The Flii molecule is also found associated with actin arcs and membrane ruffles, further establishing its importance in cellular motility, proliferation and actin dynamics (Cowin *et al*., 2007, Davy *et al*., 2000, Claudianos and Campbell, 1995, Campbell *et al*., 2002).

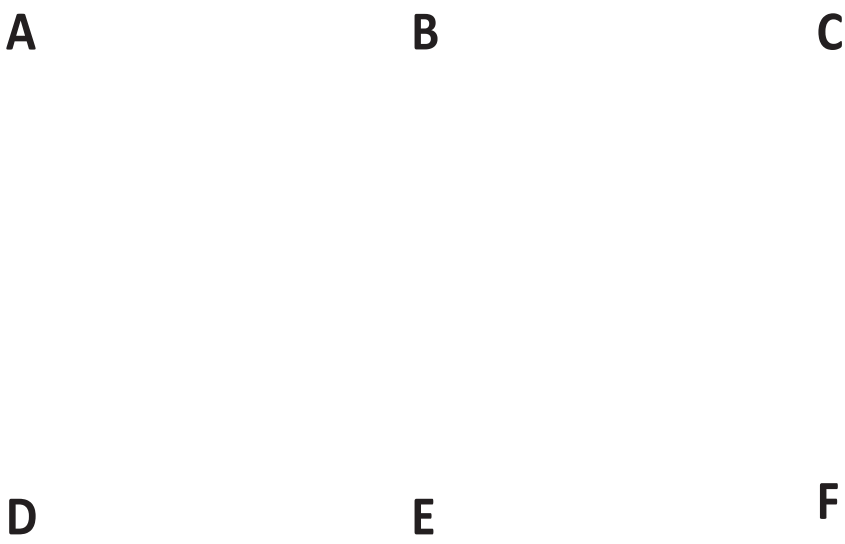
1.6.2 Flightless I biological functions

Mutations in the *Drosophila melanogaster* Flii gene lead to a loss of flight ability due to myofibrillar abnormalities in the indirect flight muscles (Campbell *et al*., 1993). *Drosophila melanogaster*s eggs lacking maternally supplied Flii undergo incomplete cellularization and defective gastrulation in embryogenesis which has been associated with disorganised actin cytoskeleton (Straub *et al*., 1996). Gastrulation defects in Flii

homozygous embryos cause lethality in early embryogenesis as Flii is required for normal cellularization of the syncytial blastoderm (Fig 1.11), indicating that the Flii protein is essential for the survival of the organism to adulthood. Although *Fliih* mutant blastocysts form an outgrowing trophoblast cell layer, egg cylinder formation fails and the embryos degenerate (Campbell *et al*., 2002). Caenorhabditis elegans Flii I homolog, fli-1, has been shown to regulate actin-dependent events during development, with roles in asymmetric cell division, cytokinesis of somatic cells and establishing anterior-posterior polarity of the zygote (Deng *et al*., 2007).

Using a genomic cosmid clone containing the complete human *Flii* gene Campbell *et al*., (2002) have constructed a Flii transgenic mouse and shown that a human Flii transgene is capable of restoring normal development to homozygous Flii mutant mouse embryos. This indicates that Flii performs essential functions during early embryogenesis in both Drosophila and mammals (Campbell *et al*., 2002). Heterozygous *Fliih* mutant mice have half the amount of Flii protein and appear phenotypically normal (Campbell *et al*., 2002). Although Flii was thought to be solely an intracellular protein there is new compelling evidence to suggest that it is also secreted by fibroblasts in culture (Cowin *et al*., 2007). Gelsolin also has a functional secretory phenotype scavenging actin that is exposed to extracellular spaces or released into the circulation following tissue injury (Lee and Galbraith, 1992).

Another important function of Flii is its role as a hormone-activated nuclear receptor (NR) co-activator. Recent findings have identified Flii as a co-activator in estrogen receptor and thyroid receptor mediated transcription (Lee *et al*., 2004). These receptors bind directly or through other proteins to specific enhancer elements associated



NOTE:
These figures are included on page 39 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.11: Gastrulation defects of Flii homozygous embryos. A WT embryo with normal gastrulation and ventral furrow formation. Mesoderm anlage has invaginated and formed a tube with all the peripheral cytoplasm incorporated into the cells. **B-C** In contrast to age matched wild-type embryos (shown in A); Flii homozygous embryos exhibit a rim of unincorporated cytoplasm (arrows). **D** Complete Flii knock-out results in embryos with varying degrees of ventral furrow formation. **E-F** In slightly older Flii knock-out embryos the ventral nuclei have moved towards the centre of the egg. pc = pole cells. Figure adapted from (Straub *et al*., 1996).

with the promoters of target genes, resulting in activation or repression of transcription (Lee *et al*., 2004). Moreover, Flii has also been identified as a substrate of the cytokine-independent survival kinase (CISK) acting downstream of PI-3kinase/CISK signaling pathway. CISK association with Flii results in Flii phosphorylation at residues Ser 436 and Thr 818 and modulated Flii activity as an estrogen receptor co-activator (Xu *et al*., 2009). Flii knockdown by RNA interference resulted in 293T cells being more sensitive to the interleukin-3 withdrawal-induced apoptosis suggesting that Flii may also involved in this cell cycle pathway. Protein post-translational modifications including phosphorylation by kinases are essential for development and cellular functions and Xu *et al*., (2009) suggest that CISK phosphorylation of Flii may impact on actin reorganisation and endosome trafficking.

The Flii molecule has been shown to interact functionally with several proteins known to enhance nuclear receptor-directed transcription, including GRIPI (p160), SRC-1, pCIP and TRAM1. These proteins engage histone-modifying activity to the promoter area by binding to the histone acetyltransferase CBP(p300) and arginine methyltransferase CARM1 (Archer *et al*., 2005). The LRR domain and gelsolin domain of the Flii molecule bind nuclear hormone receptors at the NR response element while the gelsolin-related domain also binds nuclear-actin, actin-like protein BAF53 and Arp4 all of which are components of the co-activator complex and necessary for the full co-activator function of Flii (Fig 1.12) (Lee *et al*., 2004). This co-activator function of Flii was also observed when Flii was found to bind and act in synergy with CARM1 co-activator (Lee *et al*., 2004).

Mammalian proteins possessing the LRR domain use this domain for interaction with different ligands. Flii LRR domain has the ability to form the hydrophobic curved

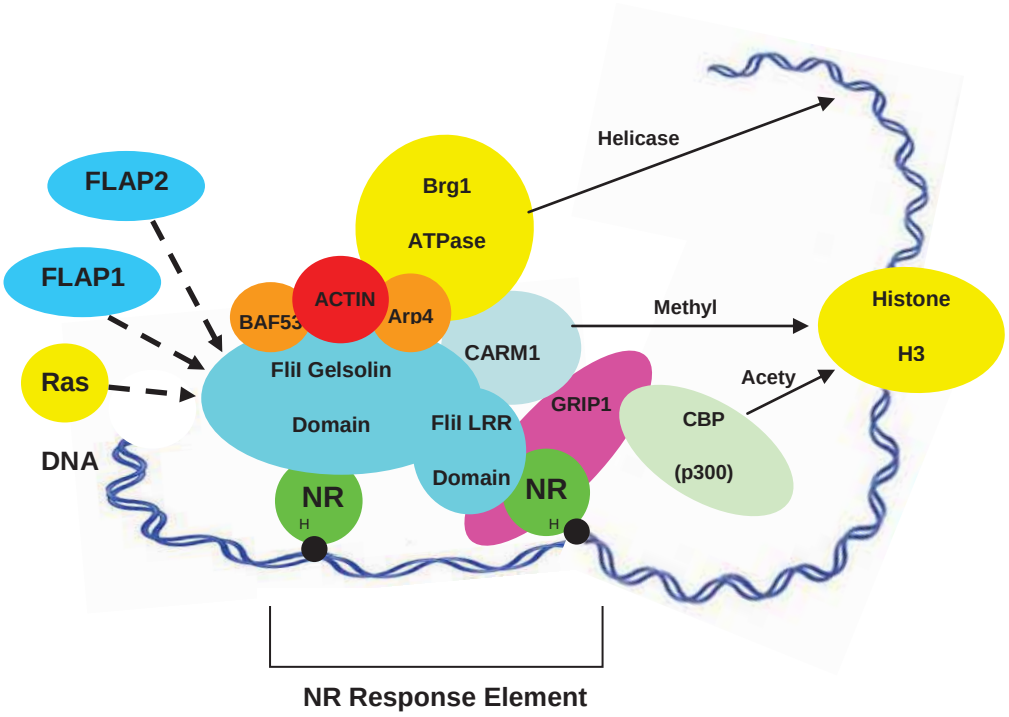


Figure 1.12: Schematic diagram of Flii role as a nuclear receptor co-activator and formation of the co-activator complexes at the promoter of an NR responsive gene. NR denotes the nuclear hormone receptor. Figure adapted from (Kopecki and Cowin, 2008).

solenoid structure which is characteristic of the proteins possessing the LRR domain. This enhances and stabilises the protein-protein and protein-lipid interactions involved in signal transduction as direct or indirect ligand binding, regulating and mediating receptor-ligand affinity (Bella *et al*., 2008, Kobe and Kajava, 2001). The identified Flii binding partners were Flii leucine rich repeat associated protein (FLAP)1 and FLAP2 and these proteins may be important in regulating Flii function (Liu and Yin, 1998) however their functions are still largely unknown.

Recent studies have shown that FLAP1 mediates the production of pro-inflammatory cytokine interferon-β via β-catenin dependant pathway (Bagashev *et al*., Dai *et al*., 2009). Flii acts synergistically in a GRIP1 co-activator complex with NRs and has an antagonistic effect on synergy between FLAP1, p300 and β-catenin, suggesting that Flii negatively regulates the activity of β-catenin co-activator complexes. Flii may therefore be involved in regulating β-catenin and LEF1/TCF-mediated transcription and that the interplay of Flii and FLAP1 may be one of the important regulation mechanisms of the ß-catenin dependent, Wnt signaling pathway (Lee and Stallcup, 2006). Further evidence of Flii involvement in regulation of β-catenin pathway was evident with studies using 3T3 fibroblasts showing direct and preferential binding of Flii to the activated form of calcium/calmodium dependant protein kinase type II (CaMK-II), hence affecting the progression of cell cycle (Seward *et al*., 2008). Inhibition of CaMK-II and over-expression of Flii resulted in suppression of transcription of β-catenin dependent transcriptional reporters while suppression of Flii led to enhanced transcriptional activity. It was hypothesized that CaMK-II influences the β-catenin dependent gene expression through Flii interaction implying Flii as a major component of this signaling pathway (Seward *et al*., 2008).

The functional activities of Flii are also evident from recent reports suggesting that the Flii homolog (Fliih) may play a prominent role in innate immune system responses where it down-regulates IL-1/TLR4 signaling of Toll-like receptor (TLR) pathway of immune system (Wang *et al*., 2006a). Flii interacts directly with MyD88, an intracellular adaptor protein immediately downstream of most Toll-like receptors. MyD88 functions to recruit multiple proteins to precisely control signaling transduction. A novel switch-on, turn-off mechanism of TLR signaling was proposed, governed by the interactions, binding strength and competition between Flii and other MyD88 binding partners, including FLAP-1 and FLAP-2. Binding of Flii to MyD88 and the Toll/IL-1 receptor domain (TIR) of the TLR molecules regulated the signaling for activation of NF-κB, a protein complex, controlling the transcription of DNA and immune response to infection. The temporal and dynamic nature of specific Flii interactions with other proteins of the TLR signaling has identified Flii as a negative regulator of the TLR4 signaling pathway (Dai *et al*., 2009, Hayashi *et al*., Wang *et al*., 2006a). A role for Flii in the immune system was also illustrated by its regulation of the pro-inflammatory caspases, namely caspase-1 and caspase-11. Flii was found to modulate the activity and intracellular localization of these caspases, distributing caspases to the actin network at the leading cell front where they can cleave and activate pro-inflammatory cytokines, including IL-1β and IL-18 (Li *et al*., 2008).

1.6.1 Role of Flightless I in wound healing

Recent studies using $Flii^{+/-}$ null mice, Flii over-expressing transgenic mice carrying the complete human Flii gene on a cosmid transgene, wild-type counterparts and well established *in vitro* models of wound repair reveal that Flii is an important negative regulator of the wound healing process (Cowin *et al*., 2007, Kopecki and Cowin, 2008).

Flii regulates wound healing through its effects on epidermal keratinocytes and dermal fibroblast cellular proliferation, motility and indirect modulation of collagen I expression. Flii$^{+/-}$ mice show improved wound healing, with increased epithelial migration and smaller, more contracted wounds, with increased expression of α-SMA positive myofibroblasts. In contrast, Flii over-expressing transgenic mice display increased wound area and dermal gape, reduced cell proliferation and delayed epithelial migration (Cowin *et al*., 2007).

Reduced levels of Flii lead to decreased collagen I expression in both *in vivo* mouse wounds as well as *in vitro* where siRNA treated fibroblasts show decreased collagen I secretion. Type I collagen is the major form of collagen produced in response to injury and its modulation greatly alters the extent of cutaneous fibrosis and scar formation (Kopecki *et al*., 2007). Slower, more organised production and deposition of collagen I often results in improved dermal architecture and reduced scarring. Indeed, Flii over-expressing mouse wounds have significantly increased levels of collagen I which are associated with extensive hypertrophic scarring in humans (Cowin *et al*., 2007, Wolfram *et al*., 2009). Aging also exacerbates the negative effect of Flii on wound repair, with larger wounds exhibited by Flii over-expressing aged mice, delayed reepithelisation and increased up-regulation of TGF-β1 (Adams *et al*., 2009). Research to date has indicated Flii as a cytoplasmic and nuclear protein but recent findings suggest that Flii is also a secreted protein released by wounded fibroblasts (Cowin *et al*., 2007) however extracellular roles for Flii are yet to be investigated.

Flii has also been shown to act as a negative regulator of wound healing in other models of wound healing besides incisional wounds. Using a partial thickness burn injury model, Flii$^{+/-}$ deficient mice exhibit improved wound healing with faster wound closure and

significantly reduced pro-scarring TGF-β1 protein and gene expression and elevated anti-scarring TGF-β3 expression while the opposite was observed in Flii over-expressing mice (Adams *et al*., 2009). Human clinical trials have shown that acute local administration of TGF-β3 significantly reduces dermal scarring in a dose dependent manner, resulting in regeneration of the skin structure (Occleston *et al*., 2008). Consequently, modulators of the TGF-β1/3 ratio and expression could be important for development of novel therapies for improved wound repair. Current studies indicate that modulation of Flii affects specific TGF-β isoform expression, where Flii deficiency results in a decreased TGF-β1 to TGF-β3 ratio while Flii over-expression resulted in a high TGF-β1 to TGF-β3 ratio and increased expression of Smad 7, a known inhibitor of the TGF-β/Smad signaling pathway. In agreement with in vivo data, Flii was also shown to interact with all TGF-β isoforms *in vitro*, and associate with activating protein-1 (AP-1), c-fos and c-jun transcription factors that regulate TGF-β expression as well as with nuclear Akt implicating a mechanistic link between Flii and regulation of TGF-β (unpublished data, Cowin A.J.).

Current research portrays the Flii molecule as an important actin-remodeling protein which plays a significant role in wound healing and as an intermediate protein linking the cytoskeleton to different signaling pathways (Adams *et al*., 2008, Cowin *et al*., 2007, Lee and Stallcup, 2006, Wang *et al*., 2006a). While exact signaling pathways and mechanisms underpinning Flii effects in wound healing are not yet defined, strong evidence identifies Flii as a potential therapeutic target for improving wound healing. The exact roles for Flii in different wound pathologies including: chronic wounds, ulcers, burns or blister wounds seen in patients suffering from fragile skin blistering diseases EB are yet to be identified however current findings clearly show a potential for Flii as a target of novel medical applications.

1.7 Epidermolysis Bullosa (EB)

EB is a complex group of genetic disorders producing various degrees of recurrent skin and mucous membrane blistering and epidermal detachment from the basement membrane. Patients with this disease experience the loss of intact epidermis, disruptions of basement membrane adhesion units and altered cellular adhesion, migration and integrin expression. Wound healing in patients suffering from EB remains a major challenge to their survival because of infection risk and fluid loss. There are four main types of Epidermolysis Bullosa which include, EB Simplex, Junctional EB, Dystrophic EB and Kindler Syndrome each characterized by different levels of blistering formation at the dermal-epidermal junction (basal layer, lamina lucida, sub-lamina densa and various respectively) and different clinical phenotypes. Another acquired immune based type of EB is called EB acquisita, where patients develop autoantibodies against ColVII resulting in blister formation at the level of ColVII anchoring fibrils (Kopecki *et al*., 2009). Advances in the understanding of the pathogenesis of EB in the last 15 years have led to the identification of several candidate genes and proteins, however present management of these diseases is still supportive and therapy symptomatic. Different therapy options are being investigated some of which are in clinical trials, including bone marrow transplant, gene therapy, cell therapy and protein based therapy. Further research focused on the development of novel therapies may lead to improved quality of life for patients suffering from EB (Kopecki *et al*., 2009).

1.7.1 Genotypes of the main Epidermolysis Bullosa types

The severity of blister expression in EB patients can range from mild blistering to severe bulla formation, erosions, scarring and mutilations. This depends on a number of factors, including the nature of the defective gene, its role in epithelial adhesion and non-

skin tissues and the type of mutation and positioning of the genetic defect within or close to critical protein domains (Mellerio *et al*., 2007, Sawamura *et al*., 2003). This disease affects 1 in 17,000 live births in all races, with an estimated 500,000 cases world-wide (Featherstone, 2007). The disease, has various forms caused by mutations in genes which code for structural proteins at the dermal-epidermal junction (DEJ) resulting in diminished adhesion of skin layers and blistering (Volz *et al*., 2007).

The clinical spectrum of severity in the four main subtypes of EB ranges from predisposition to blistering to severe morbidity and mortality (Bruckner-Tuderman, 2009). At infancy, hereditary forms of EB must be differentiated from EB aquisita which may affect both children and adults. This acquired autoimmune bullous disease is caused by injury induced by autoantibodies developed against type VII collagen, a major constituent of anchoring fibrils in the skin while the inherited counterpart, dystrophic EB is characterized by mutations in the gene encoding for type VII collagen (Varki *et al*., 2007). Over 30 distinctive EB subtypes have been identified to date however four main subtypes are most frequently encountered in clinical practices worldwide (Fine *et al*., 2000). The main types of EB with the genes and proteins involved are illustrated in Table 1.1. A common feature of these proteins is that they all contribute to the adherence of epithelium to the basement membrane zone or underlying extracellular matrix.

EB Simplex is characterized by cytolysis of basal keratinocytes and mutations in K5 and K14 resulting in inhibited formation of tonofilaments, which leads to enhanced fragility of the cytoskeleton and cytolysis of the basal keratinocytes. Different EB Simplex subtypes are classified into generalized or non-generalized EB Simplex depending on the site of blistering, however patients with EB Simplex have mainly normal life expectancy as

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of the print copy of the thesis held in
the University of Adelaide Library.

Table 1.1: Four main Epidermolysis Bullosa types and acquired Epidermolysis Bullosa, their inheritance and the different genes and proteins involved in various Epidermolysis Bullosa subtypes. AD = Autosomal dominant; AR = Autosomal Recessive. Table adapted from (Kopecki *et al*., 2009).

blistering occurs intraepidermally and wound healing takes place without scarring (Rugg *et al*., 2007). Main clinical features of EB Simplex include generalized bleeding, superficial flaccid bullae, palmo-plantar hyperkeratosis, nail dystrophy and erosions (Fine *et al*., 2000). Among all types of EB, the best phenotype-genotype correlation is seen in EBS where location of point mutations within the K5 and K14 genes determines the severity of the disease (Cummins *et al*., 2001).

Junctional EB is the most common form of this disease where the hemidesmosome-anchoring filament complex is weakened and genetic defects occur in one of the structural components including integrin α6β4, collagen XVII and the major basement membrane protein laminin-5 (Solovan *et al*., 2005). Clinical aspects of Junctional EB involve disseminated blister formation at DEJ, cleavage along the lamina lucida, polycyclic lesions with blisters and postlesional hyper-pigmentation (Bruckner-Tuderman, 2002). Degree of severity in the EB disease can be seen in different subtypes of Junctional EB which illustrate varying degrees of scarring. Junctional EB Herlitz, caused by homozygous null mutations in genes coding for hetrotrimer laminin-5, is usually lethal within the first two years after birth due to number of systemic complications, including failure to thrive, sepsis, pneumonia and tracheolaryngeal obstruction (Fine *et al*., 2008, Fine and Mellerio, 2009). In contrast, JEB progressiva, caused by non-sense, missense or insertion mutations in genes coding for hetrotrimer laminin-5, is mild with very rare extracutaneous manifestations, blister wound healing which takes place without scarring and normal patients life expectancy (Bruckner-Tuderman, 2002).

The third type of EB is Dystrophic EB where blistering occurs at the level of anchoring fibrils at the DEJ resulting in morphologically altered or absent anchoring fibrils

and generalized dermal blistering below the basement membrane, leading to disabling mutilations, mucosal involvement and scarring (Dang *et al*., 2007). Abnormalities of the anchoring fibrils and mutations in COL7A1, the gene encoding the major anchoring fibril protein collagen VII, underlie all Dystrophic EB subtypes which can be recessive or dominant. There is remarkably high number of different mutations within the COL7A1 gene which are often family-specific making development of a general gene therapy extremely difficult (Uitto and Pulkkinen, 2000). DEB is further classified into two subtypes according to the mode of inheritance, namely, autosomal dominant DEB (DDEB) or autosomal recessive DEB (RDEB) (Kopecki *et al*., 2009).

Clinically, DEB is characterized by severe recurrent blistering, pseudosyndactyly, milia and nail dystrophy, scarring and erosions and atrophic areas on lower extremities however clinical presentations of DDEB are milder than that of RDEB (Bruckner-Tuderman, 2009). The underlying genetic defect in DDEB is a glycine substitution in the collagenous domain of the pro-α-chain of the homotrimer ColVII that destabilizes the triple-helix, however as wild-type alleles are expressed equally, 12.5% of anchoring fibrils are functional, resulting in the milder clinical presentation. In RDEB, premature termination in both alleles results in a complete loss of type VII collagen gene (COL7A1) and anchoring fibrils, resulting in very severe clinical presentations and general lethality by the age of forty (Fine *et al*., 2005). To date, close to 500 different types of COL7A1 mutation have been identified, including an array of homozygous, heterozygous and compound heterozygous mutations, giving rise to a broad spectrum of phenotypes in DEB patients (Kern *et al*., 2009). Extracutaneous complications are very common in RDEB and generally include gastrointestinal involvement, growth retardation and chronic anaemia and high risk of development of aggressive squamos cell carcinoma (SCC) (Fine *et al*.,

2008, Fine *et al*., 2005, Fine *et al*., 2004). Patients with DEB are at higher risk of developing SCC than other EB patients. Patients with recessive DEB are at risk of developing SCC regardless of ColVII expression; rather the risk appears to correlate with severity of skin blistering with DEB patients having 80% of chance of developing SCC by the age of 40 and 55% of patients dying from metastatic SCC by the age of 40 (Fine *et al*., 2009, Pourreyron *et al*., 2007).

Kindler Syndrome, an autosomal recessive genodermatosis, is the fourth main subtype of EB. This syndrome results from mutations of the FERMT1 gene encoding for protein kindlin-1, a component of focal complexes found in basal keratinocytes, and clinically mimics different subtype of EB, including JEB and DEB (Fine *et al*., 2008, Ussar *et al*., 2008). Kindlin-1 protein has not been specifically shown to localise with hemidesmosome adhesion structures however it links the actin cytoskeleton to the extracellular matrix and is involved in cell signaling, growth, differentiation, apoptosis and integrin dependent cell adhesion, migration and spreading (Has *et al*., 2009, Kloeker *et al*., 2004, Larjava *et al*., 2008, Sadler *et al*., 2006). Indeed, research has shown that Kindlin-1 is required for RhoGTPase-mediated lamellipodia formation in keratinocytes (Has, 2009). Kindlin-1 is also the component of the integrin adhesion complex. Genetic and siRNA depletion of this protein in mice and cells illustrated that kindlin-1 is an essential regulator of integrin function, where depletion inhibits the integrin conformational shift from low to high affinity state of ligand binding. This led to the hypothesis that kindlin-1 co-operates with talin to regulate integrin activation and function (Moser *et al*., 2009). The main clinical features of Kindler syndrome include acral trauma-induced blistering, poikiloderma and photosensitivity thereby distinguishing this subtype from other inherited EB subtypes (Fine *et al*., 2008).

Skin disadherence and poor wound re-epithelialization are major clinical problems seen in EB patients often resulting in the loss of movement and deformity. These are especially important considerations for children, where their growth places extra demands on healing wounds. In certain forms of EB, especially EB Hallopeau-Siemens subtype, early epidermal metaplasia may lead to development of squamous cell carcinoma (SCC) which often results in amputation of the limbs (Aumailley *et al*., 2006). Examples of the four main EB subtypes with associated clinical features are shown in Figure 1.13.

1.7.2 Animal models of EB

Development of novel therapies for human disease requires preclinical validations in animal models, hence animal models of EB are of special interest. Models are available for studying the pathogenesis of EB, including both *ex vivo* and animal models (Jiang and Uitto, 2005, Sitaru *et al*., 2007). A better understanding of the cellular and molecular events of wound healing are essential to designing novel therapies for treatment and better wound management associated with different EB subtypes (Uitto and Pulkkinen, 2000). Several knockout or transgenic mice also exist for studying DEJ components and cellular receptors however problems arise as a majority of the transgenic mice mutated in adhesion molecules die neonatally, restricting the assessment of long term therapy outcomes. Current mouse models of EB include mice deficient for keratin 5, keratin 14, plectin, BP230, integrin chains α3, α6, β1 and β4, laminin-5, collagen IV, COL7A1 and COL17A1 (Bruckner-Tuderman, 2002). These animal models have helped us understand the pathophysiology of EB blistering diseases and have led to improved diagnosis and identification of novel genes and proteins involved in the molecular aspects of these diseases. However, two animal models of special interest for researchers investigating

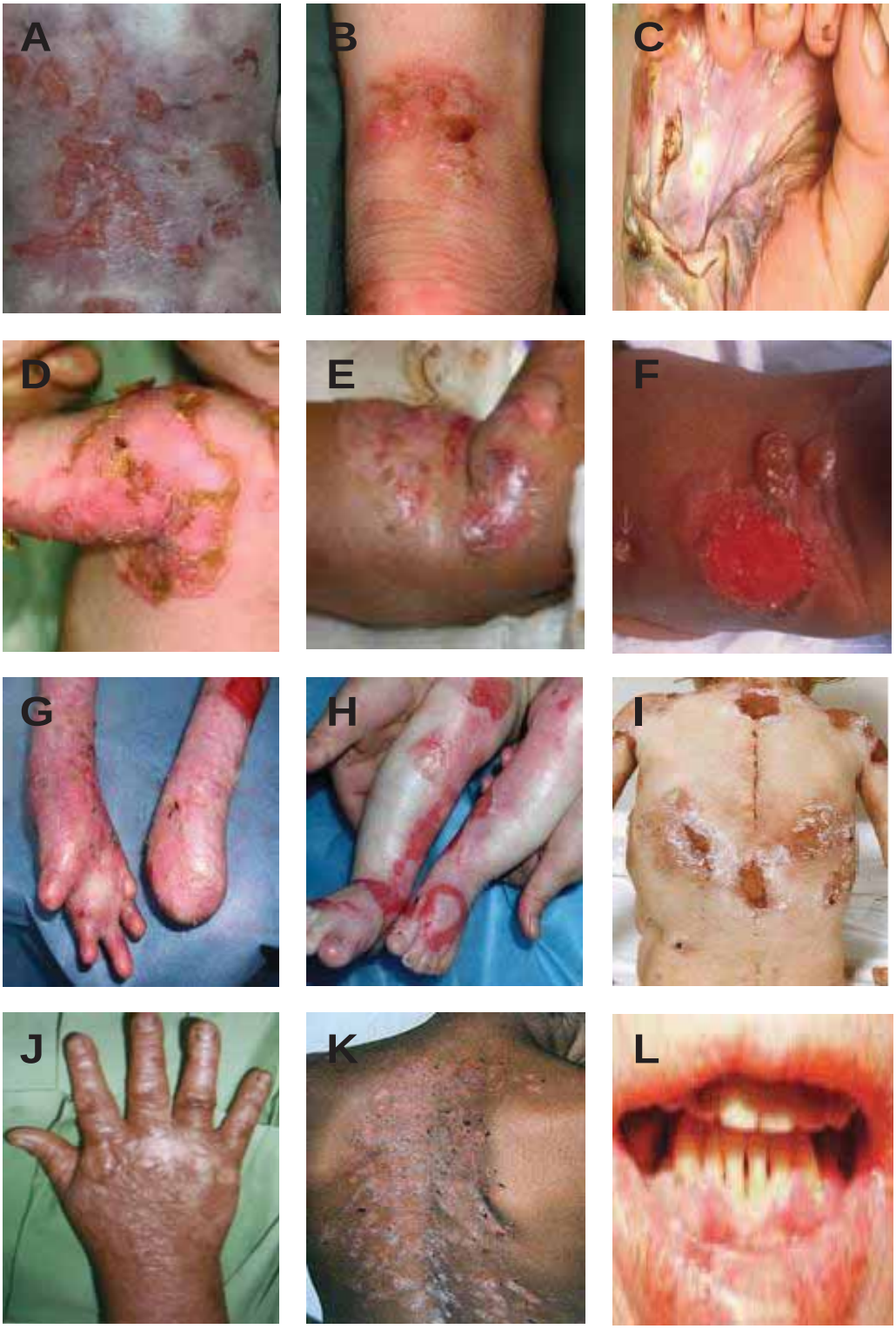

Figure 1.13: Four main Epidermolysis Bullosa types and associated clinical features. **A-C** EB Simplex with generalized blistering and superficial bullae over the body; blistering on the foot and haemorrhagic blisters and erosions on the palm **D-F** Junctional EB with extensive shoulder blistering; vesiculobullous erosions and milia formation on the abdomen and forearm **G-I** Dystrophic EB with severe scarring and pseudosyndactyly of the hands and atrophic areas on the lower extremities and the back **J-L** Kindler Syndrome with poikiloderma on the hands, erosions on the back and blistering with gingival involvement. Images courtesy of Dystrophic Epidermolysis Bullosa Research Association – Australia.

therapy options for DEB patients include a mouse model of EB aquisita (EBA) and a recently developed model of COL7A1 hypomorphic mice.

EBA is a chronic blistering disease of the skin and mucous membrane classified by sub-epidermal blistering and circulating autoantibodies at the DEJ against the main component of anchoring fibrils, collagen VII (Lehman *et al*., 2009). EBA is an acquired, antibody-mediated immune disease that can present as an inflammatory or non-inflammatory phenotype. Several studies have illustrated that anti-type VII collagen antibodies are pathogenic and responsible for clinical, histological and immunological features of EBA (Sitaru *et al*., 2006, Sitaru *et al*., 2007, Woodley *et al*., 2005). Indeed, EBA autoantibodies have been shown to recruit and activate leucocytes ex vivo, resulting in DEJ blistering in cryosections of human skin (Mihai and Sitaru, 2007). Passive transfer of autoantibodies from patients into immunocompetent mice illustrated the direct pathogenicity of human EBA autoantibodies (Woodley *et al*., 2006). Clinically, EBA skin exhibits fragility blisters, scars and milia formation, features seen in DEB. While DEB patients have reduced or absent anchoring fibrils, EBA patients have a decrease in normally functioning anchoring fibrils secondary to abnormality in an immune system, producing collagen VII autoantibodies (Remington *et al*., 2008). Passive transfer of rabbit IgG antibodies specific to type VII collagen to mice results in an EBA blistering mimicking human DEB and providing an excellent laboratory model for studying pathogenesis of DEB and development of novel therapeutic strategies (Sitaru *et al*., 2005).

A recently developed novel animal model of DEB which has attracted lots of research interest is the immunocompetent hypomorphic model of DEB. This animal model results from a conditional inactivation of ColVII expression, with collagen VII

hypomorphic mice having 10% of normal collagen VII levels in the skin. This phenotype closely resembles characteristics of severe DEB, including mucocutaneous blistering, nail dystrophy and pseudosyndactyly. Oral blistering in these mice leads to retarded growth and repeated blistering leads to excessive induction of tissue repair, prolonged inflammation and TGF-β mediated contractile fibrosis generated by myofibroblasts in the extremities, mimicking the human DEB phenotype (Fritsch *et al*., 2008). Previous animal models of targeted inactivation of COL7A1 gene generated a severely affected collagen VII knockout mouse which died weeks after birth due to severe blistering (Heinonen *et al*., 1999). However, the model described here is the first collagen VII hypomorphic model in an immunocompetent mouse which not only mimics human DEB at the genetic and phenotypic level but also survives to adulthood, allowing analysis of molecular pathogenesis of DEB and evaluation of therapeutic strategies (Fritsch *et al*., 2008). Indeed, a long term study of fibroblast therapy on a large number of these mice has shown promising results with high collagen stability and improved skin integrity and resistance to mechanical forces (Fritsch *et al*., 2008, Kern *et al*., 2009). Future research challenges include assessment of wound healing progress in these hypomorphic mice and investigations of different therapies which have a potential to improve healing outcomes of blister wounds. This which could eventually pave a way for human clinical trial on DEB patients using novel therapy approaches.

Besides the rodent models for EB, other animal models have also been established with clinical symptoms of EB and a surviving phenotype, including models in sheep and horse and a dog model of EB (Palazzi *et al*., 2000, Ruzzi *et al*., 2001). A selected list of animal models of EB developed for investigating the pathogenesis of EB types including clinical and genetic backgrounds is illustrated in Table 1.2.

EB type	Species	Mutant Gene	Clinical / Genetic Features	Reference
Simplex				
EBS	Mouse	KRT14	Basal cell cytolysis with keratin aggregates	Vassar et al., 1991
EBS	Mouse	KRT14	Inducible activation of mutant KRT14 allele	Cao et al., 2001
EBS	Mouse	KRT5	Deficient keratin IF resulting in neonatal lethality	Peters et al., 2001
Junctional				
H-JEB	Mouse	LAMA3	Targeted disruption of the gene, neonatal lethal	Ryan et al., 1999
H-JEB	Mouse	LAMC2	Targeted ablation of exon 8, neonatal lethal	Meng et al., 2003
H-JEB	Mouse	LAMB3	Spontaneous intrageneic insertion	Kuster et al., 1997
H-JEB	Horse	LAMC2	Severe blistering and exungulation	Spirito et al., 2002
NH-JEB	Dog	LAMA3	Mild blistering phenotype	Capt et al 2005
Dystrophic				
RDEB	Mouse	COL7A1	Conditional inactivation of ColVII expression	Fritsch et al, 2008
RDEB	Mouse	COL7A1	Targeted ablation of exons 46-69	Heinonen et al, 1999
RDEB	Sheep	COL7A1	Complete absence of ColVII expression	Bruckner-Tuderman et al., 1991
RDEB	Dog	COL7A1	Mutation G1906S, autosomal recessive	Baldeschi et al., 2003
DDEB	Dog	COL7A1	Reduced anchoring fibril formation	Nagata et al., 1995
DDEB	Cat	COL7A1	Reduced anchoring fibril formation	Olivry et al., 1999
Kindler Syndrome				
-	Mouse	FERMT1	Skin atrophy and intestinal epithelial dysfunction resulting in perinatal lethality	Ussar et al., 2008
EB Acquisita (EBA)				
-	Mouse	Immune induced	Destruction of ColVII anchoring fibrils resulting in subepidermal blistering	Sitaru et al., 2005

Table 1.2: Selected list of animal models of Epidermolysis Bullosa used in research, illustrating species, mutant gene and main clinical/genetic features. EBS - Epidermolysis Bullosa Simplex; H-JEB – Herlitz Junctional Epidermolysis Bullosa; NH-JEB – non-Herlitz Junctional Epidermolysis Bullosa; RDEB – Recessive Dystrophic Epidermolysis Bullosa; DDEB – Dominant Epidermolysis Bullosa; IF – Intermediate filaments. Figure adapted from (Jiang and Uitto, 2005).

1.8 Research Aims and Hypothesis

Wound healing and scar formation are an area of largely unmet medical need especially in diseases like EB where there is no cure and current therapy involves wound management. Wound healing and scarring are especially important considerations for children where surgical adjustment is required because their growth places extra demands on healing wounds and grafts. Remodelling and reorganization of actin cytoskeleton is fundamental to both non-scarring and scarring wound repair. As the actin cytoskeleton is known to modulate integrin expression and interactions during wound repair and to facilitate changes in cell adhesion and motility, tight control of the actin dynamics mediated by the gelsolin family of actin remodelling proteins is of pivotal importance. In light of recent finding by our laboratory identifying Flii protein as a negative modulator of wound healing via mechanisms of cell migration and proliferation (Adams *et al*., 2009, Cowin *et al*., 2007), studies were performed in this thesis to gain an insight into the potential role of this protein in both skin structure, cellular adhesion and wound repair in a skin blistering disease EB. In particular the aims were:

1. To determine the effects of differential Flii expression on skin structure and cellular responses including migration, spreading and adhesion.

2. To investigate whether the effects of differential Flii expression on wound repair are mediated via integrin signaling and different basement membrane proteins and describe the changes in cellular adhesion signaling following differential Flii expression.

3. To determine the role of Flii protein in the blister formation of skin disease EB by utilizing both human samples and two mouse models of skin blistering disease.

Studies presented in this thesis have identified a role for Flii protein in mediating skin architecture, actin remodelling and cellular adhesion during wound healing and have classified Flii protein as a potential therapeutic target for the development of novel therapies for patients suffering from EB. It was hypothesised that Flii is a key factor involved in cellular adhesion and that modulation of its activity would lead to improved wound healing.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

All materials unless otherwise specified were of analytical reagent grade and were obtained from Sigma-Aldrich Chemical Company, Sydney, Australia. For all experiments requiring cell culture, unless otherwise specified, Dulbecco's Modified Eagles Medium (DMEM) and Fetal Calf Serum (FCS) were purchased from JRH Biosciences, USA and Penicillin-Streptomycin was purchased from Invitrogen, Victoria, Australia. Centrifugation of the samples was performed in Hermle Z 160M bench top centrifuge.

2.1.1 Molecular Reagents

Trizol reagent, a solution of phenol and guanidine isothiocyanate used for total RNA extraction was purchased from Invitrogen, Mount Waverly, VIC, Australia. WST-1 reagent, used in the proliferation assay and TGF-β1 ligand used in the immunoprecipitation assay were purchased from Roche Diagnostics, NSW, Australia. 4-Nitrophenyl N-acetyl – β-D-glucosamidine (PNAG) 98-100% (N9376-1G) enzyme substrate used in the hexosaminidase adhesion assay and 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) nuclear stain used in immunocytochemistry were purchased from Sigma-Aldrich Chemical Company, Sydney, NSW, Australia. Extracellular matrix substrates: Fibronectin (F1141) from bovine plasma, Collagen type I (C-8919) from calf skin and Laminin (L-6274) and poly-L-lysine (P4707) were all purchased from Sigma-Aldrich, Sydney, Australia. Dispase II (#14781300) used in keratinocyte isolation was purchased from Roche Diagnostics, NSW, Australia, NSW, Australia. IgG from rabbit serum (#15006)

used as a control and Ultrapure Bovine Collagen Solution (C4243) used for a floating collagen gel contraction assay were all purchased from Sigma-Aldrich, Sydney, Australia.

2.1.2 Immunohistochemical reagents

All the antibody information including suppliers, catalogue numbers and concentrations used for immunohistochemistry and western blotting analysis in this thesis are outlined in Table 2.1. Rabbit anti-Flightless I antibody was raised against amino acids 1–300 mapping at the N terminus of Flightless I of human origin. Poly-L-lysine, Laminin from human placenta, Collagen type I from calf skin, Fibronectin from bovine plasma and Collagen Solution containing collagen extracted from bovine tendon and skin were all purchased from Sigma-Aldrich Pty. Ltd., St Louis, MO, USA. Flii neutralizing antibody (FnAb) is monoclonal IgG specifically raised against a Leucine Rich Repeat of Flii was commercially made by MabSA, SA, Australia. Rabbit anti-mouse ColVII antibody used in vivo mouse trials for induction of EB acquisita was prepared by our collaborator Prof Detlef Zillikens, as described previously (Sitaru *et al*., 2005).

In addition, the following antibodies were also used in this study: tensin and paxillin-pY^{118} (Santa Cruz Biotechnology); talin, vinculin, α-actinin and Src (Upstate Biotechnology, USA); paxillin and p130Cas (BD Transduction Laboratories); phalloidin-fluorescein isocyanate (FITC), β-tubulin antibody and DAPI nucleic acid stain (Sigma-Aldrich, Sydney, Australia); Alexa Fluor 488 donkey anti-goat IgG and Streptavidin-Cy3 Alexa Fluor 555 conjugate (Invitrogen, Australia); species-specific horseradish peroxidase-conjugated antibodies (DAKO Corporation, Botany, Australia) and species-specific

Antibody	Manufacturer	Catalogue Number	Raised in	Concentration use	
				Western	IHC
α-Actinin	Upstate Biotechnology	05-384	Mouse	1µg/ml	2µg/ml
β-Tubulin	Sigma-Aldrich	T4026	Mouse	1µg/ml	N/A
p130Cas	BD-Biosciences	610271	Mouse	1µg/ml	2µg/ml
CD151	Santa Cruz	sc-18753	Rabbit	1µg/ml	2µg/ml
Collagen IV	Rockland	600-401-106-0.5	Rabbit	N/A	2µg/ml
DAPI	Sigma-Aldrich	D9564	N/A	N/A	2µg/ml
Fibronectin	Sigma-Aldrich	F3648	Rabbit	N/A	2µg/ml
Flightless	Santa Cruz	sc-30046	Rabbit	1µg/ml	2µg/ml
Flightless	Santa Cruz	sc-21716	Mouse	1µg/ml	2µg/ml
Gelsolin	Santa Cruz	SG6406	Goat	1µg/ml	2µg/ml
Integrin α3	Santa Cruz	sc-28665	Rabbit	1µg/ml	2µg/ml
Integrin α6	Santa Cruz	sc-6596	Rabbit	1µg/ml	2µg/ml
Integrin β1	Santa Cruz	sc-8978	Rabbit	1µg/ml	2µg/ml
Integrin β4	Santa Cruz	sc-9090	Rabbit	1µg/ml	2µg/ml
Laminin	Monosan	P5040	Rabbit	1µg/ml	2µg/ml
Paxillin	Upstate Biotechnology	05-417	Goat	1µg/ml	2µg/ml
p-Paxillin	BD-Biosciences	610568	Mouse	1µg/ml	2µg/ml
PCNA	Santa Cruz	sc-56	Mouse	N/A	2µg/ml
Phalloidin-FITC	Sigma-Aldrich	P5282-0.1G	Mouse	N/A	2µg/ml
Src	Milipore	05-184	Mouse	1µg/ml	2µg/ml
Talin	Upstate Biotechnology	05-385	Mouse	1µg/ml	2µg/ml
Tensin	Santa Cruz	sc-28542	Rabbit	N/A	2µg/ml
Vinculin	Upstate Biotechnology	05-386	Mouse	1µg/ml	2µg/ml
2° Anti-rabbit Alexa Fluor 488	Invitrogen	A11008	Goat	N/A	2µg/ml
2° Anti-goat Alexa Fluor 488	Invitrogen	A11055	Donkey	N/A	2µg/ml
Biotinylated 2° Anti-mouse IgG	Vector Laboratories	BA-2000	Horse	N/A	2µg/ml
Streptavidin Alexa Fluor 555	Invitrogen	S32355	N/A	N/A	2µg/ml
Streptavidin cy3	Sigma-Aldrich	S6402	N/A	N/A	2µg/ml
HRP-Conjugated anti-mouse IgG	Dako Cytomation	P0447	Goat	1µg/ml	N/A

Table 2.1: Antibody information and concentrations used in western analyses and immunofluorescence experiments.

biotinylated IgG antibodies (Vector Laboratories, CA, USA). Anti-ColIV isolated from rabbit antiserum and cross-absorbed by immunoaffinity purification to minimize the reactivity with other collagen was purchased from Rockland (Gilbertsville, PA, USA). Monoclonal anti-RhoA or Rac1 or Cdc42 antibodies used in Rho GTPase Pull Down Activation Assay were part of the RhoA/Rac1/Cdc42 Activation Kit (Cell Biolabs, Inc.). Biotinylated species-specific anti-mouse and anti-rabbit secondary IgG antibodies were purchased from Vector Laboratories (CA, USA) while Cy3-streptavidin conjugate was purchased from Sigma-Aldrich (St Louis, MO, USA). TGF-β1 peptide (T7039) (2μg/ml) with >98% purity and cell culture tested biological activity and was purchased from Sigma-Aldrich (St Louis, MO, USA).

2.1.3 Human Tissue

Skin samples were collected by Prof Dedee Murrell from 44 patients, male and female, of all ages, with a clinical diagnosis of EBS, JEB and DEB, suitable for simple punch biopsy or excision surgery during the patient's regular visits to the Dermatology Clinic, St. Georges Hospital, Sydney, Australia. All patients signed the consent form agreeing to donate samples for research purposes and appropriate ethics was obtained from St. Georges Hospital Ethics Committee, Sydney, Australia. Classification of EB sub-types were based on clinical presentations and ultrastructural findings and gene analysis and histological site of blister formation for EB patients.

Skin biopsies were collected by Prof Leena Bruckner-Tuderman from 4 patients, male or females, aged 25-40, with a clinical diagnosis of Kindler Syndrome, suitable for

simple punch biopsy during patient's regular visits to Centre for Fragile Skin, Dermatology Clinic, University Medical Centre Freiburg, Freiburg, Germany. All patients signed the consent form agreeing to donate samples for research purposes and appropriate ethics was obtained from the Human Ethics Committee, Medical Centre Freiburg, Freiburg, Germany. Classification of Kindler Syndrome was based on clinical presentations, ultrastructural findings, gene analysis and histological site of blister formation.

The normal control group consisted of 4 skin biopsies collected from the abdomen during cosmetic surgical procedures from male and female consenting subjects of all ages with no known dermatological condition collected by Prof Rob Fitridge at Queen Elizabeth Hospital Adelaide, Australia with approval of Queen Elizabeth Hospital Ethics Committee, Adelaide, Australia.

2.1.4 Animal Tissue

Flii heterozygous ($Flii^{+/-}$), Flii over-expressing ($Flii^{Tg/+}$ and/or $Flii^{Tg/Tg}$), CD151 homozygous ($CD151^{-/-}$) and ColVII hypomorphic mice were used in this study. $Flii^{+/-}$ and $Flii^{Tg/Tg}$ mice were generated by our collaborators, Dr. Hugh Campbell and A/Prof Ruth Arkell from Molecular Genetics and Evolution Group for the Molecular Genetics of Development, Research School of Biological Sciences, Australian National University, Canberra, ACT, Australia (Campbell *et al*., 2002). $CD151^{-/-}$ mice were generated by our collaborator Prof Leone Ashman from the School of Biomedical Sciences, University of Newcastle and Hunter Medical Research Institute, Newcastle, NSW, Australia (Cowin *et al*., 2006). ColVII hypomorphic mice were generated by our collaborator Prof Leena

Bruckner-Tuderman from University Medical Centre Freiburg, Freiburg, Germany. Wild-type (WT) mice are of BALB/c genetic background. The methods of breeding are discussed below in their respective headings.

2.1.4.1 Flii$^{+/-}$ mice generation

Flii$^{+/-}$ mice were generated by loss of function mutation in the Flii gene via homologous recombination in embryonic stem cells and passage of these cells through the germ line following chimera production. Animals homozygous for Flii gene are embryonic lethal and die *in utero* at embryonic day 7 (Campbell *et al.*, 2002) hence the strain was maintained by continuous backcross of heterozygous carriers Flii$^{+/-}$ to BALB/c mice. All mice used in this study were heterozygous carriers from backcross generation 10 or later and therefore were BALB/c congenic. The generation of Flii$^{+/-}$ mice and the resulting mutation are described in detail in Campbell *et al*., (2002) and a diagram of the targeting strategy is illustrated in Fig 2.1A. The heterozygous mice were identified, by our collaborators, Dr. Hugh Campbell and A/Prof Ruth Arkell, using three primer PCR sets that amplified products specific to the wild-type or targeted allele as illustrated in Fig 2.1B. The PCR was performed on DNA extraction from ear biopsies of potential heterozygotes. The animals with one wild-type copy of the Flii gene and one mutant copy of the Flii gene express no more than 50% of the normal Flii gene expression (Campbell *et al.*, 2002).

Figure 2.1: Targeted disruption of the Flii gene. A Schematic representation of the domain structure of the targeting vector, relevant portion of the Flii gene and the targeted allele after homologous recombination. Restriction enzymes sites, *BspEI* is denoted by B, *EcoRV* by E and *NcoI* by N. Flii exons are represented by the numbered open boxes. The tk-neo and pgk-thymidine kinase casettes are indicated. **B** Three primer PCR indicating wild-type (210bp) and mutant allele (538bp) products. Animals with one wild-type copy of the Flii gene and one mutant allele expressed no more than 50% of the normal wild-type Flii expression levels. Figure adapted from (Campbell *et al*., 2002).

2.1.4.2 Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice generation

Mice carrying additional copies of the Flii gene were generated by our collaborators, Dr. Hugh Campbell and A/Prof Ruth Arkell, by introduction of a cosmid construct into the mouse genome using transgenesis. At the time of strain production, the cosmid contained the human Flii gene and the surrounding sequences with the extent of the construct being defined via restriction mapping. The availability of the mouse genome allowed for the estimation of the extent of the cosmid. Currently, it is known that the cosmid contains all of the neighbouring Smith-Magenis syndrome chromosome region candidate 7 homolog (SMCR7) gene as well as patrial coding sequence for the Topoisomerase (DNA) III alpha (TOPA3A) and Lethal giant larve homolog (LLGL1) genes (Fig 2.2.) however we have conducted extensive studies characterising our different transgenic mice to ensure that the effects on wound healing are attributed to *Flii* gene alone. This was achieved by making a second transgenic strain using only a 17.8kb BspH1 sub-fragment of the cosmid (Fig 2.2) and assessing the strain to determine whether this minimal construct is able to provide all functions of endogenous *Flii* gene. We have shown that this construct contains all necessary elements to recapitulate the function of endogenous *Flii* and that Flii protein levels are sufficiently elevated to cause altered Flii function in these mice including wound healing. A manuscript detailing these mice is currently under review at Genesis – The Journal of Genetics and Development.

The transgenic strain used in this project was backcrossed to BALB/c animals for 10 generations before being intercrossed and homozygous animals were classified via progeny testing following established protocols. The mouse colony was subsequently

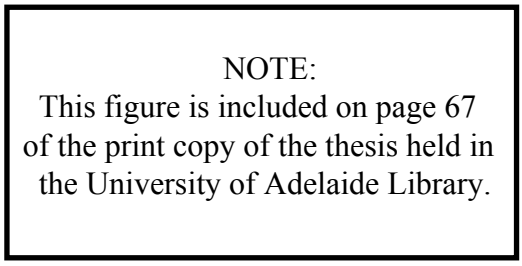

Figure 2.2: Domain structure of the cosmid containing Flii gene which is used to generate Flii transgenic mice. Cosmid contains Flii gene, SMCR7 gene, parts of TOP3A and LLGL1 genes. Restriction sites on the cosmid are also illustrated. Figure adapted from (Campbell *et al*., 2002).

maintained by intercross of animals homozygous for the transgene. These animals carry two copies of the mouse Flii gene and two copies of the human Flii transgene ($Flii^{+/+}$; $Flii^{Tg/Tg}$), denoted throughout this thesis as $Flii^{Tg/Tg}$. $Flii^{Tg/Tg}$ were out-crossed to BALB/c animals to generate the progeny that carry two copies of the mouse Flii gene and one copy of the human Flii transgene ($Flii^{+/+}$; $Flii^{Tg/+}$), denoted throughout this thesis as $Flii^{Tg/+}$.

2.1.4.3 CD151$^{-/-}$ mice generation

CD151 null mice were generated by our collaborator Prof Leone Ashman, using a contruct initiated by subcloning fragments which spanned CD151 and included adjacent sequences. Two clones, pL4C8 and pG8BC1 were isolated by primer walking (Fig 2.3A). A loxP sequence containing a HincII site was introduced into intronic sequences between exons 1c and 2 in clone pL4C8 by overlapping PCR (Fig 2.3B). Products were digested with AflII and BstEII, ligated into pL4C8 and sequenced. Minimal Flp recombinase target (FRT) sequences were introduced to facilitate the removal of the PGKNeo cassette. The construct was then prepared by subcloning the XbaI-BamHI fragment from pL4C8-HincII/loxp into pBluescript, digested with BamHI and EcoRV, ligated to BamHI-HpaI fragment from pG8BC-PGKNeo and sequenced. Homologous recombinants were identified by Southern Blotting. Chimeras were generated from targeted ES cells with standard methods and crossed with C57BL/6 mice to obtain germ line transmission. Mice heterozygous for a targeted mutant CD151 allele were crossed with C57BL/6 Cre recombinase transgenic mice to excise the loxP-flanked exons 2 to 7 of the CD151 gene in all cells including germ cells. Deletion and subsequent genotype analysis was performed by PCR with the strategy shown in (Fig 2.3B).

Figure 2.3: Targeted inactivation of the CD151 gene. A CD151 gene structure and associated overlapping genomic clones used pL4C8 and pG8BC1. **B** Targeting strategy. Schematic diagram of restriction maps for the targeting vector (top), the wild-type CD151 locus (middle) and the mutated CD151 locus after the Cre-mediated recombination (bottom). Coding exons = shaded boxes; non coding exons = open boxes; loxP sites = triangles. Figure is adapted from (Wright *et al*., 2004).

2.1.4.4 ColVII hypomorphic mice generation

In order to generate a mouse model for conditional inactivation of collagen VII expression, a targeting construct was developed similar to the descriptions above. Briefly, an 11-kb genomic fragment of *COL7A1* gene at intron 2 was replaced by a targeting construct, flanking exon 2 with 2 loxP sites. Additionally, a PGK-Neo cassette was introduced downstream of exon 2 with 2 minimal flanking Flp recombinase target (FRT) sequence to facilitate the Flp-mediated removal of the PGK-Neo cassette (Fig 2.4A). $COL7A1^{wt/wt}$, $COL7A1^{flNeo/wt}$, $COL7A1^{flNeo/flNeo}$ animals were born in a normal Mendelian ratio (Fig 2.4B) and were indistinguishable at birth. Within 24-48 hrs $COL7A1^{flNeo/flNeo}$ mice developed hemorrhagic blisters on the soles of the fore and hind paws and occasionally on the ears and mouth. Extent of blister formation ranged from small blisters on the fore paws to extensive blistering on the extremities and the trunk (Fig 2.4C). Genotyping of the $COL7A1^{flNeo/flNeo}$ mice was performed by our collaborator Prof Leena Bruckner-Tuderman, where PCR detecting the presence of the 5'loxP site, with amplicons of 269nt for the wild-type allele and 435nt for the transgenic allele in order to confirm the conditional inactivation of ColVII expression. The strain was maintained by interbreeding and $COL7A1^{flNeo/flNeo}$ mice were obtained by mating homozygous siblings. $COL7A1^{flNeo/flNeo}$ mice expressing only 10% of normal collagen VII levels are denoted as ColVII hypomorphic mice throughout this thesis.

Figure 2.4: Generation of the ColVII hypomorphic mice. A COL7A1 genomic fragment was replaced by targeting construct containing a Neo cassette (grey box) and two Frt sites (grey ovals) flanking exon 2 (E2) with two loxP sites (black triangles). **B** Genotyping of the COL7A1$^{flNeo/flNeo}$ mice was performed by PCR detecting the presence of the 5'loxPsite, with amplicons of 269nt for wild-type and 435nt for the transgenic allele. **C** ColVII hypomorphic mice develop hemorrhagic blisters on paws within 24hrs of birth. Figure adapted from (Fritsch *et al*., 2008).

2.2 Methods

Following experimental methods were used in this study. Methods adapted or modified from different published studies are appropriately referenced as stated in the text.

2.2.1 Human Samples

Human skin biopsies of blistered or unwounded skin from EB patients and control subject group were all collected under local anesthetic and embedded in OCT compound (Sakura Finetek, CA) and stored at -80ºC. Indirect immunofluorescence localization was used with a biotin - CY3 - Streptavidin amplification step. Serial cryosections (6μm) were first fixed in ice-cold acetone for 1min followed by the phosphate-buffered saline (PBS) wash and incubation for 30 min at room temperature with 3% Normal Horse Serum (NHS)/PBS blocking unspecific binding sites. Sections were then washed in PBS and incubated for 1hr with Flii or Gelsolin primary antibodies (1:100). After three washes in PBS, sections were incubated with species-specific secondary biotinylated anti-mouse antibody (1:200) for 1 hr followed by further washes in PBS and detected via CY3-conjugated Streptavidin (1:200) (Sigma-Aldrich, St. Louis, MO).

2.2.2 Murine Surgery

All experiments were approved by the Adelaide Child, Youth and Women's Health Service Animal Care and Ethics Committee and the ANU Animal Ethics Committee following the Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes. The $Flii^{+/-}$, wild-type, $Flii^{Tg/Tg}$, $CD151^{-/-}$ mice were housed in the

Women's and Children's Hospital animal house and ColVII hypomorphic mice were housed in the University of Freiburg Medical Centre animal house. All cages were cleaned and food and water replaced daily. Incisional wound surgery on CD151$^{-/-}$ mice was performed by Mr D. Adams for the purpose of a different study prior to the start of this project. Subsequently, paraffin embedded tissue and fresh frozen samples from CD151$^{-/-}$ were used in this project for both immunohistochemistry and western blotting analysis as described in Chapter 4 of this thesis.

2.2.2.1 Incisional Wound Healing Model

Full skin thickness incisional wound surgery was performed on 72 twelve week old female mice: 24 wild-type (WT), 24 Flii heterozygous (*Flii*$^{+/-}$) and 24 transgenic (*Flii*$^{Tg/+}$) mice. Four time-points: 3, 7, 14 and 21 days post-wounding were chosen to represent the different periods in wound healing. n=6 per time-point, 2 wounds created per mice. In addition, incisional wound surgery was performed on 18 twelve week old female mice: 6 wild-type (WT), 6 Flii heterozygous (*Flii*$^{+/-}$) and 6 transgenic (*Flii*$^{Tg/Tg}$) mice for experiments presented in Chapter 5 of this thesis.

Mice were weighed upon arrival, prior to surgery and post-surgery at time-points stated above to monitor their well-being. Mice that lost more than 10% of the body weight were excluded from the study. Mouse behaviour, coat appearance and movements were also observed regularly to ensure no adverse effects from surgery. Before surgery, the mice were induced with anaesthesia using 5% isoflurane at 2L oxygen per minute. Anesthesia was maintained using 2% isoflurane at 500ml oxygen per minute throughout surgery. The

mice were then shaved and the skin cleaned with Betadine solution containing 1% iodine. Two equidistant, 1cm full thickness incisions were made through the skin to the *panniculus carnosus* muscle layer on the flanks of the mice, extending 3-4cm from the base of the skull, 1cm either side of the spinal column and left to heal by secondary intention (i.e. wounds left unopposed and not sutured) (Fig 2.5). At the designated day of sacrifice, the mice were weighed and euthanized using CO_2 and cervical dislocation. The mice were then re-shaved and digital photographs of the wounds were taken prior to excision. Both left and right wounds were excised and bisected, half was used for histological analyses and the other half was snap-frozen in liquid nitrogen and stored at -80°C for biochemical analyses. Wound area was quantified by two independent blinded observers using AnalySIS software package (Soft-Imaging System GmbH, Muster, Germany). For each mouse group, unwounded skin was collected from the $Flii^{+/-}$, wild-type and $Flii^{Tg/+}$ mice and divided as stated above. Additionally, skin punch biopsies were collected from $Flii^{+/-}$, wild-type, $Flii^{Tg/+}$ and $Flii^{Tg/Tg}$ mice for electron microscopy hemidesmosome analysis, skin explant outgrowth assay and isolation of primary fibroblasts as described later in this chapter. Mouse tails were collected for isolation of primary keratinocytes and additional strips of skin 3cm by 1cm were collected from dorsal skin for tensiometry studies as detailed later in this chapter.

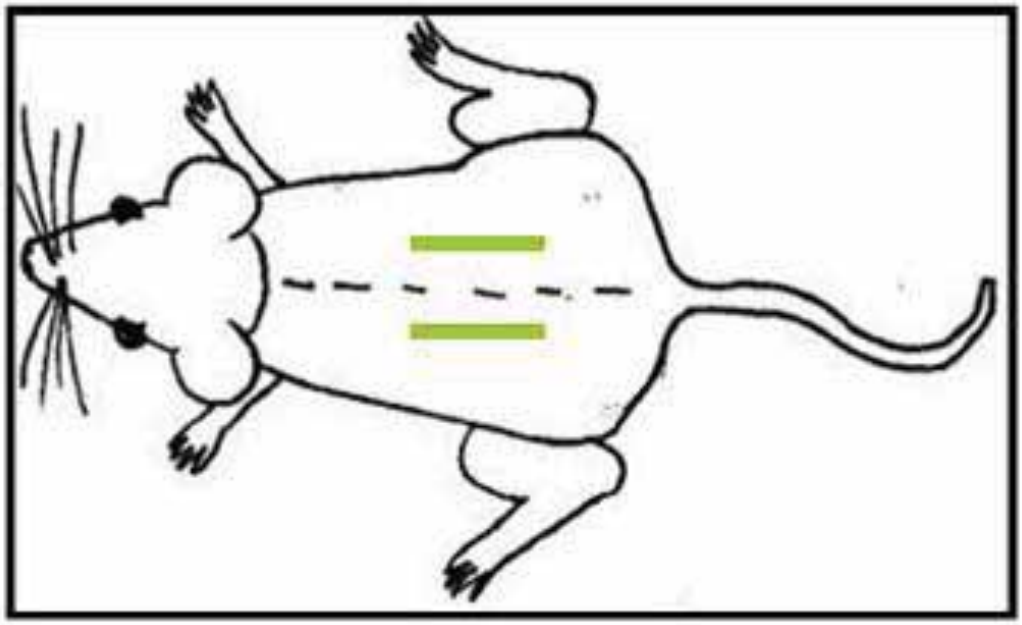

Figure 2.5: Incisional wound model used in research. Positioning of wounds on the dorsum of mice.

2.2.2.2 Epidermolysis Bullosa Acquisita (EBA) mouse model

EBA was induced in 12 female mice, 3-4weeks of age, with the BALB/c background and varying expression of Flii gene. EBA was induced in 4 Flii-deficient heterozygous null mice ($Flii^{+/-}$), 4 wild-type mice and 4 Flii over-expressing mice carrying the complete human Flii gene on a cosmid transgene ($Flii^{Tg/Tg}$). Rabbit anti-mouse ColVII antibody used in vivo mouse trials for induction of EB acquisita was prepared by our collaborator Prof Detlef Zillikens as described previously (Sitaru *et al*., 2005). Briefly, a New Zealand White rabbit was injected subcutaneously with recombinant purified mouse ColVII fragments and boosted twice at 15 day intervals with the same protein preparation. IgG from rabbit serum was isolated and affinity purified using Protein G Sepharose Fast Flow affinity column chromatography (Amersham Biosciences) according to the manufacturer's instructions. Purified and highly concentrated rabbit IgG specific to murine type VII collagen (0.3mg/g body weight) was injected subcutaneously into the backs of 3-4 week old $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ mice every second day over a period of 10 days. Mice were weighed daily and examined for evidence of cutaneous lesions (erythema, blisters and erosions) and extent of skin disease (percentage of skin surface covered by lesions). Immune response increased over time and mice were euthanised at day 16 post initial injection when the number of blisters peaked. Control animals injected with the same concentration of normal rabbit IgG failed to develop skin lesions, erythema or blisters. Blistered skin samples from front paws and back, as well as non-blistered back skin, were collected for histology, immunohistochemical analysis and snap frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

2.2.2.3 ColVII hypomorphic mouse model

Hypomorphic ColVII mice were generated using the mice of a C57BL/6 background. Within 48hrs of birth, hypomorphic ColVII mice developed extensive hemorrhagic blisters on the front paws resembling features observed in patients with Dystrophic EB. Blistered skin from the front paws, non-blistered skin from the back and inflamed skin from back paws was collected from 3 week old ColVII hypomorphic female mice and normal skin from the same anatomical sites of wild-type controls. Samples were collected for histology, immunohistochemical analysis and fresh frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

2.2.3 Skin explant outgrowth assay

2mm punch biopsies (Acupunch, ACUDERM) excised from $Flii^{+/-}$, wild-type, $Flii^{Tg/+}$ and $Flii^{Tg/Tg}$ unwounded back skin were collected in ice-cold Ham's Nutrient Mixture F12 (SAFC Biosciences), 5% Penicillin Streptomycin, 5% Fungizone (Sigma-Aldrich). Four punch biopsies were added to each well of a 6 well plate were fixed dermal side down onto glass coverslips (controls) or coverslips pre-coated with 10 mg/ml Fibronectin, 6 mg/ml Laminin or 10 mg/ml Collagen I (Sigma-Aldrich Pty. Ltd., St Louis, MO). Skin explants were cultured in 2ml 20% FCS/DMEM, 5% Penicillin Streptomycin, 5% Fungizone (Sigma-Aldrich) at 37°C, 5% CO2, replacing media after 24hrs and then every 2 days. Representative photographs of cellular outgrowth from each explant were taken daily, 3 images per explant, 8 explants per treatment group and proximal distance of cellular migration outgrowth from the dermis measured at 0–7 days post plating and analysed using AnalySIS software (Soft-Imaging System GmbH, Munster, Germany).

2.2.4 Skin tensiometry

Strips of unwounded skin measuring 3cm by 1cm were collected from the dorsum of 12 Flii$^{+/-}$, 12 wild-type, 12 Flii$^{Tg/+}$ and 12 Flii$^{Tg/Tg}$ mice and analysed for skin tensile strength using the Microprocessor Force Gauge (MFG25, M1000E, Max capacity 1000N, Mecmescin, SI Instruments Pty Ltd, Adelaide, Australia). Strips of unwounded mouse skin were clamped by the processor gauges and speed applied (3cm/min) as per the manufactures recommendations. Stretching of the skin resulted in an eventual breakage with digital recording of the force and displacement required measured in Newtons.

2.2.5 Histology processing and image analysis

Murine unwounded and wounded skin collected from surgery was fixed in 10% formalin over night, followed by processing in a Leica TP1020 tissue processor which dehydrated the tissues in a graded alcohol series (70% for 120 mins, 80% for 60 mins, 90% for 105 mins and 100% for 240 mins). They were then cleared in transitional solvent xylene for 180 mins followed by 240 mins of tissue infiltration with paraffin wax. Tissue sections (4μm) were cut from paraffin-embedded fixed tissue using a Leica RM2235 microtome. Prior to staining, skin sections were dewaxed by a series of xylene (30 mins) and graduated ethanol washes (bringing sections to water) (100% for 1 min, 70% for 1 min & 30% for 1 min) before further processing. Skin sections were either stained with Haematoxylin and Eosin (H&E) or subjected to antigen retrieval and immunohistochemistry using antibodies from Table 2.1. Staining the sections in H&E involved bringing sections to water as mentioned above, followed by staining in Lillie's-Mayer's Haematoxylin for 6min, "blueing" sections in bicarbonate water for 15 sec,

differentiating Haematoxylin in 0.25% Acid Alcohol for 6 sec, staining in alcohol based Eosin stain for 2 min, dehydrating in grated alcohol series (30% for 30 sec, 70% for 30 sec, 100% for 1 min) and clearing in transitional solvent xylene for 2 min before mounting in DePeX mounting medium.

H&E staining of unwounded skin was analysed microscopically using light microscopy. Dermal thickness was determined by measuring the distance between th basement membrane and the panniculus carnosus using Image Pro-Plus 5.1 program (MediaCyberneticsInc.). H&E stained wounds from Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice were analysed using light microscopy and wound area and gape measured as previously described (Cowin *et al*., 2007). H&E stained blistered skin from EBA induced Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice was also analysed using light microscopy for histological blister score formation, as previously described (Kawasaki et al., 2006). Briefly, blister formation was considered positive when the length of single lesion was greater than 120μm. Blisters at the edge of the section were not included in the analysis. To quantify the extent of blister formation histologically, histological blister score was calculated following the formula: histological blister score = (combined total length of all blistered regions) / (combined total length of all sections examined) x 100.

2.2.6 Electron Microscopy and Hemidesmosome analysis

Two millimeter punch biopsies (Acupunch, ACUDERM) excised from 12-week-old female Flii$^{+/-}$, wild-type, Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mouse skin were fixed overnight in 4% paraformaldehyde/1.25% glutaraldehyde/4% sucrose in phosphate-buffered saline (PBS),

pH 7.2. Tissue punch biopsies were subsequently washed for 10 min in two changes of 4% sucrose/PBS followed by post-fixation in aqueous 2% osmium tetroxide for 1 hr on a rotator. After washing, specimens were dehydrated in a graded series of ethanol (70–100%) followed by further dehydration in propylene oxide for 30 min. Tissue samples were resin infiltrated with 1:1 propylene oxide/resin solution overnight followed by an 8 hr incubation in three changes of 100% epoxy resin. Tissue samples were then embedded in fresh resin (Procure Araldite embedding kit) and polymerized at 70°C overnight. For orientation purposes, semi-thin (1mm thick) survey sections were cut on a Reichert Ultracut S ultramicrotome and stained with 1% toluidine blue in 1% borax. Ultra-thin sections were cut and mounted on 100 mesh hexagonal copper grids (Gilder Grids, Grantham, UK). Sections were stained with 2% uranyl acetate for 10 min followed by 1% lead citrate for 7min and examined with a Phillips CM100 transmission electron microscope operated at an accelerating voltage of 80kV.

Electron micrographs were taken of two different samples of unwounded and blistered skin of $Flii^{+/-}$, wild-type, $Flii^{Tg/+}$ and $Flii^{Tg/Tg}$ at different magnifications ranging from x 7, 900 to x 92, 000 with random selection of sections. Tissue skin architecture and structure was observed, particularly focusing on regions of dermal–epidermal junction. A number of different parameters were analyzed using Image Pro-Plus 5.1 program (MediaCybernetics Inc.). These included, the number of hemidesmosomes/basement membrane length (mm), number of hemidesmosomes with sub-basal dense plates and their percentage in relation to the total number of hemidesmosomes counted, average lamina lucida and lamina densa thickness of basal lamina, and average hemidesmosome length (n=150 hemidesmosomes) (Jaunzems *et al*., 1997). Examination of the differences in skin

morphology of anchoring fibers post blister induction was also examined in the blister skin samples. Density of tonofilaments and anchoring fibrils, density of collagen fibers, structural features of hemidesmosomes and desmosomes as well as other structural features of both dermis and epidermis were assessed as previously described (Jaunzems *et al*., 1997). Transition Electron Microscopy was also used to validate the sub-basal blistering in the EBA mouse model, below the intact hemidesmosome adhesion units at the level of ColVII anchoring fibrils following standard protocols as described previously (Kopecki *et al*., 2009). Electron micrographs were taken of two different blistered skin samples of EBA induced Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice at different magnifications with a random selection of sections and level of blistering and anchoring fibril assembly examined. Tissue skin architecture and structure was observed, particularly focusing on regions of dermal–epidermal junction and the structure of ColVII anchoring fibrils.

2.2.7 Immunohistochemistry and image analysis

Immunohistochemistry experiments were undertaken on all human samples, wounds collected from Flii$^{+/-}$, wild-type, Flii^{Tg+} and CD151$^{-/-}$ mice at day 0, 3, 7, 14 and 21 post-wounding and mouse blistered skin as specified in different experiments. Immunohistochemistry was also undertaken on all samples collected from Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice at day 3 post-wounding. The following method was used as the standard antigen retrieval procedure, where as Table 2.1 lists all the primary and secondary antibodies used in immunohistochemistry for different targets.

Sections were dewaxed by a series of xylene changes (30 min) and gradual ethanol washes (100% for 1 min, 70% for 1 min and 30% for 1 min), before being rinsed in 1x Phosphate Buffered Saline (PBS) and pre-treated with 250 ml Target Retrieval Solution (TRS) solution (2.8g Citric Acid, 3.76g Glycine, 0.372g EDTA, pH 5.9 in 1L 1xPBS). The sections were then microwaved for 2 min on "high" after which a "ballast" pot of water was added to help absorb some heat and pre-treatment continued for 2 x 5 min with regular "airing" to let the steam out and ensure that temperature reached 94°C but not 100°C. Sections were then cooled to 50°C on ice before they were washed in fresh 1x PBS and enzyme digestested with 0.0625g of Trypsin (Sigma-Aldrich, Sydney, Australia) dissolved in 1xPBS and pre-warmed to 37°C. Following the 3 min enzyme digestion at 37°C, sections were washed in 1xPBS and then incubated for 30 min in NHS blocking solution (3%NHS in 1xPBS). Slides were then washed in 1xPBS for 2 min and then incubated in primary antibody in a humid air tight box overnight at 4°C. Sections were then washed 3 x 2 min in 1xPBS and then incubated in biotinylated secondary antibody for 1 hr at room temperature in a humid box. Subsequently, slides were washed again 3 x 2 min in 1xPBS and Cy3 Streptavidin conjugate (Sigma-Aldrich, Sydney, Australia) for 45 min at room temperature in a dark humid box. Slides were then washed 3 x 2 min in 1xPBS to remove any unspecific binding of Cy3 and mounted in Dako Fluorescent Mounting Medium (DAKO Corporation, Botany, Australia). Slides were stored in the dark at -20°C. Integrated fluorescence intensity was determined using AnalySIS software package (Soft-Imaging System GmbH, Munster, Germany). Negative controls were included to demonstrate antibody staining specificity. Control samples undergo the exact same staining procedure outlined except omitting the primary or secondary antibody. All control sections had negligible immunofluorescence.

2.2.8 Cell Lines and Cell Culture

All cell cultures were incubated at 37ºC and 5% CO_2. Cells were serum starved in DMEM (JRH Biosciences, USA) for at least 6 hrs or as otherwise stated prior to the start of experimenting to synchronize the cells to the same phase of cell cycle. Cell cultures were performed on Human Keratinocytes (HaCaTs) obtained at passage 5 and Human Foreskin Fibroblasts (HFFs) obtained at passage 9 from liquid nitrogen stores at Women's and Children's Health Research Institute in 10% heat inactivated Fetal Calf Serum (FCS) (JRH Biosciences, USA) in DMEM containing following antibiotics unless otherwise stated: 100U/ml pencillin, 100µg/ml streptomycin and 100µg/ml Fungizone (Invitrogen, Mount Waverly, VIC, Australia). HaCaTs and HFFs were used for optimizing assay conditions and Flightless neutralising antibodies (FnAb) concentration, immunoprecipitation experiments and colocalization experiments between Flii and proteins of the cytoskeletal complex. All other assays were performed on primary fibroblasts and keratinocytes extracted from $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ mice as indicated. All primary mouse cells were used at passage 2. Primary fibroblasts were grown in 10% heat inactivated FCS in DMEM containing 100U/ml pencillin, 100µg/ml streptomycin and 100µg/ml Fungizone and primary mouse keratinocytes were grown in Progenitor Cell Targeted (PCT) Epidermal Keratinocyte-Defined Medium Cnt-07 (Millipore, Australia) containing 100U/ml pencillin, 100µg/ml streptomycin and 100µg/ml Fungizone.

2.2.9 Primary fibroblast isolation

Hair was shaved from the dorsal flank of euthanized 12-week-old female BALB/c $Flii^{+/-}$, wild-type, $Flii^{Tg/+}$ and $Flii^{Tg/Tg}$ mice and skin excised and collected in ice-cold

Ham's Nutrient Mixture F12 (SAFC Biosciences, Lenexa, KS), 1% Penicillin Streptomycin, 1% Fungizone (Sigma-Aldrich). Skin was cut into small 5 x 5mm pieces with sterile scissors in sterile conditions and placed dermal side down into 12-well plates. Explants were allowed to partially dry and fix to the bottom of the well before 2ml DMEM, 20% FCS, 1% Penicillin Streptomycin, 1% Fungizone was added and tissue cultured at 37°C, 5% CO_2. Cell media was replaced after 24 hr of initial explant culture and then every 2 days. At day 7 post explant culture once fibroblasts migrated out from the explant the skin explant was removed and cells cultured as normal in 10% FCS/DMEM, 1% Penicillin Streptomycin. Low passage cells (P 2–5) were used in all experiments.

2.2.10 Primary keratinocyte isolation

Different culture media (modified DMEM or PCT medium) and conditions coupled with different coating of the culture surfaces with extracellular matrix proteins (collagen, fibronectin, laminin) or poly-lysine were tested in order to establish a primary murine keratinocyte culture from cells obtained from dorsum skin of mice. Difficulties were faced with keeping dorsal skin keratinocytes adherent to the culture surface as well as maintaining the cell line after initial seeding. Attempts were made using dorsal skin keratinocytes from both very young and adult mice as well as a co-culture with 3T3 mouse fibroblasts. Lastly a well established protocol for isolation of primary murine keratinocytes from mice tails was used as described previously (Redvers and Kaur, 2004). Briefly, tails were collected from euthanized 12-week-old BALB/c $Flii^{+/-}$, wild-type, and $Flii^{Tg/Tg}$ mice or EBA induced mice and skin peeled from the underlying tail cartilage and incubated in Dispase II (8mg/ml^{-1}, 1.11U/mg, #14781300) (Roche Diagnostics, NSW, Australia) overnight at 4°C to dissolve the dermal–epidermal junction. Using a dissecting light

microscope, epidermis was peeled in the direction of the hair follicles and transferred to the pre-warmed 1x trypsin-EDTA (diluted from 10x stock, #T4174) (Sigma-Aldrich, Sydney, Australia) and placed on a magnetic stirring plate at 500r.p.m. for 4min. The reaction was then quenched by placing the solution on ice and adding an equal volume of trypsin inhibitor (Sigma-Aldrich, Sydney, Australia). The epidermal slurry was passed through 70mm and 40mm cell strainers (BD Biosciences) and cells centrifuged at 400g for 5min at 4°C. The cell pellet was resuspended and cells were cultured on 20μgml^{-1} Collagen IV (20μm) coated plates in PCT Epidermal Keratinocyte-Defined Medium (Cnt-07, # 0.7BM) (Cell-Tech, Millipore, NSW, Australia), 1% Penicillin Streptomycin, 1% Fungizone (Sigma-Aldrich) at 37°C, 5% CO_2. This method has been shown to result in the isolation of pure keratinocyte cell culture with no fibroblast cell contamination (Redvers and Kaur, 2004). Passages 2–5 were used in all experiments.

2.2.11 Fibroblast and keratinocyte hexosaminidase adhesion assay

Hexosaminidase adhesion assay was used to assess the adhesion properties of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ primary keratinocytes and fibroblasts extracted from non EBA and EBA induced mice following protocols previously published (May *et al*., 2001). Adhered cells produce the hexosaminidase enzyme which is involved in the hydrolysis of the terminal N-acetyl-D-hexosamine residues in the N-acetyl-β-D-hexosaminides. Addition of the 4-Nitrophenoyl-N-acetyl-β-D-glucosaminide substrate (PNAG substrate) to the adhered cells results in the hydrolysis of the PNAG by hexosaminidase enzyme resulting in a colour reaction. Hexosaminidase enzyme is directly proportional to the amount of adhered cells allowing such assay to analyse the cellular adhesion as previously described (May *et al*., 2001). Primary keratinocytes and fibroblasts were grown to 60% confluence in

PCT epidermal keratinocyte-defined medium Cnt-07 (Cell-Tech, Millipore, NSW, Australia), 1% Penicillin Streptomycin, 1% Fungizone (Sigma-Aldrich, Sydney, Australia) and 10% FCS/DMEM, 1% Penicillin Streptomycin, 1% Fungizone (Sigma-Aldrich, Sydney, Australia). Primary fibroblasts were serum-starved overnight in 0.5% FCS to synchronize the cells to the G1 phase of the cell cycle. Fibroblasts and keratinocytes were trypsinized and seeded at $5x10^4$ cells per ml onto pre-coated 96-well plates with 10µg/ml Fibronectin, 6µg/ml Laminin, or 10µg/ml Collagen I (Sigma-Aldrich, Sydney, Australia). Following the protocol described in May *et al.*, (2001) the wells were blocked with $0.5\mu g/ml^{-1}$ BSA prior to cell seeding. To account for non-specific adhesion, some wells were coated with BSA only and wells with no cells were set up as controls. Keratinocytes and fibroblasts were allowed to adhere to the extracellular matrix by incubation at 37°C for 6hr and 90min respectively, following protocols of cell adhesion and spreading published previously (Balaban *et al*., 2001, May *et al*., 2001). Following the incubation, the non-adhered cells were gently washed with PBS and 60µl of the enzyme substrate PNAG (Sigma-Aldrich, Sydney, Australia) was added to each well and incubated with the attached cells for an additional hour at 37°C. Enzyme activity was then blocked and colour reaction was developed by adding 90µl of 50mM glycine buffer. The absorbance was measured at 405nm on an ELISA plate reader. The percentage of cell adhesion to different extracellular matrix substrates was calculated by using the absorbance values and cell adhesion was expressed as a percentage of adhesion of wild-type cells.

Adhesion properties of primary wild-type fibroblasts extracted from EBA induced and IgG-treated non-induced control counterparts were also analysed in response to FnAb

addition. The protocol described above was followed with plates coated with 10µg/ml Collagen I (Sigma-Aldrich, Sydney, Australia). The negative control involved treating cells with control IgG antibody. FnAb or control IgG antibody was added to the cells both overnight post serum starvation and at the time of cell seeding. Cells were incubated at 37°C 5% CO_2 for 90 min, following which, the non-adhered cells were gently washed with PBS, and 60µl of the enzyme substrate PNAG (Sigma-Aldrich, Sydney, Australia) was added to each well and incubated with the attached cells for an additional hour in the 37°C. Enzyme activity was then blocked and colour reaction was developed by adding 90µl of 50mM glycine buffer as described above. The absorbance was measured at 405nm on an ELISA plate reader. The percentage of cell adhesion was calculated using the absorbance values and results were expressed as a percentage of adhesion of wild-type fibroblasts extracted from IgG-treated non-induced mice treated with isotype control.

2.2.12 Primary Keratinocyte Migration Scratch Assay

Primary keratinocytes isolated from $Flii^{+/-}$, wild-type, and $Flii^{Tg/Tg}$ mice skin were grown to confluence in PCT epidermal keratinocyte-defined medium Cnt-07 (Cell-Tech, Millipore, NSW, Australia), 1% Penicillin Streptomycin, 1% Fungizone (Sigma-Aldrich, Sydney, Australia) at 37°C, 5% CO_2 on glass coverslips and scratched with a P200 pipette tip, producing equal size wounds of 2 mm x 1 cm. The cells were photographed using the F-view Soft Imaging System Camera at 0, 3, 6, 9, 12, 24, 30, 36, and 48 hrs post-wounding and wound area measured using the Image Pro-Plus program (MediaCybernetics Inc.). The rate of keratinocyte migration and wound closure was quantified as a percentage of the initial wound area.

2.2.13 Proliferation assay

WST-1 Proliferation reagent (Roche Diagnostics, NSW, Australia) was used to assess the proliferation rate of HaCaTs keratinocytes and primary Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ fibroblasts. The WST-1 is a tetrazolium salt based reagent that converts the soluble formazan which directly correlates with cellular metabolic activity and proliferation rate. Cells were seeded in 96-well microtitre plates at a concentration of $1x10^5$ cells per well in 100µl of 10% or 0.5% FCS/DMEM with antibiotics. One lane of a 96-well microtitre plate was filled with media only and one lane with normal wild-type cells which did not receive any treatment or WST-1 reagent and acted as the blank controls. Cells were incubated at 37°C 5% CO_2 overnight, cell media was aspirated off and serum-free media was added to all cells to synchronize all cells to the gap phase of the cell cycle. After 6 hrs, 100µl of 10% or 0.5% FCS/DMEM with antibiotics was added to the cells and cells were incubated overnight in 37°C 5% CO_2 incubator for 24, 48 or 72 hrs depending on the time point of assessment. Following the incubation period, 10µl of WST-1 reagent was added to the cells and mixed thoroughly on an orbital shaker. The cells were then incubated at 37°C 5% CO_2 incubator for 30 min as per manufacturer's instructions where metabolic enzymes produced by cells react with WST-1 reagent to form a formazan dye product. The amount of the formazan dye produced therefore directly correlates with metabolic activity of the cells and their proliferation rate. Formazan dye was quantified where dual absorbance of 450nm and 600nm was measured on the Tecan microplate reader. Absorbance values were blanked with absorbance values of control without cells and then compared between different cells for the assessment of proliferation. Results are normalized to the untreated control sample and expressed as a percentage.

Proliferation properties of wild-type fibroblasts extracted from EBA induced or IgG-treated, non-induced control mice were analysed in response to FnAb addition. Protocol described above was followed with pre-coated 96-well plates with 10µg/ml Collagen I (Sigma-Aldrich, Sydney, Australia). The negative control involved treating cells with control IgG antibody. FnAb or control IgG antibody (50µg/ml) was added to the cells post serum starvation when all the cells synchronized to the gap phase of the cell cycle. FnAb or isotype control IgG was incubated with the cells for 24 hrs before addition of the 10µl of WST-1 regent. The cells were then incubated at 37°C 5% CO_2 incubator for 30 min as per manufacturer's instructions and absorbance measured as described previously. Percentage of cell proliferation was calculated using the absorbance values and results were expressed as a percentage of proliferation of wild-type fibroblasts extracted from IgG treated non induced mice treated with isotype control.

2.2.14 Cell Spreading

Spreading of primary keratinocytes and fibroblasts extracted from $Flii^{+/-}$, wild-type, $Flii^{Tg/+}$ and $Flii^{Tg/Tg}$ mice was analysed using immunocytochemistry for filamentous actin fibers. Keratinocytes and fibroblast spreading on coverslips coated with different extracellular matrix substrates including 10µg/ml Fibronectin, 6µg/ml Laminin or 10µg/ml Collagen I (Sigma-Aldrich, Sydney, Australia) was assessed by seeding keratinocytes in PCT epidermal keratinocyte-defined medium Cnt-07 (Cell-Tech, Millipore, NSW, Australia), 1% Penicillin Streptomycin, 1% Fungizone (Sigma-Aldrich, Sydney, Australia) and fibroblasts in 10% FCS/DMEM, 1% Penicillin Streptomycin, 1% Fungizone (Sigma-Aldrich, Sydney, Australia) at 37°C, 5% CO_2 with acetone-fixing cells at 6 hrs post-

seeding for keratinocytes and at 30, 60, 90 and 120 min post-seeding for fibroblasts, following previously published protocols (Balaban et al., 2001, May et al., 2001). Following blocking in 3%NHS, phalloidin-FITC (Sigma-Aldrich, Sydney, Australia) was added at concentration of 1:200 for 1 hr staining in the dark. Images were captured using Leica Spectral Confocal Microscope as previously described (Kopecki *et al*., 2009). Representative images of cell spreading on different extracellular matrix substrates and glass control was taken using AnalySIS software package (Soft Imaging System GmbH, Muster, Germany) illustrating filamentous actin fibers. All control sections had negligible immunofluorescence.

2.2.15 GTPase activation assay

Primary *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts were grown to 80% confluence and lysed following manufacturers protocols (Cell Biolabs, Inc). Cell lysates were analysed for levels of active small GTP binding proteins RhoA, Rac and Cdc42 using the RhoA/Rac1/Cdc42 Activation Assay (STA-405) (Cell Biolabs, Inc.). Active forms of RhoA, Rac1 and Cdc42 were detected using the Rhotekin RBD and p21-activated protein kinase PBD Agarose beads which selectively isolate and pull down the active forms of RhoA, Rac1and Cdc42 from cell lysates. The precipitated active GTP-RhoA or Rac1 or Cdc42 and total levels of RhoA, Rac1 and Cdc42 were detected by Western blot analysis using monoclonal anti-RhoA or Rac1 or Cdc42 antibody according to the manufacturer's directions (Cell Biolabs, Inc.).

2.2.16 Immunocytochemistry, image analysis and fluorescence co-localization

Immunocytochemistry was carried out as previously described (Bach *et al*., 2009, Kopecki *et al*., 2009). Briefly, primary fibroblasts isolated from Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice skin were grown to a density of 60-70% confluence on sterile glass coverslips pre-treated with poly-L-lysine (50μg/ml) (#P4707, Sigma-Aldrich, Sydney, Australia) and then coated with a mixture of extracellular matrix proteins: fibronectin (20μg/ml) and laminin (10μg/ml) or collagen (10μg/ml) (Sigma-Aldrich, Sydney, Australia) for 2 hr at 37ºC. For experiments examining incorporation of α-SMA into the stress fibers, cells were grown for 24hrs, serum starved for 12 hrs before incubation with the with TGF-β1(2ng/mL) for 48hrs and cell fixation. Cells were then washed in 1xPBS and fixed in 4% PFA (paraformaldehyde) for 10 min at room temperature, per using a permeabilising buffer (0.2% Triton-X-100 and 0.5% BSA in 1xPBS) for 5 min at room temperature prior to application of primary antibodies (1:200 or 1:500 as previously optimised) for 1 hr at room temperature. Depending on the primary antibody, further detection was achieved using fluorescent Alexa Fluor 488 donkey anti-goat IgG (2μg/ml) (Invitrogen, Oregon, USA) or species specific, biotinylated antibodies followed by signal enhancement with Streptavidin Alexa Fluor 555 conjugate (2μg/ml) (Invitrogen, Oregon, USA). Sections were washed three times in 1xPBS to remove any nonspecific binding of Cy3, mounted in Dako Fluorescence mounting medium (DAKO Corporation, Botany, Australia). For verification of staining, negative controls included replacing primary antibodies with normal rabbit IgG or normal mouse IgG. All control sections had negligible immunofluorescence.

Image analysis of immunocytochemistry involved determining the integrated fluorescence intensity using AnalySIS software package (Soft – Imaging System GmbH, Munster, Germany) and images captured using Leica Spectral Confocal Microscope as previously described (Kopecki *et al*., 2009). Briefly, images of fixed cells were captured using F-view Soft Imaging System digital camera or Olympus DP72 digital camera and a Leica Leitz DMRB fluorescent microscope with either a x40 (NA 0.70 PH2) or x100 (NA 1.30 PH3) oil objective using fluorescence filters as follows: EX 340-380nm / EM 425nm (DAPI), EX 450-490nm / EM 515nm (FITC, Alexa Fluor 488), EX 515-560nm / EM 590nm (Streptavidin-Cy3, Alexa Fluor 555). Images were pseudocoloured, overlayed and grey level adjustments prepared using AnalySIS software package (Soft – Imaging System GmbH, Munster, Germany). All image quantification and measurement procedures were carried out using AnalySIS software package (Soft – Imaging System GmbH, Munster, Germany). Focal adhesion images were prepared following background subtraction.

Phosphopaxillin images and analysis were performed as previously described (Bach *et al*. 2009). The relative phosphorylation of paxillin at individual focal adhesions was determined by drawing polygons around individual focal adhesions and obtaining pixel intensities which were used to obtain a ratio distribution graph of p-paxillin / (p-paxillin plus paxillin). Line scans of individual focal adhesions were performed on 5-pixel width lines using AnalySIS software package (Soft – Imaging System GmbH, Munster, Germany). Strict criteria were used to distinguish between focal complexes and focal adhesions based on size, location and composition. Focal complexes are smaller thin punctuate dot-like structures localized mainly at the cell periphery while focal adhesions

are much larger thicker arrow head-like structures localized both at leading cell edge of spreading cells and cell periphery of round non-spreading cells. Example of wild-type cells with focal complexes or focal adhesions clearly labelled is shown in Figure 2.6. These criteria were used to quantify the percentage of fibroblasts containing focal complexes by two blinded independent observers.

Fluorescence co-localization of Flii immunofluorescence was performed using human keratinocytes. Cells were seeded on glass coverslips, allowed to adhere and spread for 6 hrs prior to fixation in ice-cold acetone for 20 sec. Nonspecific binding was blocked by using 3%NHS for 30min prior to application of the primary antibody (a-Actinin, Paxillin, Vinculin, or Talin) (antibody information presented in Table 2.1) at a dilution of 1:200 overnight at 4°C prior to application of the rabbit Flii primary antibody (1:200) for 1hr at room temperature. Flii was detected through incubation of cells with fluorescent Alexa Fluor 488 goat anti-rabbit IgG (2μg/ml) (Invitrogen, Oregon, USA) whereas actin-associated proteins were further detected through species-specific, biotinylated secondary antibodies and signal enhancement with Streptavidin Alexa Fluor 555 conjugate (2μg/ml) (Invitrogen, Oregon, USA). Coverslips were mounted onto slides using Dako mounting medium. Fluorescence was examined using Leica Spectral confocal microscope (Adelaide Microscopy, Australia).

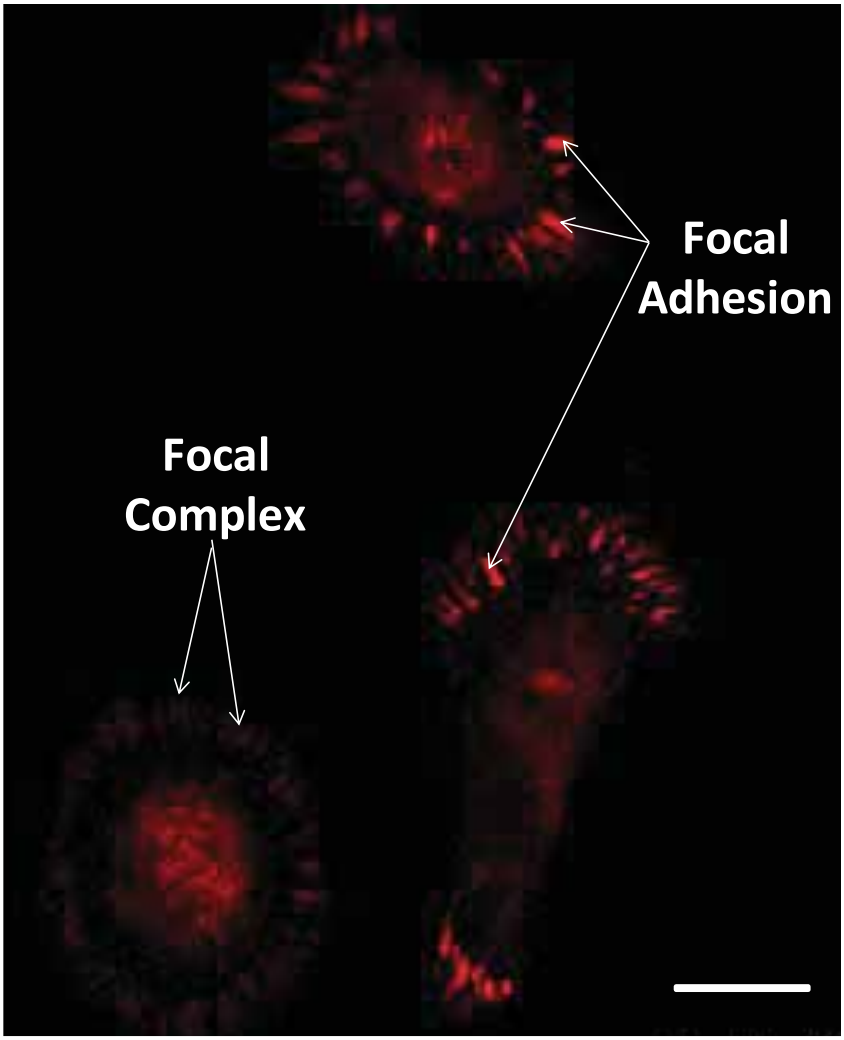

Figure 2.6: Paxillin staining of wild-type fibroblasts illustrating differences in size and structure of focal complexes and focal adhesions. Magnification x40. Scale bar = 10μm.

2.2.17 Immunoprecipitation

Immunoprecipitation was used to investigate whether Flii directly or indirectly associated with a number of different cytoskeletal proteins; integrin receptors and hemidesmosome components. Due to a large number of cells required for immunoprecipitation experiments, HaCaTs instead of precious primary mouse keratinocytes or fibroblasts were used. Briefly, cells were wounded as described in the scratch assay and allowed to start migrating for 2.5 hrs before being washed in PBS, lysed on ice with cell lysis buffer (50mM Tris, 1mM EDTA, 50mM NaCl, 0.1% Triton X-100) supplemented with Protease Inhibitor Complete Mini cocktail tablet (Roche Diagnostics, NSW, Australia). The cell lysates were precleared by centrifugation and incubated with 1mg/ml Integrin β1, Integrin β4, CD-151, anti-α-Actinin, anti-Paxillin, anti-Talin or anti-Vinculin antibodies and protein G-agarose beads (Invitrogen, Mount Waverly, Victoria, Australia) overnight at 4°C on a rocking platform. The immunoprecipitates were collected by centrifugation at 14,000g for 10 sec and supernatant discarded. Pellets were washed with lysis buffer and stored in 2xSDS sample loading buffer (25Mm Tris pH 6.8, 8% Glycerol, 1% SDS and 0.02% Bromphenol blue) prior to heating at 95°C for 3 min, loading onto a western gel and running samples using a standard western blotting protocol as previously described (Kopecki *et al*., 2009).

Western Blots were probed with Flii antibody or with gelsolin antibody as a negative control. Total cell lysate were also used as a positive control for antibody binding. Immunoprecipitation was confirmed by repeating experiments three times and also switching the antibodies used for preparation of immunoprecipitates and probing of the

western blots to confirm positive results. For immunoprecipitates where no band was shown by western blotting, validity of the antibody for use in immunoprecipitation experiments was confirmed by preparing immunoprecipitates and probing western blots with the same antibody to ensure that the antibody is efficient for use in immunoprecipitation studies.

2.2.18 Determination of the cellular F-actin content

The concentration of F-actin in primary Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ fibroblasts was determined following protocols previously described (Clark *et al*., 1998). Briefly, serum-starved cells (800,000 cells/ml) suspended in 1ml of DMEM were plated on 35mm wells pre-coated with fibronectin (20μg/ml) (Sigma-Aldrich, Sydney, Australia) for 2 hr at 37ºC and pre-blocked with 0.5% BSA 15 min, 60 min or 24 hrs at 37ºC prior to 1xPBS washes and fixation in 4% PFA in PBS for 10 min at room temperature. Cell were permeabilised with permeabilising buffer (0.5% Triton-X-100 in PBS) for 10 min at room temperature, washed in 1xPBS and stained with phalloidin-fluorescein isocyanate antibody (FITC) for 30 min at 37ºC. Cells were washed three times with 1xPBS and labeled phalloidin was released from cells by addition of 0.5ml of methanol to each well. The cells were then shaken for 2 hrs at room temperature in a dark humidified chamber and 0.3mls of the solution was analysed for F-actin content using a spectrophotometer (EX 485nm / EM 538nm).

2.2.19 Collagen Secretion Assay

Collagen secretion in wild-type and Flii$^{Tg/Tg}$ fibroblasts and keratinocytes extracted from EBA induced or control mice was quantified using the Sircol Soluble Collagen Assay kit according to the manufacturer's directions (Biocolor Ltd, Newtownabbey, U.K.). Briefly, fibroblasts and keratinocytes extracted from EBA-induced and IgG control animals were seeded as previously described and allowed to reach confluence over a period of five days. Spent media was collected from two T75 flasks of confluent cells and centrifuged at 15, 000g for 30min and supernatant collected to exclude any cell debris. A standard curve containing 0, 50, 125 and 250µg/ml of collagen and test samples was prepared. Sample supernatants (100µL) were mixed with Sircol dye (1mL) for 30 min at room temperature on the orbital rotator before being centrifuged for 10 min at 12 000g at room temperature to precipitate the collagen–dye complex. Supernatant was discarded after centrifugation and alkali reagent (1mL) was used to resuspend and completely dissolve the collagen–dye complex pellet. Sample volumes of 200µl were transferred to the 96 well microtitre plate and absorbance of the dye was measured at 540nm. Rat tail collagen I was used as a standard and all samples were measured in triplicate.

2.2.20 Floating Collagen Gel Contraction (FCGC) Assay

The floating collagen gel contraction assay was set up as previously described (Geary *et al.*, 2008). Briefly, primary fibroblasts isolated from Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ skin were trypsinized in 1 x trypsin/EDTA (#T4174) (Signal-Aldrich, Sydney, Australia) for 3min at 37ºC and quenched using DMEM followed by two washes in 0.25% BSA DMEM and cells resuspended in ice cold DMEM at 1x10^6 cells/ml. 3-D collagen gels

were prepared by mixing 8 parts of chilled collagen solution with 1 part of 10xDMEM (#D2429) (Sigma-Aldrich, Sydney, Australia) containing 10% FCS and the pH was adjusted to 7.4 using 0.1M NaOH. The fibroblasts were added to the collagen gel mixture at a concentration of $1x10^5$ cells/ml. This fibroblast-rich, collagen gel mixture (500µl/well) was added to a 48 well flat bottomed tray and allowed to set for 120 min at 37ºC 5% CO_2 after which the gel was dislodged by addition of the 1xDMEM media (1ml/well). Gels were allowed to float and images of gel contraction were captured using a Wild-M32 microscope (x 6.5 magnification) (Switzerland) with the ProgRec C5 camera (Hamburg, Germany). The degree of contraction at 24, 48 and 72 hrs post media addition was measured using Image Pro-Plus program (MediaCybernetics Inc, Maryland, USA) and results were presented as a percentage of gel contraction relative to the size of the well.

Floating collagen gel contraction assay was also used to assess the ability of fibroblasts extracted from EBA-induced mice to contract and remodel collagen with or without treatment with FnAb and TGFβ1. Primary fibroblasts from $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ EBA mice skin ($1x10^5$ cells/ml) were added to the collagen gel mixture which was added to 48 well flat bottomed plate (500µl/well) and allowed to set for 120min at 37ºC 5% CO_2. The gel was dislodged by addition of 1x DMEM media (1ml/well). Contraction and remodelling of collagen gel by $Flii^{Tg/Tg}$ fibroblasts extracted from EBA induced mice in response to FnAb treatment and by $Flii^{+/-}$ fibroblasts extracted from EBA induced mice in response to TGF-β1 (2ng/mL) was also examined were FnAb (50µg/ml) or TGF-β1 were added into both gel mixture and at time of media addition. The degree of contraction

at 72hr was determined from floating gel images using Image Pro-Plus software (MediaCybernetics Inc, MD, USA).

2.2.21 Protein Extraction and quantification

Protein was extracted from frozen unwounded, wounded, blistered and non-blistered skin obtained at sacrifice of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice. Representative tissue was selected for protein extraction, including unwounded tissue and wounded tissue at day 3, day 7 and day 14 post surgery, as well as non-blistered and blistered skin from EBA-induced and control animals. Protease Inhibitor Cocktail Tablets (Complete Mini – Roche Diagnostics, NSW, Australia) were dissolved in Lysis Buffer (1 tablet/10ml of buffer). Tissue was cut into smaller pieces and 750µl of lysis buffer containing the dissolved tablet was added to each sample. Samples were then sonicated at medium speed for 30 sec each and centrifuged twice for 10 min at 13, 000rpm in a 4°C centrifuge. After the centrifugation supernatant was collected and pellet was discarded. Samples were divided into 200µl aliquots and stored at -20°C. In order to measure the protein concentration in each sample a Pierce Bicinchoninic Acid (BCA) Quantification kit was used as per manufacturer's instructions. The absorbance was measured at a wavelength of 570nm in a Dynatech MR700 microplate reader using the BioLinx 2.0 computer software program.

2.2.22 Western Blotting

Protein amounts in each sample were equalized by dilution and heated at 95°C prior to electrophoresis. Protein fractions were run on a 10% SDS-PAGE (sodium dodecyl sulfate / polyacrylamide) gel consisting of a 10% separating solution (3.35ml 30% Acrylamide-Bis solution (37.5:1, 2.6% C, BioRad Laboratories, CA, USA), 1.25ml 3M Tris pH 8.9, 5.25ml distilled water, 125µl 10% SDS, 100µl 10% APS (Ammonium Per-sulfate)(Sigma-Aldrich Chemical Company, Sydney, Australia) and 6.25µl TEMED(N,N,N',N'-Tetramethylethylene-diamine)(Sigma-Aldrich Chemical Company, Sydney, Australia) and 4% stacking solution (0.5ml 30% Acrylamide, 0.276ml 0.5M Tris pH 6.8, 4.104ml distilled water, 50µl 10% SDS, 40µl 10% APS and 4µl TEMED). Protein fractions were run at 100V for 90 min in the electrophoresis tank. The gel was then transferred onto a 0.2µm pore nitrocellulose membrane (Advantec MFS Inc, CA, USA) by the process of Wet Transfer using the Bio-Rad Mini-ProteanII Transfer Apparatus (Bio-Rad Laboratories, NSW, Australia) and 1xWet Transfer Buffer - Tobins Buffer (3.3g Tris, 14.4g Glycine, 900ml MilliQ Water, 100ml Methanol) at 100V for 1 hr. Proteins were then stained in Ponceau Red Stain (Sigma-Aldrich, Sydney, Australia) for 10 min and then destained in MilliQ water and washed in PBS Tween (0.3% Tween/PBS) (50ml 20x PBS, 3ml Tween, 947ml MilliQ water). The gel was subjected to Coomassie Staining (Sigma-Aldrich, Sydney, Australia) for 30 min and destained in 40% Methanol, 10% Acetic Acid and 50% MilliQ water overnight to check for the transfer efficiency.

Membranes were then blocked in 5% skimmed milk in 0.3% Tween/PBS for 1hr and hybridized with appropriate primary antibody at 1µg/ml concentration, diluted in

blocking buffer prior to addition to the membrane overnight at 4°C. This was followed by 4 x 10 min membrane washes in 5% skim milk, 0.3% Tween/PBS blocking buffer and application of appropriate secondary antibody conjugated to horse radish peroxidise (HRP) at 1μg/ml and diluted in blocking buffer and applied to membranes in dark for 1 hr at room temperature. This was followed by further membrane washes of 4 x 10 min in 0.3% Tween/PBS and signal detection using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, USA) and signal capture using GeneSnap analysis software program (Syngene, Maryland, USA). Membranes were stripped by incubation in Stripping Buffer (5ml 20% SDS, 350μl 2-Mercaptoethanol (#M7522, Sigma-Aldrich, Sydney, Australia) , 6.25ml 0.5M Tris/HCl pH 6.7, 38.4ml MilliQ Water) for 30 min with gentle shaking every 10 min followed by re-probing of membranes with β-tubulin (Sigma-Aldrich, Sydney, Australia) as a loading/transfer control.

2.2.23 RNA extraction

RNA used in this study was extracted from blistered and non-blistered skin collected from the back and front paws of ColVII hypomorphic mice and wild-type control animals. Samples of mouse skin were cut to the same size and sonicated upon addition of Trizol reagent (Invitrogen, Mount Waverly, Victoria, Australia). Samples were centrifuged to pellet the genomic DNA for 10 min at 12,000 x g at 4°C in an Eppendorf 5415 R cold centrifuge. The supernatant was removed to a fresh tube and samples were incubated at room temperature for 5 min before addition of 200μl of chloroform to each tube and vigorous shaking for 15 sec by hand. Samples were incubated at room temperature for another 3 min before further centrifugation at 12,000 x g at 4°C for 15 min. The clear

aqueous phase was transferred to a fresh tube and 500µl of isopropanol was added to each tube to precipitate the RNA and mixed. Samples were incubated at room temperature for 15 min before further centrifugation at 12,000 x g for 10 min at 4°C. The supernatant was then transferred and the pellet washed with 1ml of 75% ethanol to clean the RNA of possible contaminants. Samples were centrifuged at 7, 500 x g for 5 min at 4°C, ethanol was removed, the pellet was dried and RNA was dissolved in 50µl DEPC-water and stored in -80°C.

2.2.24 DNase treatment and RNA Quantification

Samples obtained from RNA extraction were subjected to DNase treatment to remove any remaining contaminating genomic DNA (DNA-free DNase Treatment and Removal Kit Ambion, Austin, TX, USA) as per manufacturer's instructions. To 100µl, of RNA 0.1 volume of 10 x DNase I Buffer and 1µl of rDNase I were added, mixed well and incubated at 37°C for 30 min. Following the incubation, 0.1 volume of DNase Inactivation Reagent was added to each sample and mixed. Samples were left for 2 min at room temperature, occasionally mixed and then centrifuged at 10, 000 x g for 90 sec. The supernatant was removed into fresh tubes and RNA was quantified by dilution of the samples 1 in 20 with RNase-free water and 100µl duplicates were quantified against the RNase-free water applied as a blank using a Pharmacia Biotech GeneQuant RNA/DNA Calculator. Absorbance of the samples were measured at 260nm and 280nm wavelength which quantify the RNA absorbance as µg/ml concentration while the purity of the RNA was confirmed by considering the A_{260}/A_{280} ratio. A ratio in between 1.7-2.0 indicates that RNA is uncontaminated by protein and is of good quality.

2.2.25 Complementary Deoxyribonucleic Acid (cDNA) Synthesis

cDNA was synthesized from RNA samples by reverse transcription where 1µg of RNA, 4µl 2.5µM dNTPs (dATP, dCTP, dTTP and dGTP, 100mM each, Promega, Madison, WI, USA) and 2µl Oligo$(dt)_{12\text{-}18}$ Primer (25µg at 0.5µg/µl, Invitrogen, Mount Waverly, Victoria, Australia) were all combined in an Eppendorf tube, heated at 85°C for 3 min and returned on ice for a few minutes. Then, 2µl 10x Stratascript Buffer (Stratagene), 1µl RNasin (Promega) and 1µl Stratascript Reverse Transcriptase (Stratagene) were all added to the tube and heated for 60 min at 42°C followed by heating for 10 min at 92°C and cooling on ice for a few minutes before the cDNA was stored at -20°C. One sample was also chosen and set up for cDNA synthesis as above except Reverse Transcriptase was not added and this sample was then used as a Polymerase Chain Reaction (PCR) negative control.

2.2.26 Real-Time quantitative-Polymerase Chain Reaction (RTq-PCR)

Absolute qPCR SYBR Mix (Abgene Limited, UK) was used in PCR reactions where sample cDNA was set up in 25µl PCR reaction tubes with a final reaction of 20µl containing 15.5µl Absolute qPCR SYBR Mix, 0.5µl cDNA template and 4µl of Primers (forward and reverse primers mixed together, 5.625µM each). The following primers were used: Flii forward: 5-CCT CCT ACA GCT AGC AGG TTA TCA AC-3, reverse: 5-GCA TGT GCT GGA TAT ATA CCT GGC AG-3; Cyclophilin A forward: 5-GGT TGG ATG GCA ATG TG-3, reverse: 5-TGC TGG TCT TGC CAT TCC TG-3 (Gene Works, Adelaide, South Australia). Polymerase chain reactions were run in a Rotor-Gene 2000 Real-Time Cycler (Corbett Research, distributed by AdelLab Scientific, Adelaide) by a

following cycle – temperature profile. Denature cycle at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 25 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec during which the product signal (Cycling A (Sybr)) was acquired using RotorGene software v5.0.47. A further extension at 72°C for 5 min was followed by a melt cycle from 72-99°C, holding 30 sec on first temperature point (72°C) and 5 sec on all other temperature points, acquiring melting point of product again on Melt A (Sybr). A final step of 60°C for 1 min occurred to re-anneal the PCR products. Obtained values were normalized to Cyclophilin A and changes in *Flii* gene expression graphed as a fold change relative to the expression of Flii in non-blistered back skin of wild-type control animals.

2.2.27 Statistical Analysis

Statistical differences were determined using the InStat program v3.05 (GraphPad Software, USA) using the unpaired Student's t-test or ANOVA. For data not following a normal distribution, the Mann-Whitney U test was performed. A P value of less than 0.05 was considered significant.

Chapter 3

EFFECT OF FLIGHTLESS ON SKIN ARCHITECTURE

Parts of this chapter have been published in following journal:

Kopecki Z, Arkell R, Powell BC, Cowin AJ. Flightless I regulates hemidesmosome formation and integrin-mediated cellular adhesion and migration during wound repair. J *Invest Dermatol.* 2009 Aug;129(8):2031-45. **IF 5.5 RIF 1/39.**

3.1 Introduction

Wound healing is a complicated and highly regulated series of cellular events that results in the restoration of the skin. The end result of wound healing is highly dependent on rate of wound reepithelialisation, which in turn is reliant on cellular ability to dissociate anchoring attachments from the basement membrane and cellular ability to interact with dermal fibroblasts and the extracellular matrix proteins during cellular migration across the wound bed (El Ghalbzouri and Ponec, 2004). Re-establishment of the hemidesmosome anchoring adhesion sites following wound reepithelialisation is also a vital process linking the actin cytosketon to the extracellular matrix, allowing cells to respond to changes in the environment and also enabling adhesion of skin layers and strong cellular attachment to the basement membrane (Tsuruta *et al*., 2003).

Processes underpinning cellular migration, spreading and adhesion are all dependent on the actin cytoskeleton and interactions of different proteins acting as both structural, signaling, adaptor and regulating components of different processes during wound healing. Mutations in these proteins may lead to fragile skin, disadhesion of skin layers, impaired cellular migration and wound healing and skin blistering, as observed with patients suffering from EB, while over-expression of certain proteins like collagen type I may lead to excessive scarring and skin hypertrophy (Gurtner *et al*., 2008). Consequently, proteins which have the ability to modulate the actin cytoskeleton and cellular responses of migration and adhesion during wound healing are of pivotal importance to the development of novel therapies aimed at improved wound healing of patients.

We have previously documented that Flii, a highly conserved member of the gelsolin family of actin-remodelling proteins is an important negative regulator of wound

healing (Adams *et al*., 2009, Adams *et al*., 2008, Cowin *et al*., 2007, Kopecki and Cowin, 2008). Our studies revealed that Flii-deficient mice have improved wound healing, with increased epithelial migration and enhanced wound contraction. In contrast, Flii over-expressing mice have impaired wound healing with larger, less contracted wounds, reduced cell proliferation, and delayed epithelial migration. Importantly, we have shown that application of neutralizing antibodies raised against the leucine rich domain of Flii improves human fibroblast and keratinocyte cell migration and proliferation and improves the appearance and reduces the size of murine wounds *in vivo*, illustrating the potential for development of a new therapy by which wound healing could be improved.

Here, we investigate the differential effect of Flii gene expression on skin structure and strength. For the first time we extract primary murine keratinocytes and fibroblasts and examine the effect of differential Flii expression on cellular migration, spreading and adhesion on different extracellular matrix substrates. We further demonstrate the potential for use of Flii neutralizing antibodies in development of novel therapy for improved wound healing by examining the effect of these antibodies on proliferation of primary murine keratinocytes. Results presented in this chapter illustrate a novel role for the Flii protein, where its over-expression impairs the formation of stable adhesion sites and decreases skin tensile strength. Moreover, these results show an important role of Flii protein in both keratinocyte and fibroblast adhesion, spreading and migration on different extracellular matrix substrates which may help us to understand the differences in wound healing observed between $Flii^{+/-}$, wild-type and $Flii^{Tg/+}$ mice.

3.2 Results

3.2.1 Flightless over-expressing mice have thinner and more fragile skin

The effect of Flii gene expression on skin architecture was first investigated using light microscopy of H&E-stained unwounded dorsal skin from 12 week old female heterozygous ($Flii^{+/-}$), wild-type and Flii over-expressing mice carrying one ($Flii^{Tg/+}$) or two ($Flii^{Tg/Tg}$) complete human genes as described previously in (Campbell *et al*., 2002, Cowin *et al*., 2007). All skin sections showed normal skin architecture including a stratified epidermis; dermis composed of loosely arranged connective tissue, blood vessels and hair follicles, subcutaneous layer of adipose tissue in the deep dermis and an underlying panniculus carnosus muscle layer (Fig 3.1). Further examination of the unwounded skin sections illustrated intact basement membrane, regular arrangement of glands, blood vessels and hair follicles and no difference in the thickness of the epidermis layer between different skin sections (Fig 3.1A-D). No significant difference was observed in epidermal thickness of unwounded skin between $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ mice (Fig3.1A-D). However, measuring the distance between the basement membrane and the panniculus carnosus, which includes both the papillary and sub-dermal regions, showed that Flii over-expressing mice had significantly thinner skin than both wild-type and $Flii^{+/-}$ mice (Fig 3.1A-E). To determine if $Flii^{Tg/Tg}$ skin was more fragile, strips of skin were tested for their breaking strength using a tensiometer. Unwounded skin of $Flii^{Tg/Tg}$ mice was significantly weaker, and had lower tensile strength compared to unwounded skin of $Flii^{+/-}$, wild-type and $Flii^{Tg/+}$ mice (Fig 3.1F). To further characterise the effect of differential Flii gene expression on the skin, ultra-structural examination of the unwounded skin structures was performed using transmission electron microscopy.

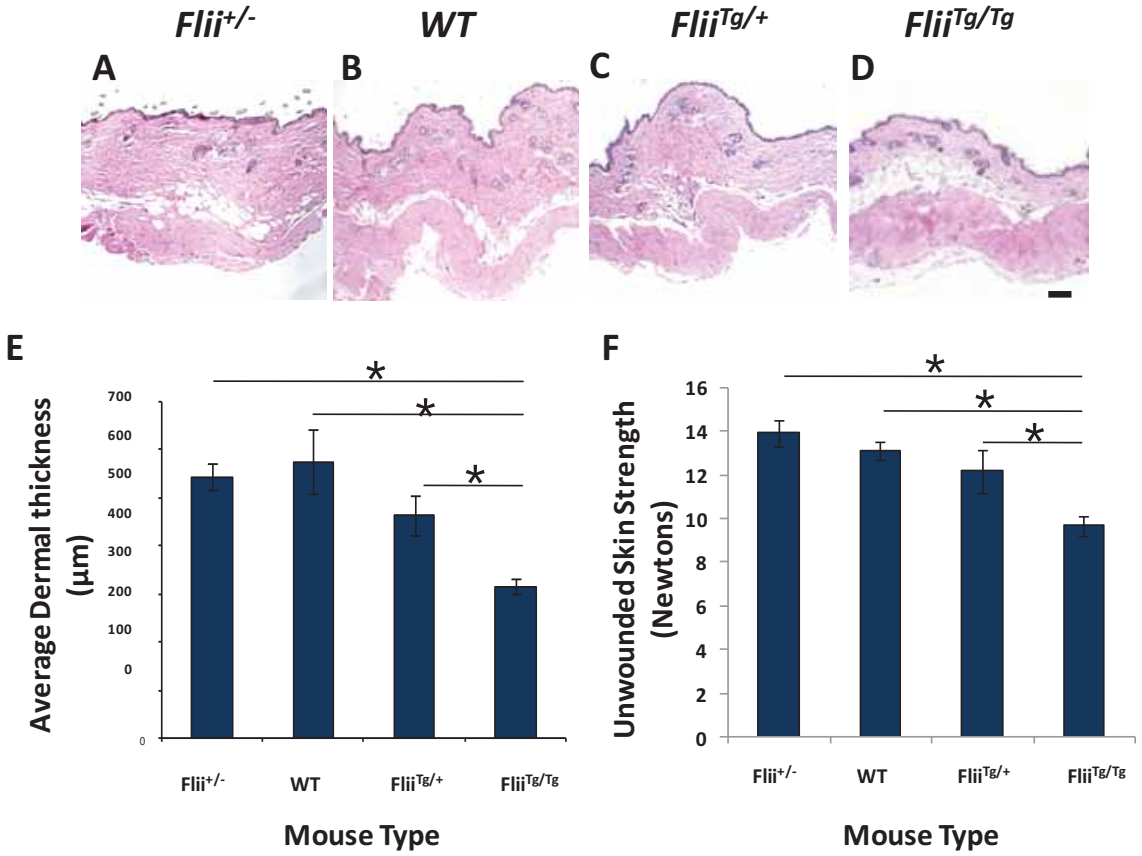

Figure 3.1: Flii over-expressing mice have significantly thinner and more fragile skin structure. A-D Representative images of haematoxylin and eosin stained unwounded skin of 12 weeks old female Flii$^{+/-}$, WT, Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice illustrating significantly decreased dermal thickness in Flii$^{Tg/Tg}$ unwounded skin compared to other mice groups. Magnification x 4. Scale bar = 500µm and refers to all images. n=9. **(E)** Graph showing the effect of Flii gene expression on dermal thickness. Flii$^{Tg/Tg}$ mice have significantly thinner dermis compared to Flii$^{+/-}$, WT and Flii$^{Tg/+}$ mice. n=9. **F** Tensiometer analysis of Flii$^{+/-}$, WT, Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice skin strength. Flii$^{Tg/Tg}$ mice skin has significantly weaker tensile strength compared to Flii$^{+/-}$, WT and Flii$^{Tg/+}$ counterparts. n=12. Mean +/- SEM. *P<0.05.

3.2.2 Over-expression of Flightless impairs the formation of hemidesmosome adhesion sites

Transmission electron microscopy revealed that the skin from Flii$^{+/-}$ mice had significantly higher numbers of hemidesmosomes at the dermal-epidermal junction compared to wild-type counterparts (Fig 3.2A-D). Examining hemidesmosome adhesion sites, linking the basal keratinocytes to the basement membrane showed that Flii over-expressing (Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$) mice had significantly decreased numbers of hemidesmosomes (21% and 28% decrease, respectively) compared to wild-type mice. In contrast, Flii$^{+/-}$ mice had increased numbers of hemidesmosomes compared to wild-type mice (40% increase) (Fig 3.2A-D and Fig 3.3). Examining the numbers of desmosome adhesion sites in-between the basal keratinocytes in the epidermis in Flii$^{+/-}$, wild-type, Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice skin showed no apparent difference between all four mice groups, suggesting a specific role for Flii in formation of hemidesmosome adhesion structures (Fig 3.2E-H).

Higher magnification (x 64, 000) revealed that Flii over-expression not only resulted in a decreased number of hemidesmosomes but also affected their structural formation. No significant difference in hemidesmosome structure was observed between Flii$^{+/-}$ and wild-type mice (Fig 3.4A-B), however Flii over-expression resulted in hemidesmosomes with a reduced thickness and length of the inner plaque, a significantly reduced number of hemidesmosomes with sub-basal dense plates (Fig 3.4A–H and Fig 3.5A), and significantly shorter hemidesmosome adhesion sites (Fig 3.4G-H and Fig 3.5B). Examination of the arrangement of collagen fibers in the dermis of Flii$^{+/-}$, wild-type, Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice showed no apparent differences between all four groups (data not shown). However, although Flii$^{+/-}$ and wild-type mice showed a number of intracellular networks of dense cytokeratin tonofilaments in the epidermis and anchoring fibrils in the

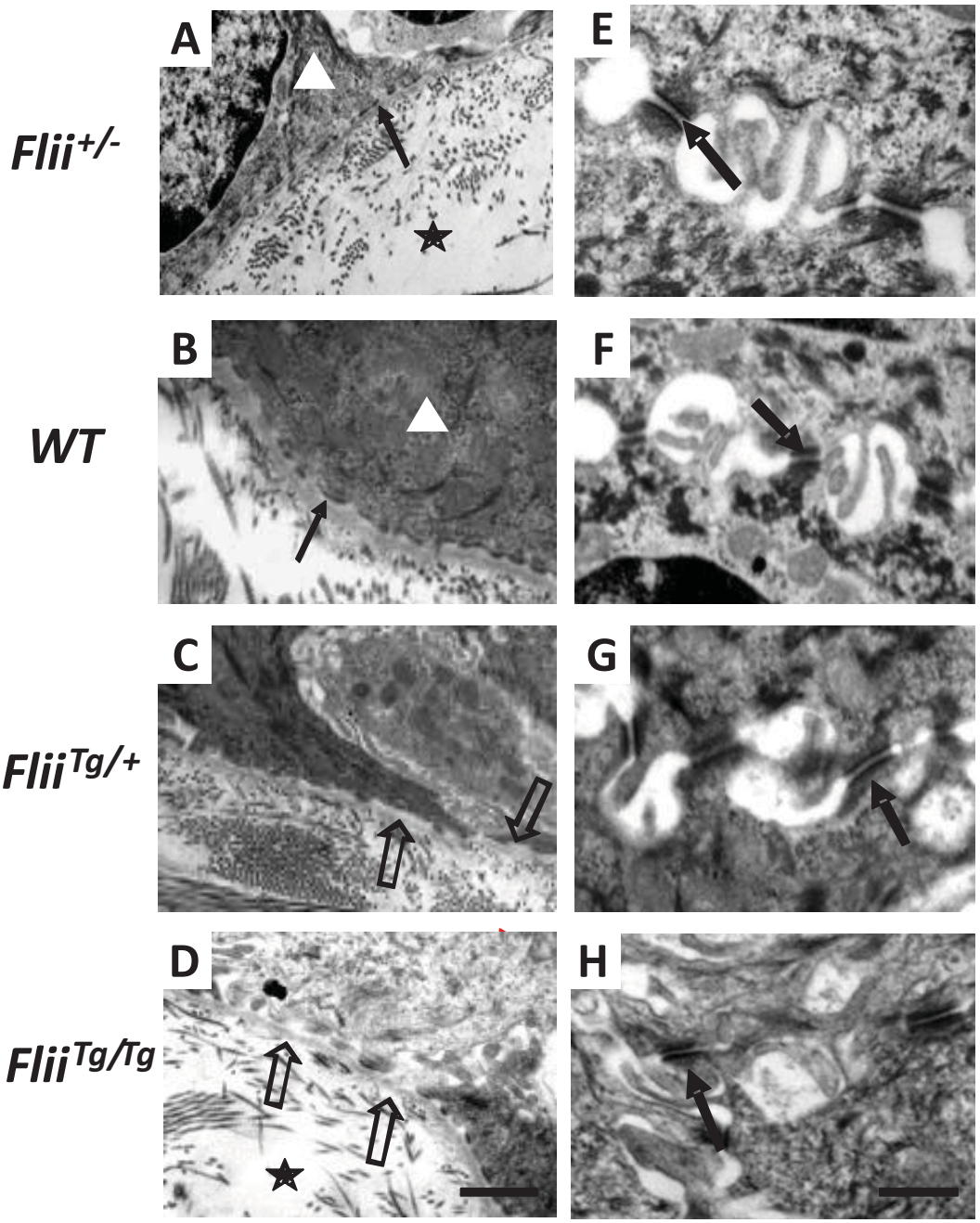

Figure 3.2: Flightless over-expressing mice have reduced hemidesmosome formation. A–D Transmission electron microscopy reveals that unwounded skin from Flii$^{+/-}$ mice has significantly higher numbers of hemidesmosomes at the dermal–epidermal junction compared to WT, Flii$^{Tg/+}$, and Flii$^{Tg/Tg}$ mice. Hemidesmosomes = solid black arrow. Region without hemidesmosomes = clear arrow. Extracellular matrix = star. Basal keratinocyte = triangle. Magnification x 7, 900. Scale bar in D refers to A–D and = 2μm. n = 2 mice. **E-H** Examination of desmosomes adhesion sites between basal keratinocytes shows no difference in structure or number between Flii$^{+/-}$, WT, Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice skin. Magnification x 25, 000. Scale bar in H refers to E-H and = 6μm. n = 2 mice.

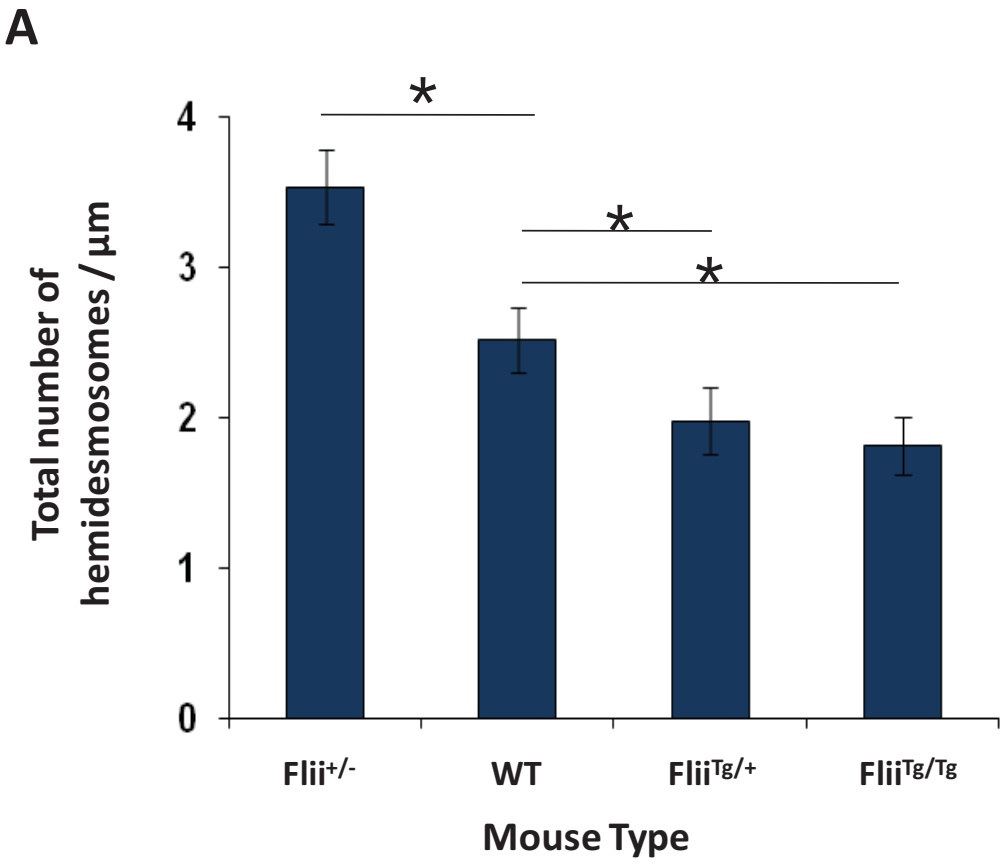

Figure 3.3: Decreasing Flightless expression results in increased hemidesmosome formation. Graph showing number of hemidesmosomes at the basal keratinocyte to basement membrane location in unwounded skin with different levels of Flii gene expression. $Flii^{+/-}$ have a significantly higher number of hemidesmosomes, whereas both $Flii^{Tg/+}$ and $Flii^{Tg/Tg}$ have a significantly decreased number of hemidesmosomes compared to WT counterparts. n=2. 150 hemidesmosomes were analysed. Mean +/- SEM. *P<0.05.

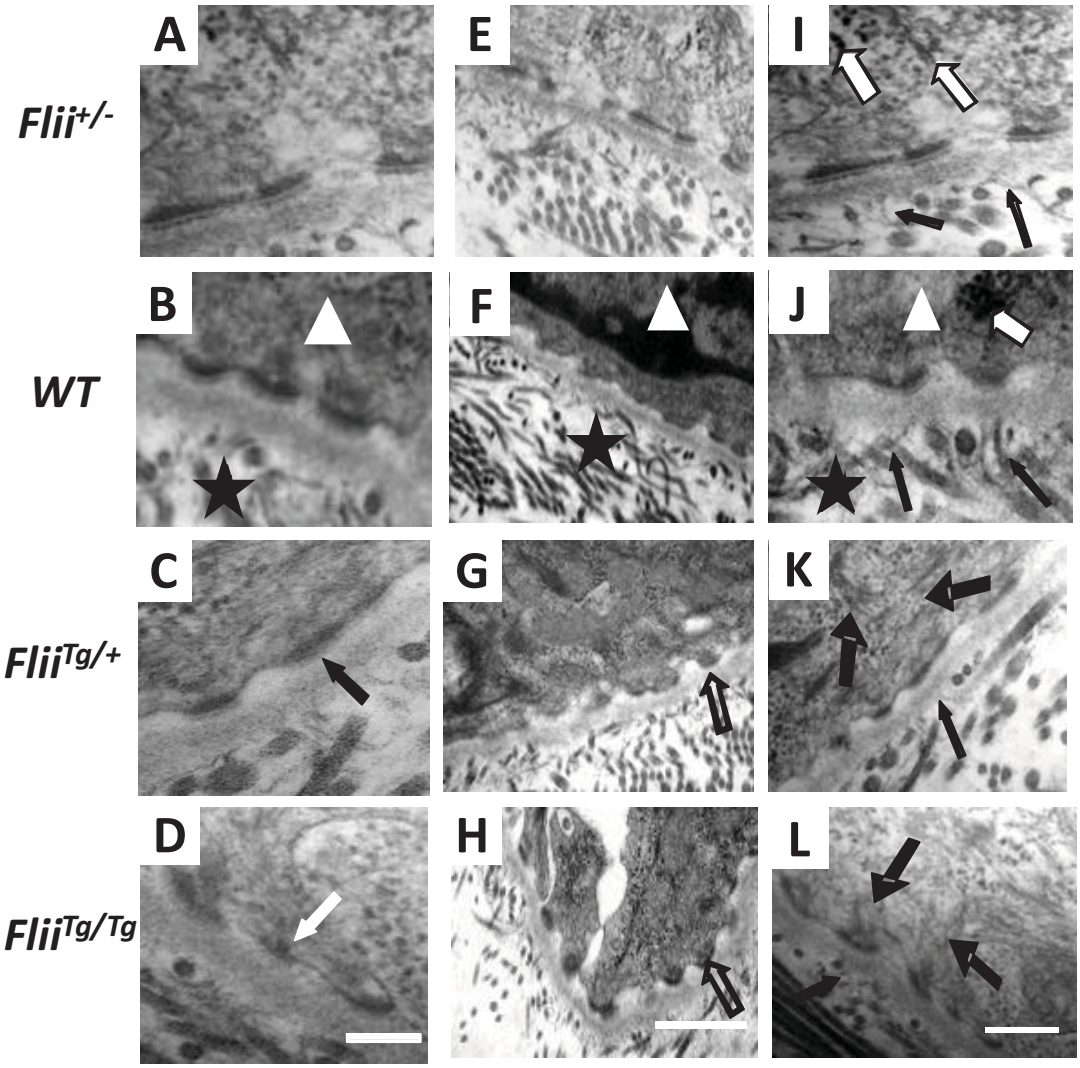

Figure 3.4: Flightless over-expressing mice have impaired hemidesmosome structure. **A–D** Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ show reduced inner plaque (white arrow) and significantly decreased number of hemidesmosomes with sub-basal dense plate (black arrow) at the basal keratinocyte to basement membrane location. Magnification x 64,000. Scale bar = 200 nm. **E-H** Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice have shorter hemidesmosome adhesion sites (clear arrow). Magnification x 25,000. Scale bar = 500nm. **I-L** Flii$^{+/-}$ and WT mice show intracellular networks of dense tonofibrils (white arrow) and anchoring fibrils (thin black arrow) whereas Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice have diffuse tonofilaments (thick black arrow) and decreased network of anchoring fibrils (thin black arrow). Black star = extracellular matrix. White triangle = basal keratinocyte. Magnification x 64,000. Scale bar = 200 nm. n = 2 mice.

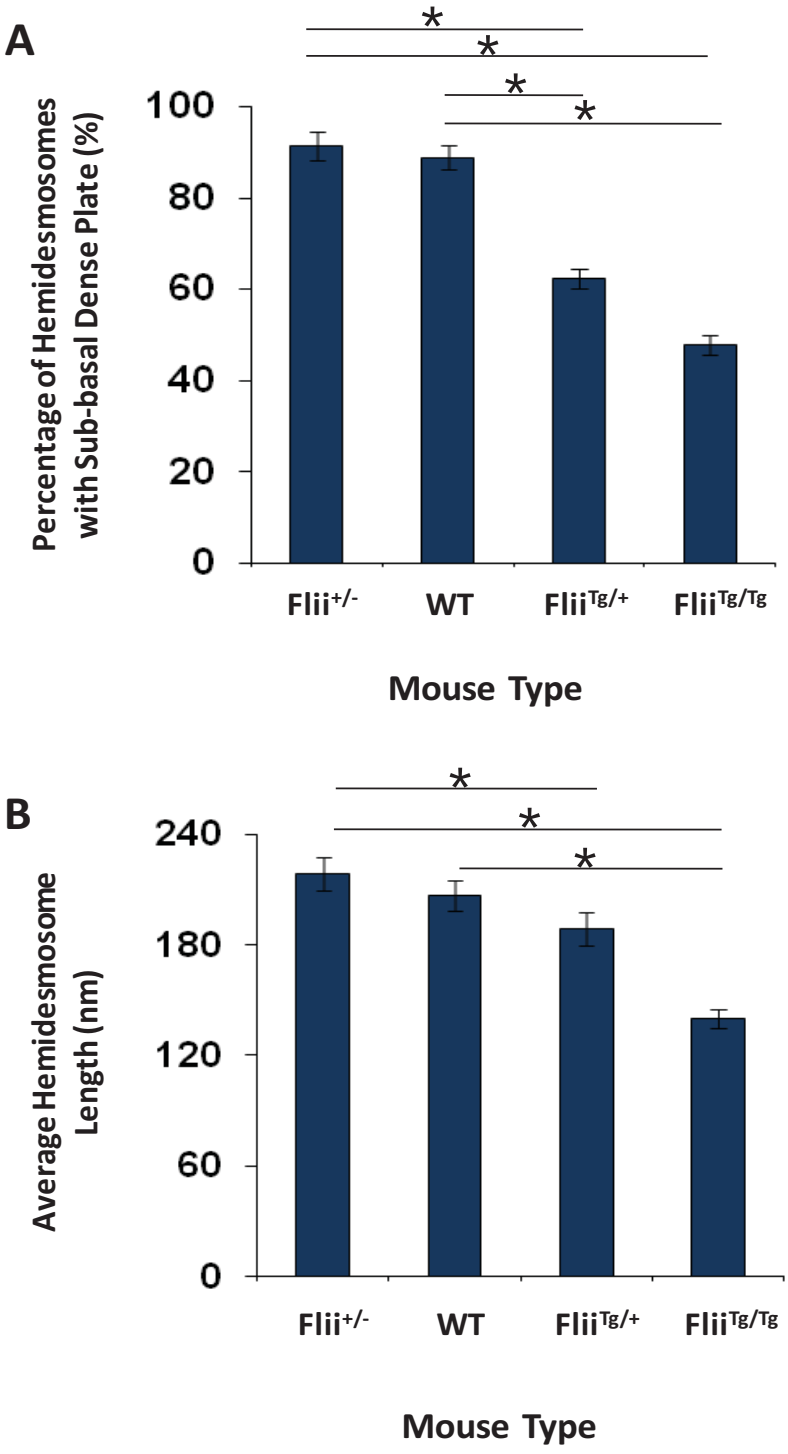

Figure 3.5: Flightless over-expressing mice have shorter hemidesmosome length.
A Graphical analysis of percentage of hemidesmosomes with sub-basal dense plate at the basal keratinocyte to basement membrane location. Both Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice have a significantly reduced number of hemidesmosomes with sub-basal plate when compared to WT and Flii$^{+/-}$ mice. **B** Graphical analysis of hemidesmosome length. Flii$^{Tg/+}$ mice have significantly shorter hemidesmosome length compared to Flii$^{+/-}$ mice, whereas Flii$^{Tg/Tg}$ mice have significantly shorter hemidesmosome length compared to Flii$^{+/-}$, WT, and Flii$^{Tg/+}$ mice. 150 hemidesmosomes were measured per group. Mean +/- SEM. *P<0.05.

dermis, Flii over-expressing mice (both Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$) had sparse tonofilaments and a decreased network of anchoring fibrils (Fig 3.4I–L). Electron microscopy further revealed intact basal lamina separating the epidermis from the dermal connective tissue in all groups and no significant difference in thickness of lamina lucida and lamina densa of the basement membrane (data not shown).

3.2.3Flightless regulates keratinocyte and fibroblast cell adhesion and spreading

The impaired hemidesmosome structure observed in response to different levels of Flii expression suggested that Flii might affect cellular adhesion and spreading. Keratinocytes and fibroblasts derived from Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ skin were seeded onto three extracellular matrix substrates, laminin, collagen I and fibronectin, and the effect on adhesion was determined (Fig 3.6A-B). Dose-dependent effects of Flii were observed on all three substrates investigated. Flii$^{+/-}$ keratinocytes showed significantly increased adhesion on both laminin and collagen I compared with wild-type cells, whereas both Flii$^{+/-}$ and wild-type keratinocytes had significantly increased adhesion compared to Flii$^{Tg/Tg}$ keratinocytes on all three substrates (Fig 3.6A). Decreased Flii expression in keratinocytes derived from Flii$^{+/-}$ mice skin showed improved keratinocyte adhesion on laminin and collagen extracellular matrix substrates, suggesting that integrin cell receptors for these extracellular matrix substrates might be important in better understanding the role of Flii in mediating cell adhesion.

A similar pattern of cellular adhesion to laminin, collagen and fibronectin extracellular matrix substrates was observed with primary fibroblasts extracted from Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice skin. Flii$^{+/-}$ fibroblasts have significantly improved adhesion

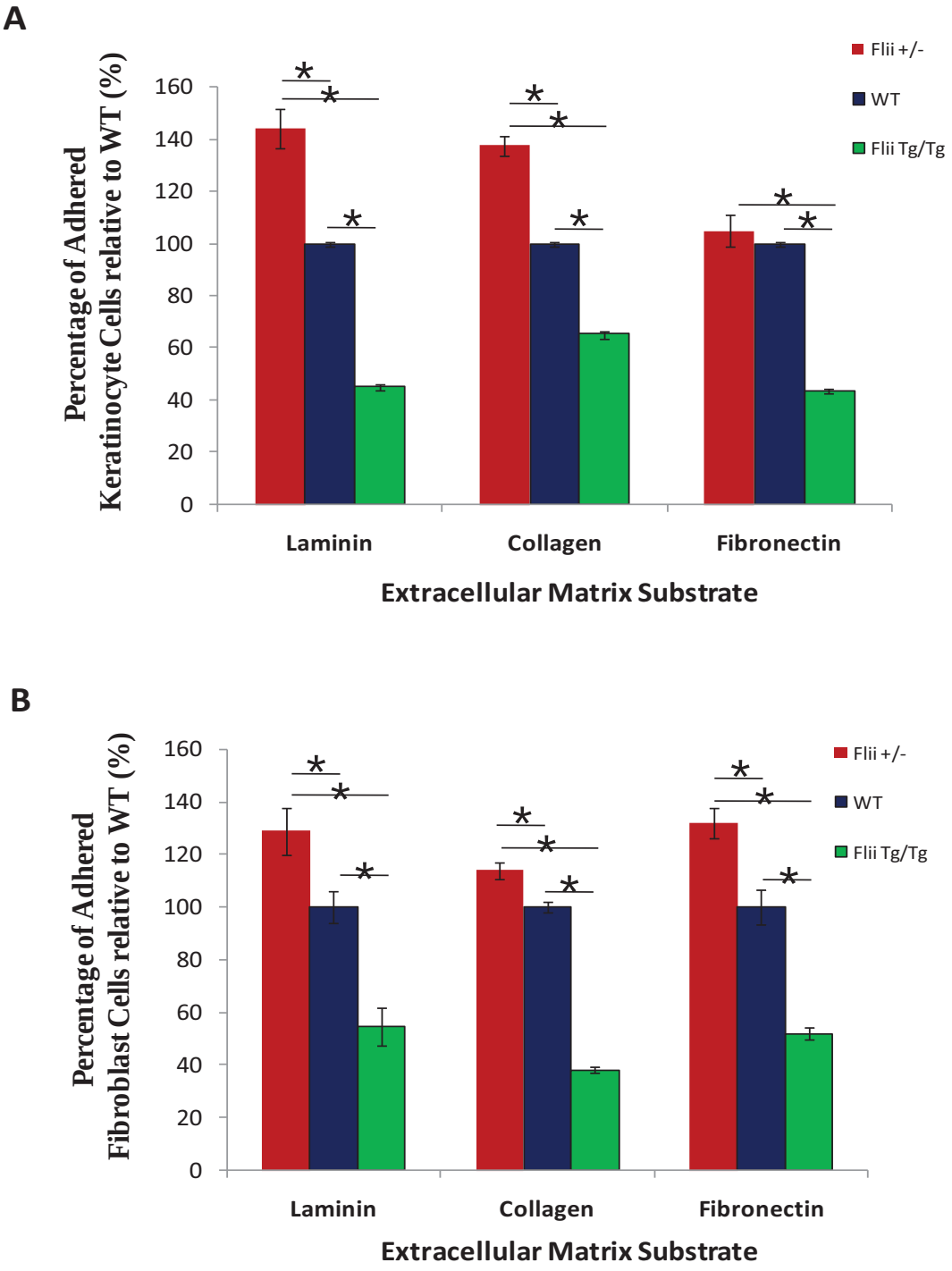

Figure 3.6: Flightless regulates keratinocyte and fibroblast cell adhesion. Adhesion properties of primary murine Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ keratinocytes and fibroblasts were examined using a hexosaminidase adhesion assay. **A** Graph showing keratinocyte adhesion on laminin, collagen I and fibronectin. Flii$^{Tg/Tg}$ fibroblasts have significantly impaired adhesion on all three different substrates compared to both WT and Flii$^{+/-}$ keratinocytes. **B** Graphical analysis of fibroblast cell adhesion on different substrates. Flii$^{+/-}$ fibroblasts have significantly improved cell adhesion compared to WT and Flii$^{Tg/Tg}$ counterparts on all substrates. n=6. Mean +/- SEM. *P<0.05.

compared to both wild-type and Flii$^{Tg/Tg}$ fibroblasts while over-expression of Flii in Flii$^{Tg/Tg}$ fibroblasts results in a significantly impaired fibroblast adhesion on all three substrates (Fig 3.6B).

We further examined the effect of Flii on cellular spreading on different extracellular matrix substrates and a glass-only control by staining keratinocytes (Fig 3.7A–L) and fibroblasts (Fig 3.7M–X) with fluorescein isothiocyanate-phalloidin and examining the filamentous actin cytoskeleton organization. Addition of the substrate improved cellular spreading compared to the glass-only control in all groups of both cell types. While Flii$^{+/-}$cells appeared larger than wild-type keratinocytes and fibroblasts no significant difference was observed between spreading on different extracellular matrix substrates, both illustrating defined actin rings, developed filamentous actin networks and lamellipodial protrusions, indicative of effective cellular spreading. On examining the spreading of Flii$^{Tg/Tg}$ fibroblasts and keratinocytes, we observed an impaired spreading phenotype with decreased spreading, more round cell morphology and less filopodia-like processes compared to both Flii$^{+/-}$ and wild-type counterparts (Fig 3.7A–X).

3.2.4 Keratinocyte migration and cellular outgrowth are regulated by Flightless

Previous results from our laboratory have shown that decreasing Flii expression improves migration of both primary murine fibroblasts and a human keratinocyte cell line, while Flii over-expression results in impaired cellular migration (Cowin *et al*., 2007). Here we extracted primary murine keratinocytes and examined the effect of differential Flii gene expression on keratinocyte migration and cellular outgrowth on different extracellular matrix substrates. Confluent monolayers of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ keratinocytes

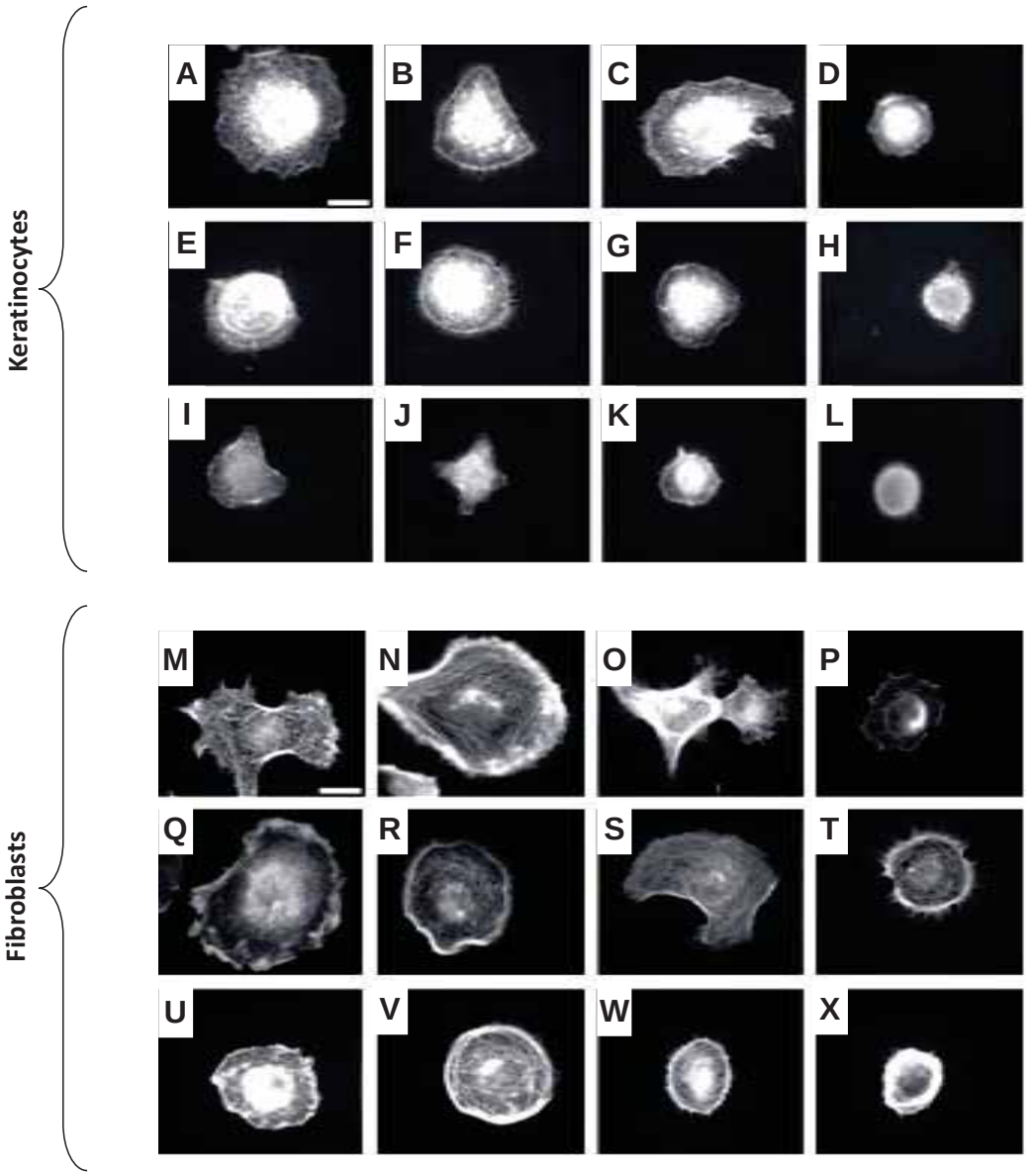

Figure 3.7: Flightless affects cellular spreading on different extracellular matrix substrates. Keratinocyte **A-L** and fibroblast **M-X** spreading on laminin, collagen I and fibronectin extracellular matrix substrate and glass control was examined by staining with phalloidin-FITC. Representative images of cell spreading on different substrates and the associated cell actin network during cell spreading are shown in **A-X**. Magnification x 100. Scale bar in **A** and **M** = 10μm and refers to all images.

grown on glass coverslips were scratch-wounded and residual wound area examined over a 48 hours period (Fig 3.8). In agreement with our previous results showing that decreased Flii expression improves cellular migration, here we also found that Flii$^{+/-}$ keratinocytes had a significantly improved cellular migration post *in vitro* wounding and a smaller wound area compared to both wild-type and Flii$^{Tg/Tg}$ keratinocytes from 6hrs to 48hrs post wounding (Fig 3.8A-D). At 48hrs post wounding, scratched wounds of Flii$^{+/-}$ keratinocytes have closed, and while wounds of wild-type keratinocytes still remained slightly open, the wounds of Flii$^{Tg/Tg}$ keratinocytes were still open for over 50% of the initial wound area, suggesting that Flii over-expression impairs wound healing of primary murine keratinocytes (Fig 3.8A-D).

To further investigate the effect of Flii gene expression on cell migration on laminin, collagen I, and fibronectin extracellular matrix substrates, punch biopsy skin explants from Flii$^{+/-}$, wild-type, Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice were used in vitro to analyse cell migration. Cellular outgrowth from the explants was measured as average distance of cellular migration over a period of seven days. Results were similar on all three different extracellular matrix substrates investigated and while differences in cellular migration between Flii$^{+/-}$, wild-type and Flii over-expressing cells were subtle during the first two days of the experiment the effect of modulating Flii expression on cellular outgrowth was clearly evident during the remainder of the experiment. Representative images of cellular outgrowth from skin explants on collagen I at day 6 post culture are illustrated in Figure 3.9A. An extensive halo of cells was apparent around the explants of Flii$^{+/-}$ and wild-type skin after 2 days culture whereas Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ explants showed a reduced patchy cellular outgrowth. Although initially a mixed population of skin cells, light microscopy revealed that fibroblasts rapidly became the most predominant cell type growing out of all

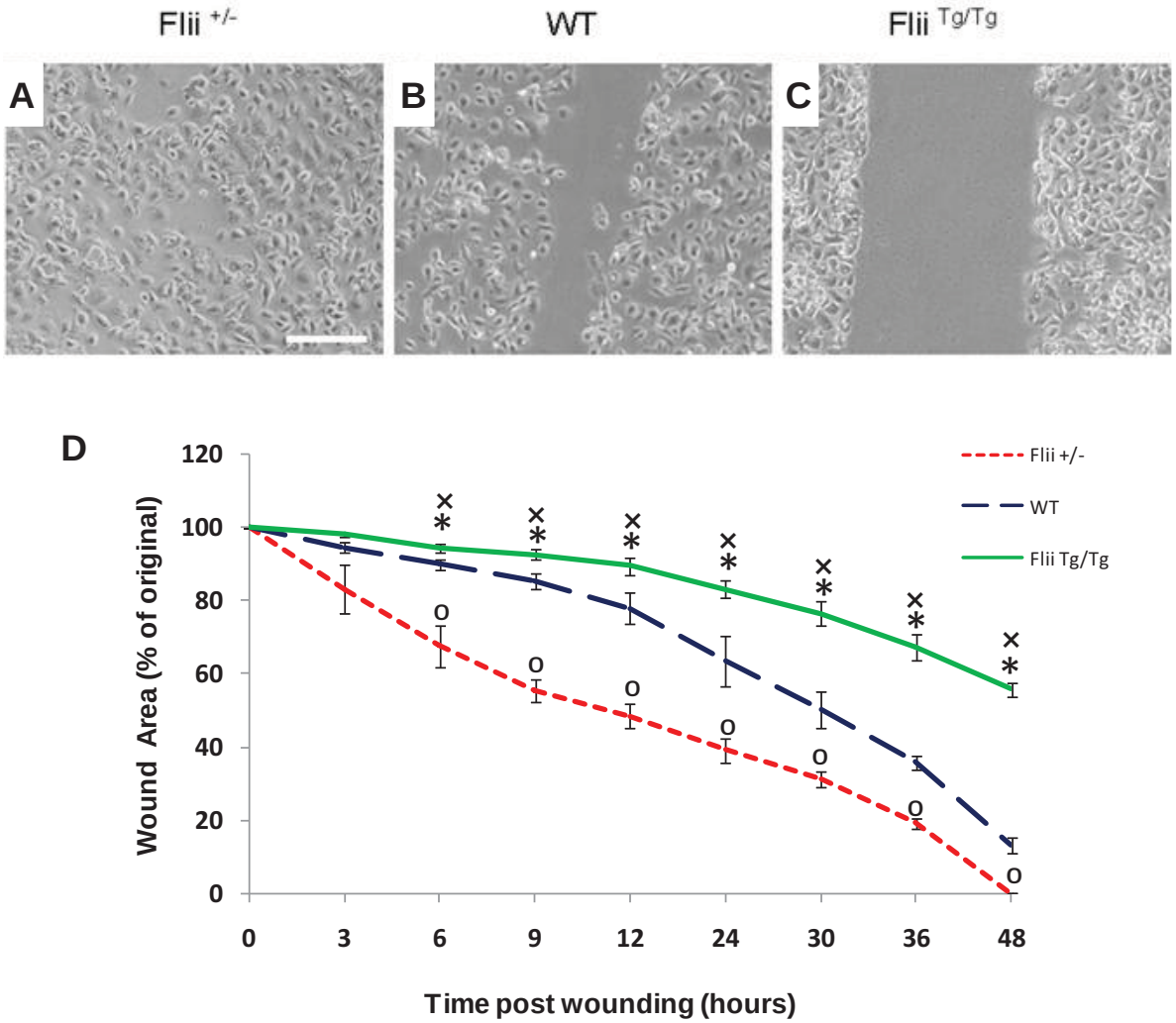

Figure 3.8: Flightless regulates keratinocyte cell migration. Scratch wound model was used to assess the differential effect of *Flii* gene expression on primary keratinocyte migration. Wound areas were measured using the Image Pro-Plus program, and the rate of closure quantified as a percentage of the initial wound area. **A-C** Representative images of scratch wound areas in Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ keratinocytes at 48hrs post wounding. Magnification x 4. Scale bar = 500µm. **D** Graph showing changes in wound area over a 48hr period of keratinocyte migration. x = Flii$^{Tg/Tg}$ significantly different to WT. * = Flii$^{Tg/Tg}$ significantly different to Flii$^{+/-}$. º = Flii$^{+/-}$ significantly different to WT. n = 6. Mean +/- SEM. x, º,*P<0.05. Scale bar in **A** = 100µm and refers to all images.

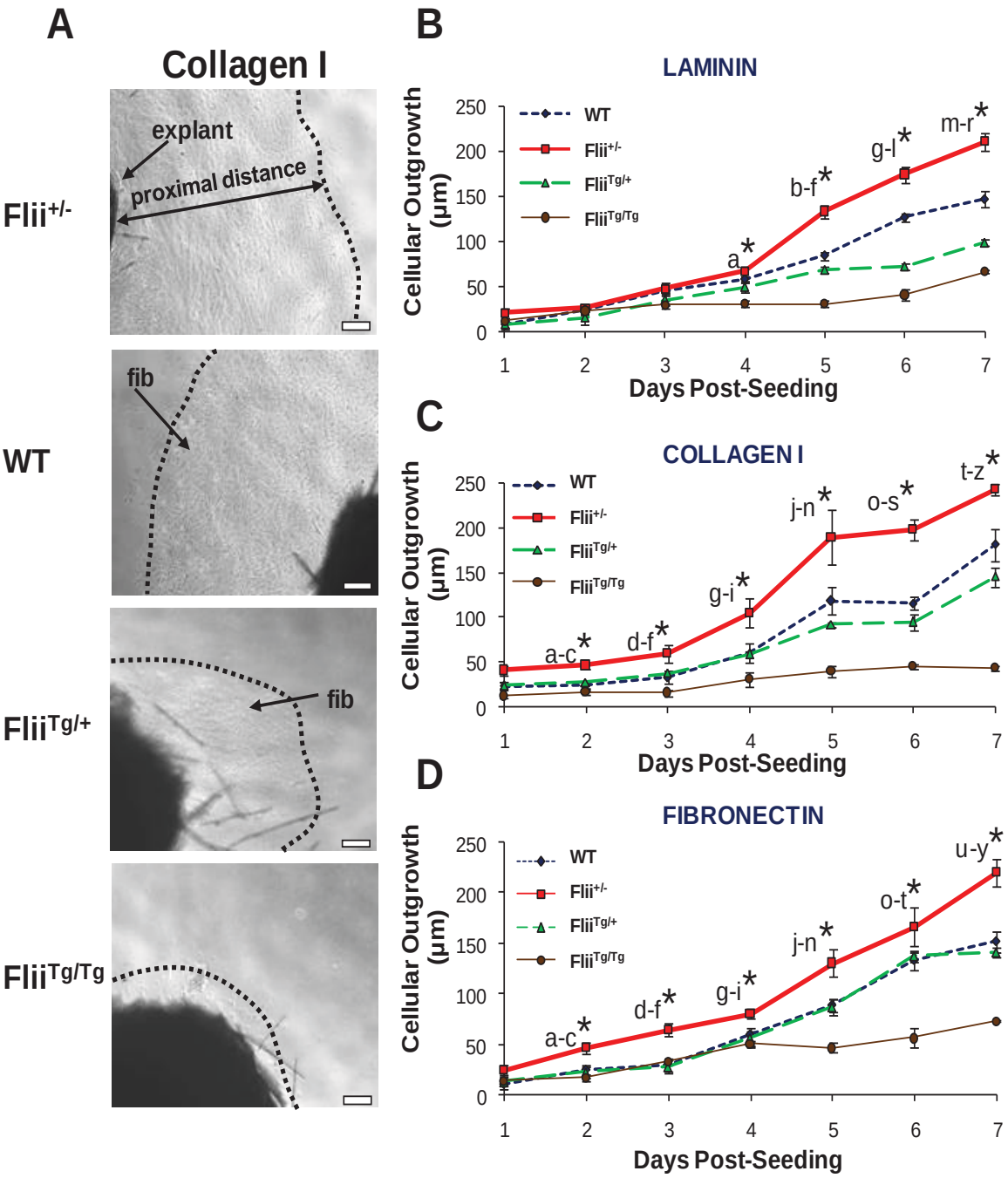

Figure 3.9: Differential effects of Flii on fibroblast outgrowth from skin explants. 2mm punch biopsies from Flii$^{+/-}$, WT, Flii$^{Tg/+,}$ and Flii$^{Tg/Tg}$ on collagen I, laminin, and fibronectin-coated coverslips were cultured over 7 days and cellular outgrowth measured as average distance of migration. **A** Representative images of cellular outgrowth on collagen I taken at day 6 post seeding. Fib = fibroblasts. Magnification x 4. Scale bar = 100µm. **B** Flii$^{+/-}$ cells have significantly increased outgrowth on laminin compared to Flii$^{Tg/Tg}$ fibroblasts from day 4 post-seeding, and compared to both WT and Flii$^{Tg/+}$ cells from day 5 post-seeding (*a–r) respectively. **C** Cellular outgrowth on collagen I shows a similar pattern to laminin, where Flii$^{+/-}$ cells have increased outgrowth compared to WT, Flii$^{Tg/+,}$ and Flii$^{Tg/Tg}$ fibroblasts (*a–z), respectively. **D** Cellular outgrowth on fibronectin is similar to those on laminin and collagen I, except no significant difference is observed between WT and Flii$^{Tg/+}$ fibroblasts outgrowth (*a–y) respectively. Mean +/- SEM. n = 8. *P<0.05.

the explants. A dose-dependent effect of Flii gene expression on cellular outgrowth on laminin (Fig 3.9B), collagen I (Fig 3.9C), and fibronectin (Fig 3.9D) was observed, with $Flii^{+/-}$ cells migrating faster than wild-type, which in turn were faster than $Flii^{Tg/+,}$ with $Flii^{Tg/Tg}$ cells being the least able to migrate from the explant. As observed with our *in vitro* scratch assay examining keratinocyte migration (Fig 3.8A-D), here we also see that decreasing Flii expression resulted in an approximately 25% increased cellular outgrowth on different extracellular matrix substrates, while Flii over-expression in $Flii^{Tg/Tg}$ cells resulted in an approximately 60% decreased cellular migration (Fig 3.9A-D). These data illustrate the role for Flii in regulation of cellular migration.

2.2.5 Application of Flightless neutralizing antibodies significantly improves the proliferation of primary murine keratinocytes

Our previous studies have shown that Flii is a secreted protein and that the exogenous application of Flightless neutralising antibodies (FnAb) raised against the leucine rich repeat (LRR) of Flii significantly improve wound repair via mechanisms involving cellular migration and proliferation (Cowin *et al*., 2007). A range of FnAb concentrations and dose-matched IgG control were added to a sub-confluent population of human keratinocytes and proliferation assessed using a WST-1 assay in order to investigate the optimal FnAb concentration. Using human keratinocytes we demonstrate the effect of FnAb on cellular proliferation in a dose dependant matter and show that concentrations higher than 1:1000 are effective in significantly increasing keratinocyte proliferation compared to the untreated control (Fig 3.10A). Addition of the control dose matched IgG showed no effect on keratinocyte proliferation, confirming that neutralizing Flii expression using FnAb is an effective mean to increased keratinocyte population (Fig 3.10B).

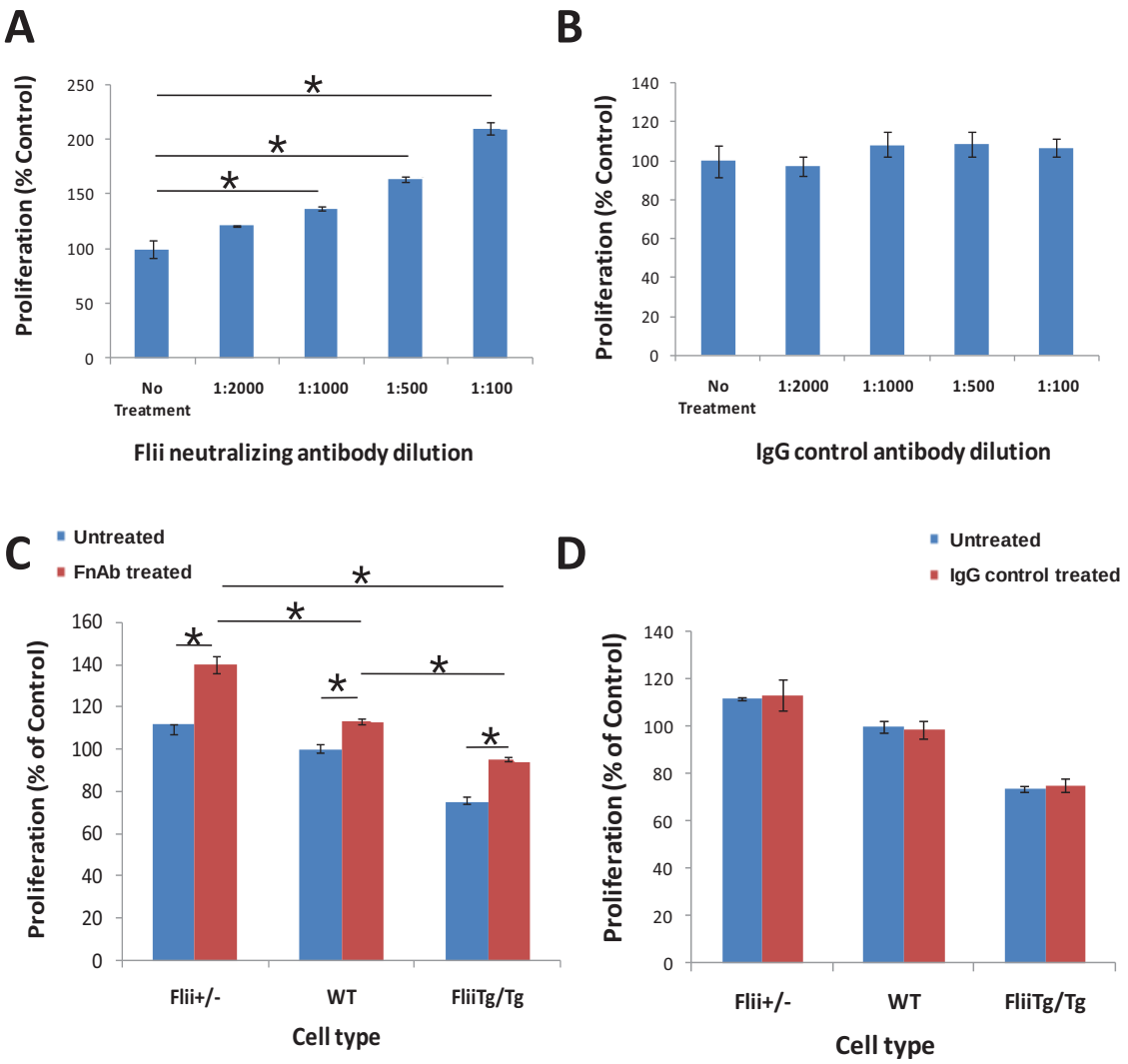

Figure 3.10: Flightless neutralizing antibodies increase keratinocyte proliferation. Human keratinocytes were incubated with increasing concentrations of Flii neutralizing antibodies (FnAb) **A** and control IgG antibodies **B** and the effect on cellular proliferation at 24hr period examined using a WST-1 proliferation assay. $Flii^{+/-}$, WT and $Flii^{Tg/Tg}$ primary mouse keratinocytes were incubated with FnAb **C** and dose matched IgG control **D** and the effect on cellular proliferation at 24hr period examined using a WST-1 proliferation assay. Treatment of primary keratinocytes with IgG control antibodies showed no effect on cellular proliferation however treatment with FnAb resulted in significantly increased keratinocyte proliferation in all cell groups. Nevertheless, $Flii^{+/-}$ keratinocytes still maintained increased proliferation compared to both WT and $Flii^{Tg/Tg}$ keratinocytes, which displayed impaired cellular proliferation compared to their counterparts. n = 6. Mean +/- SEM. *P<0.05.

Using primary murine keratinocytes extracted from Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice skin we now also examined the effect of FnAb on proliferation of keratinocytes with varying levels of Flii expression (Fig 3.10C). We also used a dose matched IgG control on these primary murine keratinocytes illustrating no significant difference between treated or untreated keratinocytes (Fig 3.10D). Untreated primary keratinocytes showed a pattern of proliferation similar to our previous results using primary fibroblasts, where decreased Flii expression resulted in improved cellular proliferation compared to wild-type cells which, in turn, displayed an improved cellular proliferation compared with Flii$^{Tg/Tg}$ cells in which Flii expression was elevated (Fig 3.10D). Treatment of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ primary keratinocytes with FnAb resulted in decreased Flii expression and significantly improved cellular proliferation across all cell types, however treated cells still showed the differences in cellular proliferation where Flii$^{+/-}$ keratinocytes had significantly improved proliferation compared to wild-type cells which, in turn, showed significantly improved proliferation compared to Flii$^{Tg/Tg}$ keratinocytes (Fig 3.10C).

3.3 Discussion

Effective wound healing is dependent on a balanced compromise between cellular migration and adhesion. Both cellular migration and adhesion require a complex re-organization of the actin cytoskeleton in a spatially and temporally coordinated manner. These processes are controlled by both extracellular and intracellular processes, including control at the level of actin cytoskeletal remodelling and at sites where cells adhere to the extracellular matrix. Consequently, proteins which have the ability to modulate the actin cytoskeleton and affect the expression of cell surface receptors and their ligands are important regulators of wound healing. Indeed, our previous results have shown that Flii, an actin remodelling protein, is a negative regulator of wound healing, where mice heterozygous for the Flii gene have enhanced wound healing, while mice over-expressing Flii have larger wounds and delayed wound reepithelialisation (Cowin *et al*., 2007). Using both Flii siRNA and Flii neutralising antibodies it has been shown that decreasing Flii expression in human fibroblasts and keratinocytes improves their migration and proliferation, however mechanisms behind these findings are yet to be elucidated (Cowin *et al*., 2007).

In an attempt to better understand the role of Flii in skin biology and changes which take place during wound healing enabling this actin remodelling protein to act as a negative regulator of wound healing it was decided to examine the role of Flii in unwounded skin. This was achieved using the mice with differential expression of the Flii gene. Here it was shown that over-expression of Flii results in a significantly thinner and more fragile skin, similar to the skin of patients which suffer from a skin blistering disease EB where mutations in different proteins involved in maintaining strong adhesion of skin layers results in skin blistering. These results suggested that Flii might be involved in the formation or maintenance of stable adhesion of skin layers via adhesion sites called

hemidesmosomes. Efficient hemidesmosome disassembly upon wounding and quick formation of new stable adhesion sites are important events in wound repair, allowing cellular migration over the provisional wound bed and strong adhesion at the dermal–epidermal junction (Jones *et al*., 1998). Hemidesmosome adhesion structures have dual roles, they are composed of both structural proteins and signaling integrin receptors present at the dermal epidermal junction and consequently provide both a stable adhesion between the skin layers and a link between the extracellular matrix and actin cytoskeleton, allowing cells to sense signals in response to wound healing and change from a stable adhesion to a migratory phenotype (Kirfel and Herzog, 2004). Indeed, recent advances in integrin receptor structure have revealed an increasing number of integrin-binding cytoskeletal proteins involved in integrin-dependent adhesion signaling pathways (Arnaout *et al*., 2007). In this chapter it has been demonstrated that Flii might regulate both stable adhesion and motility and might have contradictory roles in intact unwounded skin compared to healing wounds. Flii is not only a negative regulator of wound healing but also plays a role in the assembly of adhesion structures, illustrating the diversity in function of this unique protein.

These results suggest that Flii plays an important role in the formation and maintenance of stable adhesion sites of the basement membrane at the dermal–epidermal junction. Under normal conditions, hemidesmosomes have a triangular cytoplasmic inner plaque, a sub-basal dense plate just below and external to the basal membrane, and thin extracellular anchoring filaments which extend from the plate into the basement membrane (Jaunzems *et al*., 1997). Using transmission electron microscopy, it was observed that epidermal basal cells of mice deficient in Flii expression assemble more hemidesmosome structures than the wild-type counterparts, yet wounds of these mice heal significantly faster than those of wild-type mice. The opposite was true for Flii over-expressing mouse

skin which had significantly decreased tensile strength and fewer numbers of hemidesmosomes than wild-type and Flii$^{+/-}$ mice yet these mice had impaired wound healing. Moreover, shorter hemidesmosomes, with reduced inner plaque and a reduced percentage of hemidesmosomes with sub-basal dense plates was observed in Flii$^{Tg/+}$ and Flii$^{Tg/Tg}$ mice skin. This was somewhat surprising because conditions in which wound healing is inhibited would presumably favour increased hemidesmosome formation. Several mechanisms could explain how over-expression of Flii protein results in inhibited hemidesmosome formation as well as delayed wound healing. Flii is both an intracellular and a secreted protein (Cowin *et al*., 2007) consequently its expression may regulate both integrin-ligand interactions, integrin heterodimer expression as well as the expression of proteins that regulate the activity of integrin receptors. Flii could therefore contribute to both formation of stable adhesion sites and affect cellular adhesion and migration during wound healing. Flii contains a unique structure; besides the gelsolin related domain, Flii also contains a leucine rich repeat, a known protein-protein interacting domain (Kopecki and Cowin, 2008) suggesting possible interaction of Flii with proteins present at the dermal epidermal junction and integrin cell receptors. Interaction of Flii with other proteins through its leucine rich repeat domain may be important in its role as a negative regulator of wound healing as application of neutralizing antibodies raised against the leucine rich domain of Flii result in faster wound healing that that observed in untreated controls (Cowin *et al*., 2007). Neutralising antibodies against Flii may either from a Flii-antibody complex marking the protein for degradation or their binding to the leucine rich repeat of Flii may interfere with the functions of the different Flii domains.

Moreover, a decreased number of hemidesmosomes and shorter hemidesmosome length, observed here in Flii over-expressing mice would result in decreased hemidesmosome contact along the dermal–epidermal junction aspect of basal

keratinocytes, leading to weakened hemidesmosome to tonofilament interaction, delayed formation of new adhesion sites, and impaired reepithelialisation, as was observed in these mice (Cowin *et al*., 2007). Results presented here also suggest that Flii may modulate the balance or expression of integrin receptors. Flii involvement in integrin-signaling pathways may contribute to changes in keratinocyte and fibroblast migration, adhesion, and spreading. Our results which suggest a role for Flii in integrin mediated cellular adhesion and migration and Flii interaction with other structural and signaling proteins present at the dermal epidermal junction will be discussed in Chapters 4 and 5 of this thesis.

In order to investigate whether the effects of Flii expression on migration appear to depend on cell type or specific extracellular matrix substrate we examined cellular migration on the three most common proteins found in wound beds, namely laminin-332, collagen I and fibronectin. Laminin-332 is the integral component of hemidesmosome adhesions and the first extracellular matrix protein encountered by migrating cells across the wound bed (Hamill *et al*., 2009). The inhibition of hemidesmosome structure observed in Flii over-expressing mice wounds suggest that Flii over-expressing cells might also have impaired cellular migration and adhesion on laminin substrate. In agreement with altered hemidesmosome structure, $Flii^{Tg/Tg}$ keratinocytes and fibroblasts showed significantly decreased adhesion and spreading on laminin substrate and also on fibronectin and collagen I extracellular matrix substrates compared with wild-type counterparts. In addition, both $Flii^{+/-}$ keratinocytes and fibroblasts had significantly improved adhesion and spreading on laminin and collagen I extracellular matrix substrates. Rate of cellular adhesion and spreading on the extracellular substrate is often affected by the activity or expression of integrin receptors for the particular extracellular matrix substrate (Cowin *et al.*, 2006; Critchley *et al.*, 1999). Results presented in this chapter show that decreased Flii gene expression also increases the rate of keratinocyte migration and improves cellular

outgrowth from Flii$^{+/-}$ skin explants on laminin, collagen I and fibronectin substrates, indicating the important role of Flii in regulating cellular adhesion and migration. Similar patterns of cellular adhesion and migration were observed on laminin, collagen I and fibronectin extracellular matrix substrates for all cell groups, suggesting that integrin cell receptors for all three of these substrates might be affected by differential Flii expression where Flii over-expression leads to blocking of the ligand binding or direct binding to the integrin chains causing a conformational change in the receptor expression. This will be further discussed in Chapter 4 of this thesis.

In this study, further evidence for the effectiveness of FnAb as potential therapy for improved wound healing has been provided. In agreement with previous results (Cowin *et al*., 2007) it is clear that application of FnAb to cell culture significantly improves keratinocyte proliferation compared to untreated or IgG treated control cells. Flii$^{+/-}$ keratinocytes still maintained the significantly improved proliferation compared to wild-type cells, which in turn, proliferated at a significantly higher rate than Flii$^{Tg/Tg}$ keratinocytes. However, treatment of Flii$^{Tg/Tg}$ keratinocytes with FnAb increased their proliferation close to the levels of cellular proliferation seen in wild-type untreated control cells. The application of FnAb improved keratinocyte proliferation in all cell types by ~25% and, if similar results were to be achieved in human patient's wounds, this would be clinically significantly and may result in improved wound healing. Future studies need to elucidate the molecular mechanisms behind increased cellular proliferation, however our current results suggest that the Flii effects on cellular adhesion and migration might be more important during wound healing.

In summary, this study has demonstrated the importance of Flii protein in skin structure and formation and maintenance of hemidesmosomes. It has shown that Flii is not

only an important regulator of wound healing but also plays a role in unwounded intact skin structure and function. Over-expression of Flii not only impedes wound healing but also results in thinner and more fragile skin structure. Furthermore, these studies suggest that the Flii effect on wound healing may primarily be mediated by both cellular adhesion and migration, and demonstrate that Flii is a potential target for development of therapy aimed at improved wound healing.

Chapter 4

EFFECT OF FLIGHTLESS ON BASEMENT MEMBRANE PROTEINS DURING WOUND HEALING

Parts of this chapter have been published in following journal:

Kopecki Z, Arkell R, Powell BC, Cowin AJ. Flightless I regulates hemidesmosome formation and integrin-mediated cellular adhesion and migration during wound repair. *J Invest Dermatol*. 2009 Aug;129(8):2031-45. **IF 5.5 RIF 1/39.**

4.1 Introduction

Integrin transmembrane receptors are principal cell surface adhesion receptors which mediate cell-matrix adhesion and communication, promoting the attachment and migration of cells on surrounding extracellular matrix (Berrier and Yamada, 2007). Integrin heterodimers form functional receptors that connect the cytoskeleton to the extracellular matrix and act both as traction sites and mechanosensors altering actin cytoskeleton dynamics (Hehlgans *et al*., 2007). Integrin cytoplasmic domains form multi-molecular complexes with proteins involved in cell signaling and with adapter proteins which connect the actin cytoskeleton, thereby controlling the tension forces necessary for cellular adhesion, movement, cell–matrix interactions and tissue organization (Hynes, 2002). In the previous chapter we have shown that Flii protein modulates keratinocyte and fibroblast cell adhesion and migration while also playing a role in the formation of hemidesmosome adhesion structures, suggesting the possible involvement of Flii in integrin mediated cellular adhesion and migration.

Integrin chains, α3, α6, β1 and β4, combine to form three functional heterodimers, α3β1, α6β1 and α6β4, which bind laminin isoforms and have been shown to be particularly important in epidermal integrity (Watt, 2002). Laminin-binding integrin receptors have also been shown to associate with the tetraspanin CD151. Tetraspanin CD151 promotes cellular migration by regulating integrin trafficking (Liu *et al*., 2007), plays an important role in wound reepithelialisation (Cowin *et al*., 2006) and is involved in hemidesmosome assembly (Litjens *et al*., 2006). Integrin α6β4 binds directly to CD151 to form stable adhesion sites called hemidesmosomes. Consequently, proteins like Flii which affect the formation of stable hemidesmosomes and affect cellular migration during wound healing may act through direct or indirect interactions with integrin receptors, integrin ligands

including tetraspanin CD151 or extracellular matrix substrates like laminin, as well as other structural and signaling proteins that regulate activity of integrin receptors.

Moreover, we have shown that expression of Flii affects cellular migration over different extracellular matrix substrates, further implying that Flii may affect different integrin receptors used by cells for migration across the wound bed and binding to the extracellular matrix substrates. Integrin receptors have been shown to greatly mediate interactions between keratinocytes and different extracellular matrix proteins, including laminin, collagen, and fibronectin (Hynes, 2002). Integrin binding to extracellular ligands is mediated by conformational changes induced by the interactions of the cytoplasmic domains with cytoskeletal and adaptor proteins including paxillin, talin, vinculin which further couple to downstream signaling proteins including Src, FAK and p130Cas. Following the binding of the extracellular ligand, the complex multiprotein assembly of the cytoskeletal and signaling proteins is initiated, linking the integrin to the actin cytoskeleton, conveying the signal into the cell and forming the focal adhesion sites (Hehlgans *et al*., 2007).

In this chapter we examine the effect of Flii on integrin receptors and other components of hemidesmosome complexes, including tetraspanin CD151 and laminin extracellular matrix substrate, in response to wounding using mice with differential expression of both Flii and tetraspanin CD151 proteins. Furthermore, we use human keratinocytes to investigate potential Flii binding to different hemidesmosome components and adaptor proteins involved in adhesion mediated integrin signaling, including paxillin, talin, vinculin and α-actinin.

4.2 Results

4.2.1 Flightless negatively regulates tetraspanin CD151 expression during wound healing

Tetraspanin CD151 is a key component of hemidesmosomes and plays an important role during wound re-epithelialisation by selectively interacting with different laminin-binding integrin receptors (Cowin *et al*., 2006). In order to investigate the effect of Flii on CD151 expression and relationship between Flii, a cytoskeletal protein, and CD151, a transmembrane protein, we used mice with differential gene expression of Flii or tetraspanin CD151.

Using $Flii^{+/-}$, wild-type and $Flii^{Tg/+}$ mice we found no significant difference in expression of CD151 in unwounded skin of these mice, however an effect of Flii in modulating CD151 expression was observed in response to wounding. CD151 expression was increased in response to wounding in the epidermis, peaking at day 3 and decreasing by day 7 post-wounding in wild-type and Flii over-expressing wounds (Fig 4.1A-B). In contrast, $Flii^{+/-}$ wounds showed significantly elevated CD151 expression at day 3 and maintained this elevated expression through to day 7 post-wounding, before returning to basal levels by day 14 post-wounding where no significant difference was observed between $Flii^{+/-}$, wild-type and Flii over-expressing mice wounds (Fig 4.1A-B). These results suggest that Flii may negatively regulate CD151 expression during wound healing.

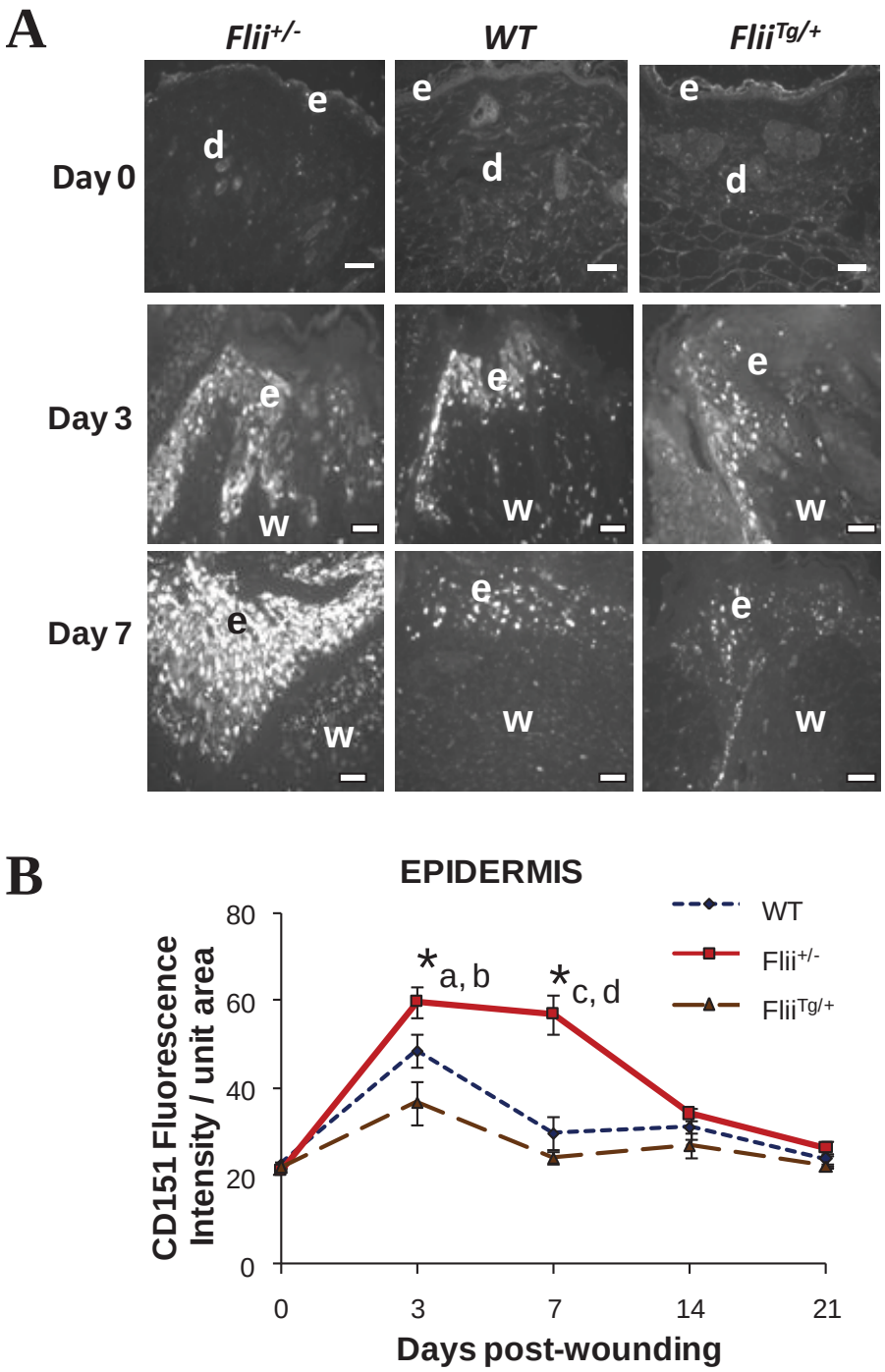

Figure 4.1: Tetraspanin CD151 expression is increased in Flightless deficient mice during wound repair. A CD151 expression detected as a white fluorescence in the epidermis and dermis of wounded Flii$^{+/-}$, WT and Flii$^{Tg/+}$ skin at day 0, 3 and 7 post-wounding. Epidermis = e. Wound matrix = w. Magnification x 40. Scale bar = 100µm. **B** Graphical representation of CD151 immunofluorescence intensity in the epidermis during wound healing. Both WT (*a) and Flii$^{+/-}$ (*b) mice wounds have significantly increased CD151 expression in epidermis at day 3 post-wounding when compared to Flii$^{Tg/+}$ counterparts. Flii$^{+/-}$ wounds also have significantly increased CD151 expression compared to WT (*c) and Flii$^{Tg/+}$ (*d) wounds at day 7 post-wounding. Mean +/- SEM. n = 6 mice per group per time-point. *P<0.05.

Examining Flii expression in wounds of mice with deficient CD151 gene expression also suggested a possible correlation between expression of Flii and tetraspanin CD151 during wound healing (Fig 4.2). While no significant difference was observed in the Flii expression in unwounded mice skin between $CD151^{-/-}$ mice or wild-type counterparts, Flii expression was increased in response to wounding in both the epidermis and the wound matrix of $CD151^{-/-}$ and wild-type mice (Fig 4.2). Flii expression peaked at day 7 post-wounding and returned to basal levels by day 14 post-wounding in both $CD151^{-/-}$ and wild-type mice skin. $CD151^{-/-}$ mice wounds had significantly increased Flii expression in the epidermis both at 3 and 7 days post wounding (*a-b) respectively (Fig 4.2A-B).

4.2.2 Expression of CD151, Flightless and laminin protein levels during wound healing as determined by Western Blotting

In order to further examine the correlation between Flii and CD151 expression and the effect of Flii on different components of hemidesmosome adhesion structure, protein extracts from $Flii^{+/-}$, wild-type, $Flii^{Tg/+}$ and $CD151^{-/-}$ wounds were examined for protein expression using Western Blotting (Fig 4.3). In agreement with the immunohistochemistry results, we found that $Flii^{+/-}$ mice wounds had increased CD151 expression both at day 3 and 7 post-wounding compared with wild-type counterparts (Fig 4.3A). Using protein extracts from $CD151^{-/-}$ and wild-type mice wounds at day 0, 3 and 7 post-wounding we found that Flii expression was increased in response to wounding and was higher in $CD151^{-/-}$ mice wounds at both day 3 and 7 post-wounding (Fig 4.3B). Further investigation of the effect of Flii on components of hemidesmosome structures showed that Flii deficient mice wounds expressed increased levels of laminin protein at day 3 and 7 post-wounding compared to wild-type counterparts while Flii over-expression had reduced laminin

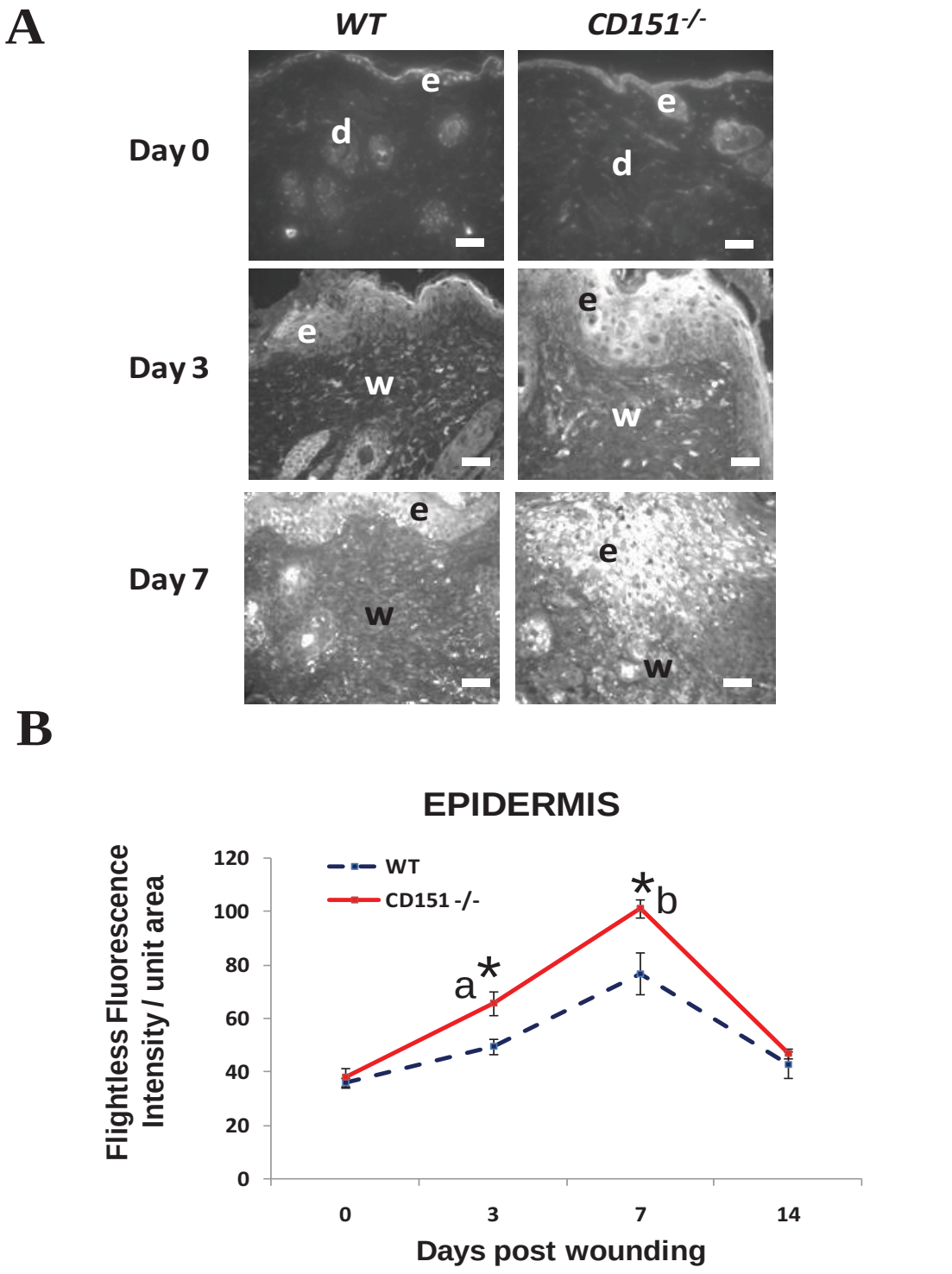

Figure 4.2: Flightless expression is increased in CD151 deficient mice during wound repair. A Flii expression detected as a white immunofluorescence in the epidermis and dermis of wounded WT and CD151$^{-/-}$ mouse skin at day 0, 3, 7 and 14 post-wounding. Epidermis = e. Wound matrix = w. Magnification x 40. Scale bar = 100μm. **B** Graphical representation of Flii fluorescence intensity during wound healing. CD151$^{-/-}$ mice skin have significantly increased Flii expression compared to WT counterparts at both day 3 (*a) and day 7 (*b) post-wounding, respectively. Mean +/- SEM. n = 6 per mice per time-point. *P<0.05.

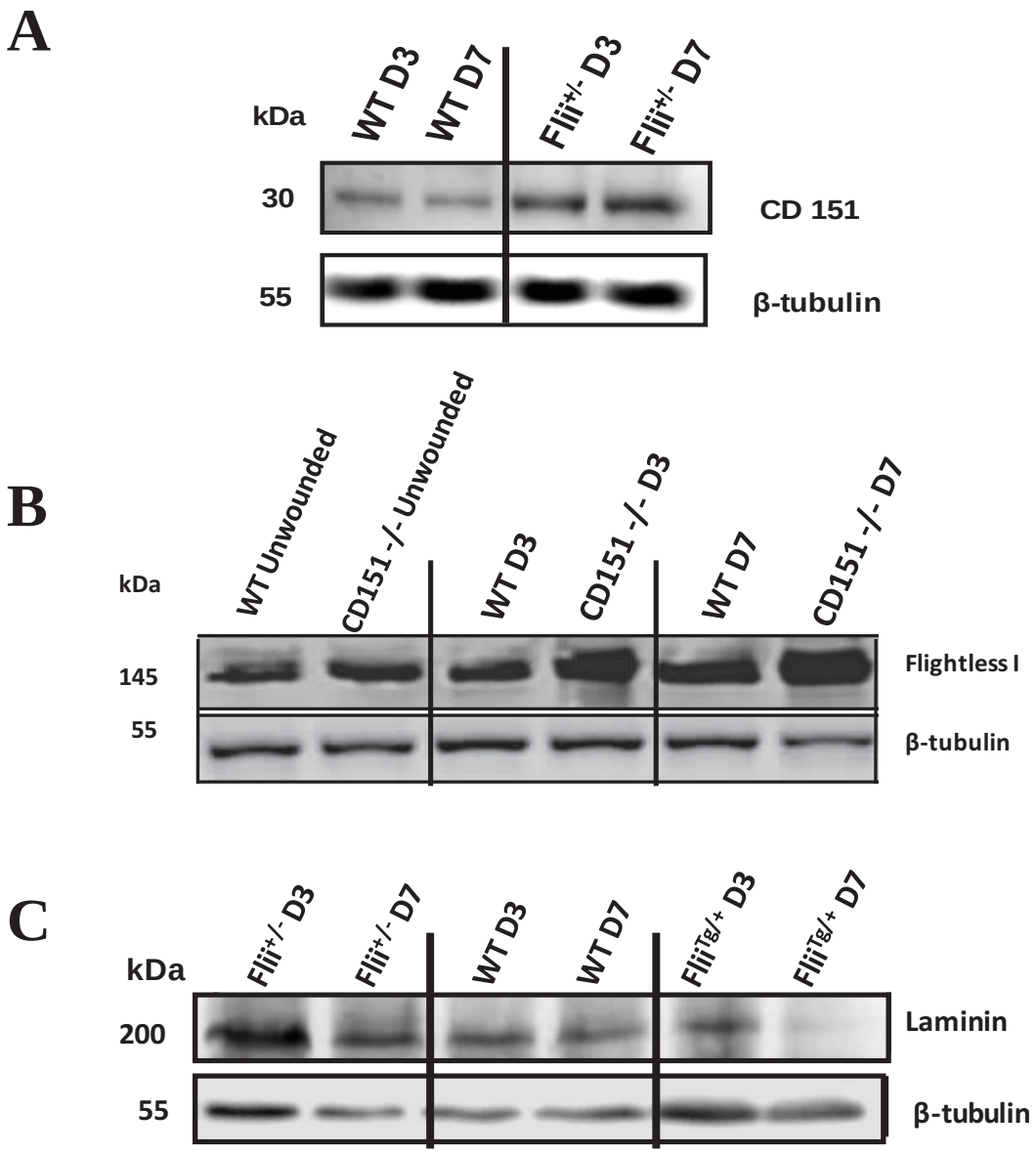

Figure 4.3: CD151, Flightless and laminin protein expression during wound repair as determined by Western Blotting. Using protein extracts from Flii$^{+/-}$, WT, Flii$^{Tg/+}$ and CD151$^{-/-}$ mice wounds (containing the epidermis and the dermal wound bed) protein expression of CD151, Flii and laminin was investigated. All results were normalized to β-tubulin loading control. **A** Flii$^{+/-}$ deficient mice have increased expression of CD151 in response to wounding at both day 3 and 7 post-wounding. **B** Flii expression is increased in response to wounding and is higher in CD151$^{-/-}$ mice wounds compared to WT counterparts at both day 3 and 7 post-wounding. **C** Flii$^{+/-}$ deficient mice have increased laminin expression at day 3 post-wounding while Flii over-expression resulted in reduced Flii expression at day 7 post-wounding. Blots are representative of three independent experimental repeats. n = 3 samples from different mice per time-point.

expression, especially at day 7 post-wounding which may explain the differences we observe in hemidesmosome structure and wound healing between these mice (Fig 4.3C).

4.2.3 Manipulation of Flightless gene expression alters the expression of laminin binding integrin subunits

The effect of differential Flii gene expression on cellular migration and components of hemidesmosome adhesion structures including CD151 and laminin protein suggested that Flii might mediate cellular responses through its effects on integrin receptors. To explore the potential of Flii in mediating cellular adhesion and migration during wound repair, immunohistochemical analysis of laminin integrins α3, β1, α6, and β4 subunits, collagen integrin α2, α1 subunits and fibronectin integrin α5 subunit were undertaken on $Flii^{+/-}$, wild-type, and $Flii^{Tg/+}$ wounds. Moreover we also examined the effect of Flii on the expression of the main basement membrane collagen, collagen IV and a wound matrix extracellular substrate fibronectin. No significant difference in the expression of collagen integrin α2, α1 subunits and fibronectin integrin α5 subunit or basement membrane collagen IV and fibronectin extracellular matrix substrate were observed in wounds of $Flii^{+/-}$, wild-type, and $Flii^{Tg/+}$ mice. However differential Flii gene expression affected different integrin subunits of the laminin ligand, namely integrin α3, β1, α6, and β4 expression during wound healing of $Flii^{+/-}$, wild-type and $Flii^{Tg/+}$ mice (Fig 4.4). Representative images of integrin α3, β1, α6, and β4 staining at day 3 post-wounding are shown in Figure 4.5A. Integrin α3 in unwounded skin was significantly higher in $Flii^{+/-}$ compared with wild-type, which in turn was higher than in $Flii^{Tg/+}$. Expression was localized within both basal and suprabasal keratinocytes and wounding caused similar decreases in α3 expression in all three groups, returning to baseline conditions by day 14 (Fig 4.4A).

The cytoplasmic domain of integrin β1 directly connects to the actin cytoskeleton and its degree of stabilization must be tightly regulated to provide optimal traction forces required for cellular migration and spreading. Integrin β1 is expressed on basal keratinocytes and its expression in response to wounding was increased in suprabasal keratinocytes along the newly forming epidermis at the wound margin, returning to basal levels by day 14 post-wounding (Fig 4.4A-B). A dose-dependent effect of Flii gene expression on integrin β1 expression was observed with $Flii^{+/-}$ having a significantly elevated expression in contrast to wild-type and $Flii^{Tg/+}$, in which integrin β1 was significantly decreased (Fig 4.4A-B). Similar expression was seen in fibroblasts in the wound matrix with integrin β1 expression remaining elevated until day 7 post-wounding and decreased by day 14 post-wounding. Western blotting, confirmed this dose-dependent effect of Flii gene expression on β1 levels in response to wounding (Fig 4.4C).

Integrin α6 plays an important role in both cellular migration and adhesion during wound repair where it couples to integrin β1 and β4 during formation of a functional laminin receptor and stable hemidesmosome adhesion sites, respectively (Watt, 2002). Examining the immunostaining of $Flii^{+/-}$, wild-type, and $Flii^{Tg/+}$ mice wounds using an integrin α6-specific antibody revealed increased expression in response to wounding in all groups, with similar expression patterns in both the epidermis (Fig 4.4A) and wound matrix (data not shown). Integrin α6 was predominantly expressed on basal keratinocytes attached to basement membrane and on fibroblasts of newly formed granulation wound tissue (Fig 4.4A). No significant difference in integrin α6 expression was observed between wild-type wounds and $Flii^{Tg/+}$ wounds at day 3 post-wounding; however, wild-type wounds had significantly higher integrin α6 expression at day 7 post-wounding

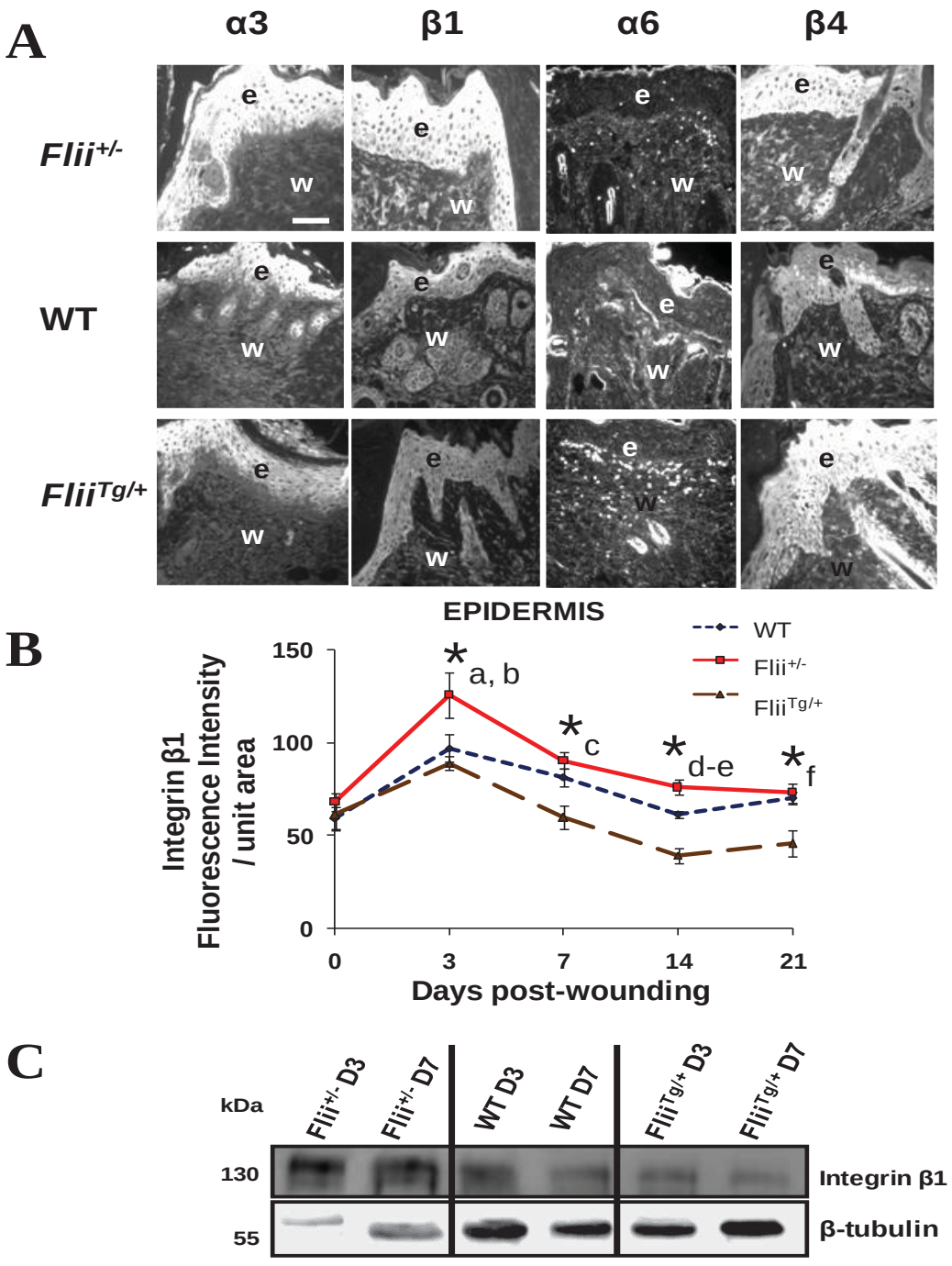

Figure 4.4: Manipulation of Flightless gene expression results in altered expression of laminin-binding integrin α3, β1, α6, β4 subunits during wound healing. A α3, β1, α6, and β4 integrin expression detected as white immunofluorescence in the epidermis and dermis of wounded skin at day 3 post-wounding. Epidermis = e. Wound matrix = w. Scale bar in **A** refers to all images = 100μm. **B** Graph showing quantitative analysis of integrin β1 expression in the epidermis of Flii$^{+/-}$, WT, and Flii$^{Tg/+}$ wounds. Flii$^{+/-}$ wounds have significantly increased β1 integrin expression compared to WT wounds at day 3(*a) and 14 (*d) post-wounding and compared to Flii$^{Tg/+}$ wounds at day 3(*b), 7 (*c), 14 (*e), and 21 (*f) post-wounding. **C** Western blot analysis of β1 integrin in protein extracted from Flii$^{+/-}$, WT and Flii$^{Tg/+}$ skin at day 3 and 7 post-wounding. Mean +/- SEM. n = 6 mice per group per time-point. *P<0.05.

compared with Flii$^{Tg/+}$ wounds (Fig 4.4A and 4.5B). Flii$^{+/-}$ wounds appeared to have delayed the expression of α6; however, its expression remained elevated for a long time and remained significantly increased compared with wild-type and Flii$^{Tg/+}$ at day 21, when the wounds were completely re-epithelialised (Fig 4.5B).

Integrin β4 is expressed on cell surfaces as a heterodimeric complex with integrin α6, forming a functional laminin integrin receptor α6β4 which associates with CD151 and plectin to stabilize the hemidesmosome adhesion complex and plays an important role in mediating cellular migration (Watt, 2002). Expression of integrin β4 was also altered in response to wounding with different levels of Flii gene expression. In unwounded skin, integrin β4 is mainly expressed on basal keratinocytes and upon wounding the expression increases and eventually upon hemidesmosome disassembly integrin β4 relocates to suprabasal keratinocytes of the migrating tongue and lamellipodial structures mediating cellular migration (Santoro and Gaudino, 2005, Watt, 2002). This pattern of increasing integrin β4 expression was seen in response to wounding in Flii$^{+/-}$, wild-type, and Flii$^{Tg/+}$ wounds in both the epidermis and wound matrix (Fig 4.4A and 4.5C) although temporal differences were observed. Integrin β4 expression increased in wild-type wounds at day 3, peaking at day 7 before returning to basal levels by day 14 post-wounding. Interestingly, in both Flii$^{+/-}$ and Flii$^{Tg/+}$ wounds integrin β4 expression peaked earlier at day 3, decreased by day 7 and returned to basal levels by day 14 post-wounding (Fig 4.5C). Integrin β4 expression was significantly higher in Flii$^{+/-}$ and Flii$^{Tg/+}$ wounds at day 3 post-wounding compared with wild-type counterparts (Fig 4.4A). At day 7 post-wounding, integrin β4 expression was significantly decreased in Flii$^{+/-}$ and Flii$^{Tg/+}$ wounds compared to wild-type counterparts and Flii$^{Tg/+}$ wounds had significantly higher integrin β4 compared with Flii$^{+/-}$ wounds suggesting that this integrin subunit may not be directly

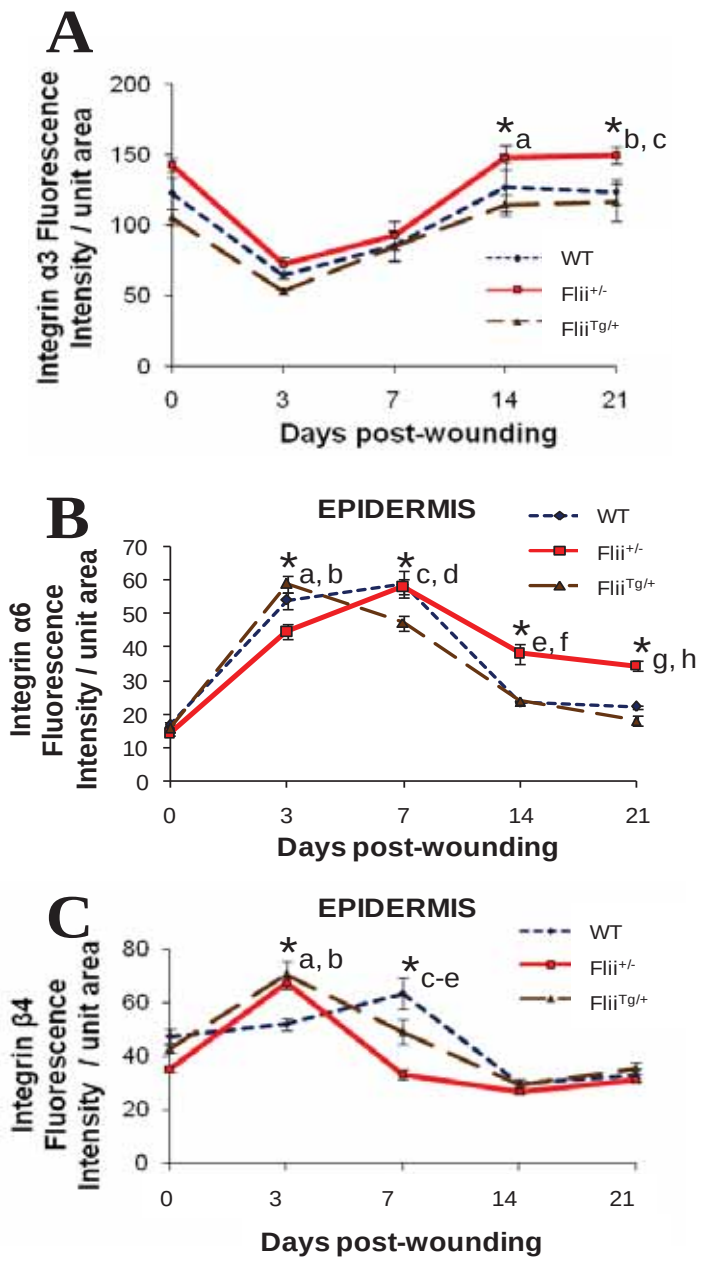

Figure 4.5: Graphical representation of α3, α6, and β4 integrin optical density in the epidermis of Flii$^{+/-}$, WT, and Flii$^{Tg/+}$ wounds. A Flii$^{+/-}$ wounds have significantly increased α3 integrin expression compared to Flii$^{Tg/+}$ (*a) wounds at day 14 post-wounding and compared to both WT (*b) and Flii$^{Tg/+}$ (*c) wounds at day 21 post-wounding. **B** Flii$^{+/-}$ wounds have significantly decreased α6 integrin expression compared to Flii$^{Tg/+}$ (*a) and WT (*b) wounds at day 3 post-wounding. At day 7 post-wounding both wild-type (*c) and Flii$^{+/-}$ (*d) wounds have significantly increased α6 integrin expression compared to Flii$^{Tg/+}$ wounds. Flii$^{+/-}$ wounds have significantly higher α6 integrin expression compared to WT and Flii$^{Tg/+}$ mice wounds at day 14 (*e, f) and 21 (*g, h) post-wounding, respectively. **C** Both Flii$^{+/-}$ (*a) and Flii$^{Tg/+}$ (*b) wounds have significantly higher β4 integrin expression at day 3 post-wounding when compared to WT counterparts. At day 7 post-wounding, WT wounds have significantly higher expression of β4 integrin compared to Flii$^{Tg/+}$ (*c) wounds whereas Flii$^{+/-}$ wounds have significantly decreased β4 integrin expression compared to both WT (*d) and Flii$^{Tg/+}$ (*e) wounds. n = 6 mice per group per time-point. Mean +/- SEM. *P<0.05.

affected by differences in Flii gene expression. By day 14 and 21 post-wounding integrin β4 levels had returned to basal levels (Fig 4.5C).

4.2.4 Flightless co-localizes with focal complex proteins and may modulate integrin-dependent signaling

Examining the effect of differential Flii gene expression on laminin binding integrin subunits and hemidesmosome components during wound healing suggested that Flii may modulate cellular adhesion and migration in an integrin dependent manner. Nevertheless, Flii interaction with different integrin subunits or hemidesmosome components may be in a direct or indirect manner, as proteins containing leucine rich repeat domains are known to interact with multiple structural, signaling and adaptor proteins involved in different cell signaling pathways (Bella *et al*., 2008, Dai *et al*., 2009, Kobe and Kajava, 2001, Lee and Stallcup, 2006, Liu and Yin, 1998). To gain a mechanistic insight into the role of Flii in the hemidesmosome structure and function, we used a well-established simple wound healing model using human keratinocytes (HaCaTs) to investigate possible Flii-binding partners. Using immunoprecipitates and cell lysates from wounded keratinocytes we firstly investigated the possible binding between Flii and tetraspanin CD151 and integrin subunits β1 and β4, as these subunits form dimmers with multiple integrin α subunits to form functional laminin, collagen and fibronectin receptors. Flii was not observed to co-immunoprecipitate with integrin chains β1, β4, and CD151 (Fig 4.6A). We confirmed these results using dual immunofluorescence and confocal microscopy and while Flii expression was strong at the cell periphery where there is a strong expression of both trasnsmembrane protein CD151 and different integrin subunits there was no colocalization between Flii and integrin chains β1, β4 or CD151 suggesting that the Flii effect on integrin-mediated cellular adhesion and migration

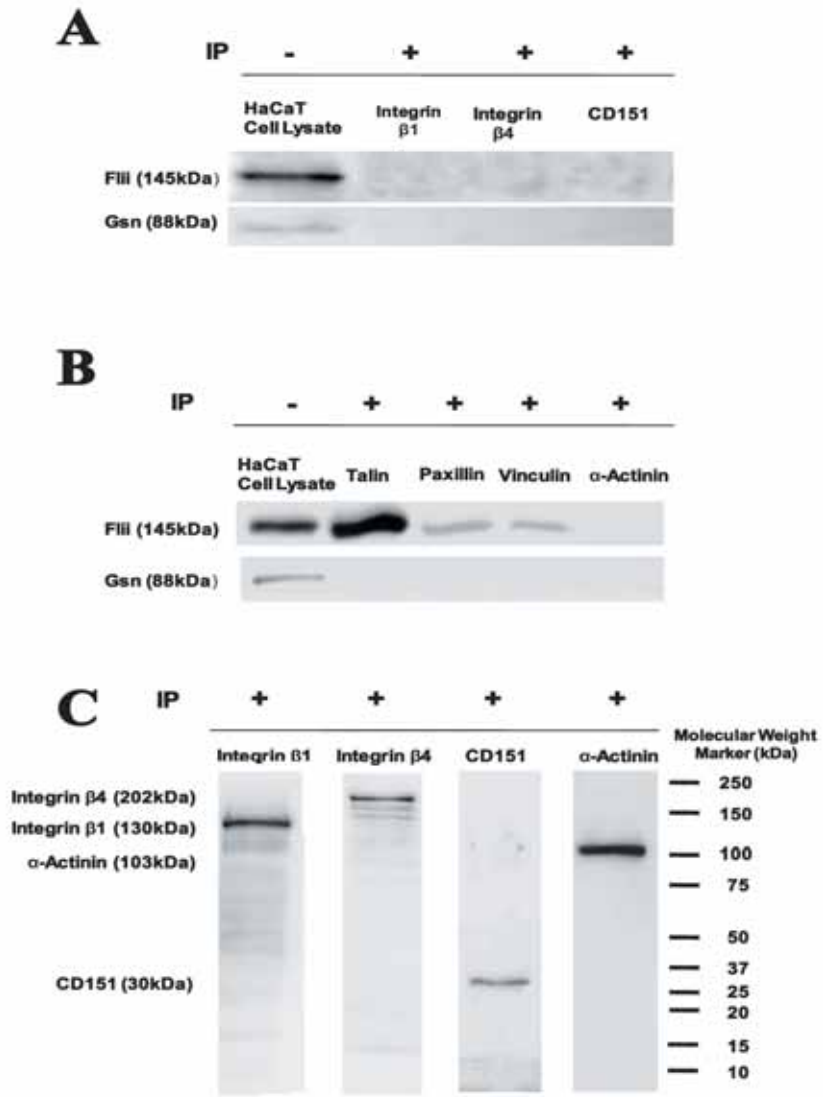

Figure 4.6: Flightless co-localization with integrin-binding proteins involved in adhesion-dependent signaling pathways. Anti-integrin β1, β4, and tetraspanin CD151 **A** and anti-talin, paxillin, vinculin, and α-actinin **B** immunoprecipitates (IP) were prepared from wounded HaCaTs keratinocytes and immunoblotted with Flii or gelsolin (positive control) antibodies. Flii co-immunoprecipitates with talin, paxillin and vinculin actin-binding cytoskeletal proteins. No co-immunoprecipitation was observed with Flii and integrins β1, β4, tetraspanin CD151 or α-actinin. Specific bands were observed in HaCaT cell lysate controls. **C** Anti integrin β1, β4, tetraspanin CD151 or α-actinin immunoprecipitates (IP) prepared from wounded HaCaTs keratinocyte and run using western blotting in **A-B** were stripped and re-probed with antibody used for preparation of immunoprecipitates to confirm antibody specificity and results observed in **A-B**. All data are representative of three independent experiments.

might not be mediated through direct binding to CD151 or integrin β1 and β4 subunits or might be a transient interaction.

In order to further investigate the mechanism behind Flii function in cellular adhesion and migration we investigated possible Flii binding with both structural and adaptor focal complex proteins known to associate directly with integrin β1 and form cytoskeletal complexes involved in integrin dependent signaling pathways including; talin, α-actinin, vinculin, and paxillin. Using immunoprecipitates and cell lysates from wounded human keratinocytes and western blotting we found that Flii does immunoprecipitate with talin, paxillin, and vinculin, but not α-actinin focal complex protein (Fig 4.6B). Gelsolin was present in the total cell lysate but it did not immunoprecipitate with any of the focal complex proteins. This suggested that co-immunoprecipitation between Flii and talin, vinculin and paxillin was specific. Flii showed no association with α-actinin. In order to confirm that Flii does not associate with Integrin chains β1 and β4, tetraspanin CD151 and α-actinin we tested the specificity of our antibodies by probing the specific immunoprecipitation pull-downs with the antibody used in preparation of these immunoprecipitation pull-downs. This showed strong positive bands, integrin chains β1 and β4, tetraspanin CD151 and α-actinin, indicating that these antibodies are good for immunoprecipitation studies and that specific proteins were present in the appropriate immunoprecipitation pull-downs (Fig 4.6C). This confirmed that the absence of the Flii binding with integrin chains β1 and β4, tetraspanin CD151 and α-actinin as illustrated in (Fig 4.6A-B) are a valid result. Using dual labelled immunofluorescence and confocal microscopy on human keratinocytes we further confirmed the association between Flii and talin, vinculin and paxillin focal complex proteins. Flii protein exhibited specific co-localization with talin, vinculin and paxillin but not α-actinin (Fig 4.7).

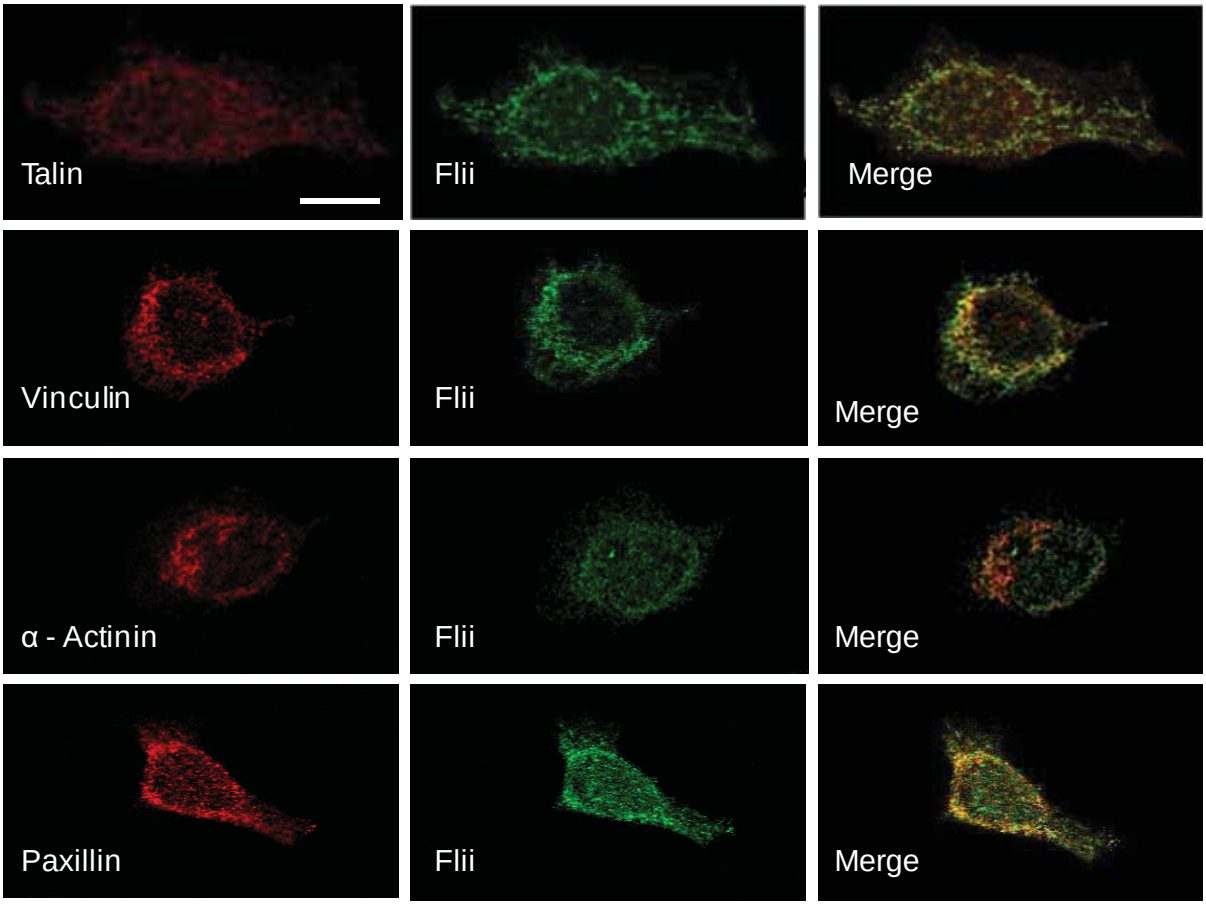

Figure 4.7: Flightless co-localization with focal complex proteins involved in integrin adhesion signaling. Representative confocal images of dual immunofluorescence on sub-confluent HaCaT keratinocytes showing Flii (green) and different actin-binding protein (red) staining. Merged images show Flii co-localization with talin, vinculin, and paxillin but not α-actinin. Magnification x100. Scale bar = 10μm and refers to all images. Data are representative of three independent experiments.

4.3 Discussion

Hemidesmosome intermediate filament structures are multi-protein complexes acting as stable adhesion and anchoring sites for the keratin cytoskeleton, however their assembly/disassembly during wound healing is presently unclear. In the previous chapter we have shown that Flii over-expression results in altered hemidesmosome structure and impaired cellular adhesion, spreading and migration. Components of the hemidesmosomes including tetraspanin CD151, laminin and laminin binding integrin subunits play important roles in mediating cellular migration across the wound bed and stable adhesion to the new matrix. Studies have shown that deletion of laminin binding integrin chains α3 and β1 result in early embryonic lethality or death soon after birth (DiPersio *et al*., 1997, Fassler and Meyer, 1995) whereas wound healing in β1 integrin conditional knockout mice is delayed due to impaired cell migration (Grose *et al*., 2002), hence illustrating the importance of these receptors in cellular adhesion and migration during wound repair. Moreover, studies to date have illustrated that the function of integrins greatly depends on tissue and cell context and their role in cell adhesion or migration are modulated in response to receptor interactions with a number of different proteins and extracellular matrix ligands. Integrin receptors are also involved in the activation of latent TGF-β and act as potential regulators of the Smad-signaling pathway, illustrating that these receptors are more than simple adhesion molecules and their role in cell signaling during wound healing is becoming increasingly important (Reynolds *et al*., 2008, Wipff and Hinz, 2008).

Wounding represents a complex model of cell migration where cells change from a resting to a migratory phenotype. This involves changes in integrin expression profile and requires a dramatic re-organization of the actin cytoskeleton and junctional complexes (Kirfel and Herzog, 2004, Santoro and Gaudino, 2005). Using Flii$^{+/-}$, wild-type, Flii$^{Tg/+,}$ and

$CD151^{-/-}$ mice we now provide insight into the role of Flii in wound repair and show that Flii directly regulates integrin-mediated adhesion and migration through interaction with focal complex proteins. In this way, Flii could modulate adhesion-dependent signaling pathways involved in wound repair. Flii role in adhesion signaling pathways will be discussed further in Chapter 5 of this thesis.

The tetraspanin CD151, an integral component of hemidesmosomes, is expressed in both keratinocytes and fibroblasts. Although its effects on migration depend on both cell type and extracellular matrix substrates, studies have shown that CD151 activity enhances integrin cell adhesion structures by providing hemidesmosome and intercellular junction stability (Chometon *et al*., 2006). Decreased expression of CD151 impairs the function of its primary interaction partners, laminin binding integrin receptors α3β1 and α6β4 (Chometon *et al*., 2006, Geary *et al*., 2008). We have previously shown that CD151 is involved in wound re-epithelialization, affecting both cellular adhesion and migration, and that CD151 null mice exhibit retarded epidermal and dermal contraction (Cowin *et al*., 2006, Geary *et al*., 2008). CD151 regulates adhesion by being an integral part of hemidesmosomes, where it associates with integrin α3β1 and laminin-5 to form pre-hemidesmosomal integrin clusters serving as a nucleation site for the assembly of stable hemidesmosomes by the integrin α6β4 (Litjens *et al*., 2006). Recent studies indicate that CD151 catalyzes cell migratory activity by regulating the glycosylation of integrin α3β1 (Baldwin *et al*., 2008) and indirectly associates with collagen and fibronectin integrin receptors, α2β1, α5β1 respectively, to influence fibroblast mediated dermal contraction (Geary *et al*., 2008, Liu *et al*., 2007, Sincock *et al*., 1999).

Flii$^{+/-}$ mice have significantly increased CD151 and laminin protein expression in response to wounding which may result in up-regulated activity of integrin receptors and contribute to improved cellular migration and wound contraction through association of tetraspanin CD151 with different integrin receptors and laminin extracellular matrix substrate. Increased CD151 and laminin expression may result in an enhanced hemidesmosome assembly upon wound re-epithelialization, as illustrated in unwounded skin of Flii$^{+/-}$ mice in Chapter 3 of this thesis, as CD151 and laminin are integral protein components of hemidesmosome adhesion structures. Laminin-5 is a major component of the basement membrane and its processing is required for stable hemidesmosome formation and control of cellular behaviour by interaction with integrin receptors in a bidirectional signaling between the extracellular matrix and the cell interior (Dogic *et al*., 1998, Hintermann and Quaranta, 2004). The expression of laminin-binding integrin receptors are key determinants of rapid wound re-epithelialization. Moreover, laminin-5 provides a surface over which cells migrate, potentially promoting faster wound healing as we have previously observed in Flii$^{+/-}$ mice (Cowin *et al*., 2007). Furthermore, Flii$^{Tg/+}$ mice wounds had significantly decreased CD151 and laminin expression compared with wild-type counterparts which may have affected the observed irregular formation of poorly defined hemidesmosomes by decreasing the number of pre-hemidesmosomal integrin clusters and delayed re-epithelialization and wound contraction observed in these mice (Cowin *et al*., 2007).

Tetraspanin CD151 has also been shown to modulate integrin outside-in signaling and stabilise the affinity conformation of integrin α3β1 required for effective interaction of this integrin with its laminin ligand while its indirect association with both collagen and fibronectin integrin subunits has also been show to have functional consequences (Geary *et al*., 2008). In agreement with these results, the correlation between Flii and CD151

expression during wound healing was further examined in CD151$^{-/-}$ mice wounds. This revealed significantly increased Flii expression compared to wild-type counterparts, and these CD151$^{-/-}$ mice also exhibit impaired wound healing (Cowin *et al*., 2006). Increase of Flii expression in CD151$^{-/-}$ mice might reflect the impaired wound healing of these mice or impaired expression of integrin receptors normally activated by tetraspanin CD151. These results suggest that Flii may regulate wound repair through its association with CD151 and subsequent effects on integrin mediated downstream adhesion signaling.

We further investigated the effects of Flii gene expression on different integrin subunits and basement membrane and extracellular matrix proteins important during wound repair. The expression levels of collagen and fibronectin integrin subunits, collagen IV or fibronectin showed no significant difference in wounded Flii$^{+/-}$, wild-type and Flii$^{Tg/+}$ skin. However, examining the expression of the predominant binding partners of CD151 in epithelial tissue, laminin-binding integrin chains α3, β1, α6, β4 suggested a role for Flii in regulating integrin-mediated cellular adhesion and migration. Integrin receptor α3β1 binds to unprocessed laminin and plays an important role in determining the incorporation of laminin into its higher order structure within extracellular matrix, hence stimulating the migration of the first row of keratinocytes over the provisional wound matrix which then secrete more laminin for subsequent cells to migrate and cover the wound (deHart *et al*., 2003, Nguyen *et al*., 2000). Although we observed elevated α3 expression in unwounded skin of Flii$^{+/-}$ versus wild-type versus Flii$^{Tg/+}$ mice, all subsequent effects of wound healing were in proportion to these basal levels.

In contrast, reduced Flii gene expression resulted in increased integrin β1 protein levels, whereas over-expression of Flii led to decreased levels of integrin β1, which may have resulted in a decreased expression of different integrin receptors or their affinity for

extracellular matrix ligands and could adversely affect cellular migration and fibroblast-mediated wound contraction. Integrin β1 chain expression is vital during wound repair, as it associates with different integrin α chains to form laminin, collagen, and fibronectin receptors and its loss or decrease results in impaired cell migration, decreased cell proliferation and aberrant deposition of extracellular matrix proteins namely, laminin and collagen (Brakebusch and Fassler, 2005). Integrin β1 also directly links to the actin cytoskeleton and a cytoskeletal complex of structural, adaptor and signaling proteins, providing a degree of stabilization which is tightly regulated to provide optimal traction forces for cellular migration (Chometon *et al*., 2006).

Mouse wounds displayed an altered expression of integrin α6 and β4 chains in response to different Flii gene levels. These two integrin chains combine to form a functional α6β4 integrin receptor which binds to processed laminin and stabilizes the cell–substrate interactions and induces formation of hemidesmosomes once the wound is re-epithelialized (Jones *et al*., 1998). However, laminin – α6β4 complex may also lead to the assembly of immature hemidesmosomes present at early stages of wound healing, which may be more dynamic that the mature stable hemidesmosomes in allowing cell migration during wound healing while also providing stability for the cell-wound bed interactions (Tsuruta *et al*., 2003). Integrin α6 staining was observed in basal keratinocytes and fibroblasts within the wound matrix. Interestingly, at day 3 post-wounding, wild-type and Flii$^{Tg/+}$ wounds had significantly increased integrin α6 expression compared with Flii$^{+/-}$ wounds, suggesting that Flii$^{+/-}$ mice may have weaker cell–substrate interactions. At day 7 post-wounding, once the wound was re-epithelialized, Flii$^{Tg/+}$ wounds had a significantly reduced expression of α6, which may lead to impairment in the formation of new hemidesmosomes.

In contrast to integrin α6 chain expression, we found that both Flii$^{+/-}$ and Flii$^{Tg/+}$ wounds displayed an earlier, increased response in integrin β4 chain expression upon wounding compared with wild-type wounds, where integrin β4 chain expression peaked at day 7 post-wounding. We observed a redistribution of integrin β4 chain to the basolateral/suprabasal surface in agreement with studies in α6 knockout mice, where integrin β4 chain is not concentrated in basal keratinocytes, suggesting that polarized expression of integrin β4 is dependent upon the presence of an integrin a6 chain (Georges-Labouesse *et al*., 1996, Manohar *et al*., 2004). The long cytoplasmic chain of integrin β4 recruits a number of signaling molecules involved in cellular proliferation (Mainiero *et al*., 1997, Raymond *et al*., 2005) and increased expression of integrin β4 in the absence of integrin α6 chain suggests an involvement of integrin β4 in signaling cascades independent of its adhesive function. However, contradictory responses in integrin β4 expression with varying levels of Flii gene expression shown here suggest that integrin β4 chain expression may not be directly affected by Flii.

In agreement with our previous results (Cowin *et al*., 2007) and results presented in Chapter 3 and 4 of this thesis indicate a role for Flii in cellular migration, adhesion and proliferation, however the exact mechanisms by which Flii affects wound healing are yet to be elucidated. Although Flii is secreted in response to wounding and its extracellular roles are yet to be identified (Cowin *et al*., 2007), Flii intracellular roles include actin remodelling and nuclear transcription, implicating Flii involvement in different signaling pathways. Flii is associated with actin arcs and membrane ruffles where it co-localizes with different molecules involved in regulating cytoskeleton re-organization, including Ras and Cdc42, and has both cytoskeletal and nuclear functions suggesting its involvement in numerous different pathways (Campbell *et al*., 2002, Davy *et al*., 2000, Lee *et al*., 2004). It has been speculated that Flii may link signal transduction and cytoskeletal regulation (Fong

and de Couet, 1999). In addition, Flii is a transcriptional co-activator with the estrogen and the thyroid receptors (Lee *et al*., 2004) and studies have revealed that it interferes with transcription by competing with β-catenin and FLAP1, an activator of Tcf/Lef-dependent genes (Lee and Stallcup, 2006). These studies illustrate that proteins like Flii which contain the leucine rich repeat domain could engage in numerous protein-protein interactions. Consequently, we examined the association of Flii with both integrin cytoplasmic chains and downstream cytoskeletal proteins.

Focal adhesions are important sites of signal transduction, where numerous proteins are involved in coordinating the integrin signals to downstream effectors. Indeed, a number of actin-binding cytoskeletal proteins, including talin and α-actinin, have been shown to be present at the epidermal–dermal interface and interact directly with the integrin β1 cytoplasmic domain of the hemidesmosome protein complex, forming a cytoskeletal complex and modulating different signal transduction pathways and cellular behaviour (Gonzalez *et al*., 2001, Zhang *et al*., 2008). Here, we show that Flii does not bind directly to integrin β1, β4, and tetraspanin CD151 during wounding; however, it does interact with talin, paxillin, and vinculin actin-binding proteins, which form the cytoskeletal complex directly linking the integrin receptors to the actin cytoskeleton (Critchley *et al*., 1999). Most interesting of these results is the association between Flii and talin and Flii and paxillin, suggesting possible mechanisms of Flii function in mediating cellular responses during wound healing.

Talin is a cytoskeletal linker protein that interacts directly with the cytoplasmic tails of all β-integrin subunits except integrin β4 subunit. Consequently, talin binds to a number of different integrin receptors involved in cellular responses of adhesion and migration during wound healing. This integrin-talin interaction “hyper-activates” integrin receptor

function and is required for efficient receptor responses to changing environment during tissue injury and wound repair (Harburger and Calderwood, 2009). This interaction was briefly discussed in the literature review section and is illustrated in Figure 1.8 of this thesis (Moser et al., 2009). Flii association with talin cytoskeletal protein suggests that Flii may modulate integrin-mediated cellular adhesion and migration through the regulation of the pool of talin available for binding and activating integrin receptors (Kligys and Jones, 2009). Over-expression of Flii could lead to sequestration of talin, resulting in decreased integrin activation and impaired wound healing as previously described in our studies (Cowin *et al*., 2007). On the contrary, decreasing Flii expression could increase the pool of talin available to activate integrins, possibly explaining the enhanced wound healing seen in Flii deficient mice. This proposed mechanism does not account for enhanced number of hemidesmosomes observed in Flii$^{+/-}$ mice skin (Chapter 3) as talin does not regulate the activity of integrin α6β4, however increased α3β1 activity would result in improved cellular migration. Moreover, increased CD151 expression as consequence of Flii deficiency could lead to enhanced nucleation and/or stabilization of hemidesmosomes in unwounded skin, independent of integrin activation induced by talin during wound healing.

The mechanism of talin regulation is not known. Recent studies show that talin interacts with other proteins like kindlin-1 and this interaction is required for efficient integrin activation (Moser *et al*., 2009). The cross-talk between talin and kindlin-1 and cooperative regulation of integrin affinity suggests that other proteins like Flii that do not directly bind to cytoplasmic tails of integrin β1 may also regulate integrin activity through modulation of binding between talin and kindlin-1, however this remains to be further investigated.

Our observation of the association between Flii and paxillin also suggests a possible mechanism behind the Flii effect on cellular adhesion and migration (described in Chapter 3). Paxillin is known to bind to many down-stream signaling proteins effecting the changes in the organization of the actin cytoskeleton required for cell motility and wound repair. Association of Flii with paxillin and its subsequent effect on paxillin phosphorylation and on downstream adhesion signaling, as well as formation of stable adhesion sites, will be described in detail in Chapter 5 of this thesis as these events are vital in determining cell adhesion and migration properties during wound healing.

In summary, our results in this Chapter suggest that Flii may be directly involved in integrin-mediated adhesion by binding to associated cytoskeletal complexes, hence suppressing the function of the activated ligand-bound integrin receptors and mediating integrin-dependent adhesion signaling pathways. Flii over-expression may result in decreased integrin clustering, altered hemidesmosome adhesion complex and inhibition of the delicate mechanical connection between ligand-bound integrin receptors and the actin cytoskeleton required to impel different adhesion-dependent signaling pathways. Consequently, Flii may cause altered expression levels of integrin receptors, laminin protein, and tetraspanin CD151, as observed in the impaired healing response of Flii over-expressing mouse wounds. Results presented here now demonstrate that Flii is a potentially important regulator of wound healing through processes involving hemidesmosome formation and integrin-mediated cellular adhesion and migration.

Chapter 5

FLIGHTLESS AFFECTS PAXILLIN PHOSPORYLATION AND SUPRESSES ADHESION SIGNALING PROTEINS

Parts of this chapter are accepted for publication in the Journal of Investigative Dermatology Feb 2011:

Kopecki Z, O'Neill G, Arkell R and Cowin AJ. Regulation of focal adhesions by Flightless I involves inhibition of paxillin phosphorylation via Rac1 dependent pathway 2010. Accepted for publication *J Invest Dermatol.* Feb 2011. **IF 5.5 RIF 1/39.**

5.1 Introduction

Numerous proteins present at the cytoplasmic face of focal adhesions are involved in both focal adhesion structure and downstream signaling pathways. Clustering of the signaling and structural adaptor proteins to the cytoplasmic tail of integrin subunits allows for the formation of multi-molecular complexes and transmission of the signals from the extracellular matrix to the actin cytoskeleton, resulting in a controlled balance between cellular adhesion and migration during wound healing (Sastry and Burridge, 2000). Integrin subunits at sites of focal adhesions provide bi-directional cross-talk across the cell membrane and dynamically link the intracellular and extracellular microenvironments, resulting in precisely regulated cellular morphology, adhesion and migration (Berrier and Yamada, 2007).

Initial integrin mediated cell-matrix adhesions, termed focal complexes, develop underneath lamellipodia and are driven by actin polymerization. A proportion of focal complexes develop into focal adhesions which are associated with contractile stress fibers (Berrier and Yamada, 2007). Focal adhesions function as both adhesion sites and signal transduction mechanosensors allowing cells to adapt to the changing extracellular matrix protein composition and cellular environment encountered during wound healing (Bershadsky *et al*., 2006). Focal adhesions have the ability to transform into fibrillar adhesions that can modify the structure and rigidity of the extracellular matrix, hence contributing to tissue remodeling during wound healing (Arnaout *et al.*, 2007). A vital function of focal adhesions is to anchor the polymerized actin filament stress fibers into bundles which provide the contractile force required for effective translocation of the cell body during cellular migration, however impaired turnover of focal adhesions results in

inhibited cellular migration (Bach *et al*.). Proteins like Flii that are involved in regulating actin organization and polymerization are essential mediators of membrane protrusions and cell migration. Previous studies have shown that Flii colocolizes with molecules involved in regulating cytoskeletal reorganisation, including members of the Rho family of GTPases: Ras and Cdc42, suggesting a common functional pathway in Flii mediated cytoskeletal regulation (Davy *et al*., 2001). It is currently unclear whether this interaction is direct as these proteins can lie downstream or upstream of Flii and can link to Flii via proteins like talin, paxillin and vinculin (Davy *et al*., 2000). Rho family of GTPases act as GTP-dependent molecular switches that bind and modulate the activities of effector molecules regulating actin cytoskeleton including formation of focal adhesions and actin stress fibers (Tominaga *et al*., 2000). Closely regulated activation of Rho family of GTPases and turnover and phosphorylation of focal adhesion proteins allows cells to respond appropriately to their environment. Focal adhesion proteins like paxillin provide a point of signal convergence, a platform for protein kinases such as Src which are stimulated by various growth factors like to interact with other signaling proteins hence allowing downstream signaling pathways to proceed (Goetz, 2009).

Studies have shown that paxillin has the ability to bind numerous proteins that are involved in actin cytoskeleton organization during cell motility and results described in the previous chapter have demonstrated that Flii also associates with paxillin, suggesting a potential mechanism for Flii regulation of cellular adhesion and migration. Indeed, various proteins use paxillin as a substrate and a docking site to regulate, disturb or bypass the normal adhesion and growth factor signaling cascades required during controlled cell migration, adhesion and proliferation (Kopecki *et al*., 2009, Schaller, 2001, Turner, 1998). Flii over-expressing fibroblasts have impaired cellular adhesion, spreading and delayed

wound scratch closure (Cowin *et al.*, 2007, Kopecki *et al*., 2009) (see Chapter 3). However, it is unclear whether the effects of Flii on actin remodelling play a role in its ability to regulate wound healing. Current studies suggest that its actin remodelling properties are important for processes involved in early development while its ability to regulate integrin-mediated adhesion and interaction with adaptor proteins involved in focal adhesion complexes, namely talin, paxillin and vinculin, are vital during skin wound repair (Kligys and Jones, 2009, Kopecki *et al*., 2009) (see Chapter 4). This chapter further describes the effect of differential Flii gene expression on integrin-mediated focal adhesion formation and turnover and association with adaptor, structural and signaling proteins involved in focal adhesion dynamics and downstream adhesion signaling.

5.2 Results

5.2.1 Flightless over-expressing fibroblasts have increased actin stabilization and focal adhesion formation

Previous studies have identified that over-expression of Flii protein results in impaired fibroblast adhesion, spreading and migration (Cowin *et al*., 2007, Kopecki *et al*., 2009) (see Chapter 3). Flii associates with focal complex proteins like paxillin which is involved in cellular adhesion signaling and actin cytoskeleton modulation (Kopecki *et al*., 2009) (see Chapter 4). Consequently, using fibroblasts extracted from $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ mice we examined effect of differential Flii gene expression on actin distribution and focal adhesion formation by coimmunostaining of phalloidin and paxillin respectively. In agreement with observations in Chapter 3 of this thesis, both $Flii^{+/-}$ and wild-type fibroblasts showed effective cellular spreading, developed a filamentous actin network and lamellipodial crawling on the extracellular matrix with actin meshwork at the leading

edges of the cell, suggestive of directional cell movement (Fig 5.1). In contrast, phalloidin staining of filamentous actin fibers showed increased filamentous actin in Flii$^{Tg/Tg}$ fibroblasts with formation of prominent ventral stress fibers linking focal adhesion sites across the cell periphery (Fig 5.1) suggesting that Flii over-expression may result in increased filamentous actin content. Flii$^{Tg/Tg}$ fibroblasts illustrated individual focal adhesion staining around the entire cell periphery and no evident focal complexes (Fig 5.1). In agreement with observations of increased stress fibers in Flii$^{Tg/Tg}$ fibroblasts, significantly increased filamentous F-actin content was measured in Flii$^{Tg/Tg}$ fibroblasts compared to wild-type fibroblasts at 15min post seeding and compared to both Flii$^{+/-}$ and wild-type fibroblasts at 60min and 24hrs post seeding respectively (Fig 5.2).

The effect of Flii on cellular adhesion was further investigated by examining the focal adhesion and focal complex formation by Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ fibroblasts. Paxillin is an important focal adhesion protein that is phosphorylated in response to integrin-matrix interaction where it functions as an adaptor protein facilitating focal adhesion turnover and downstream signaling required for effective cellular migration. Paxillin staining of focal adhesions revealed formation of focal complexes and focal adhesions at the leading edges of Flii$^{+/-}$ and wild-type fibroblasts and increased peri-nuclear expression of paxillin. Assessment of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ fibroblasts revealed a significantly increased number and larger focal adhesions in Flii$^{Tg/Tg}$ fibroblasts compared to both Flii$^{+/-}$ and wild-type counterparts (Fig 5.3A-B). Using paxillin immunohistochemistry the effect of differential Flii expression on fibroblast formation of focal complexes was also investigated. Examining the number of focal complex positive cells showed that over-expression of Flii results in a significantly decreased percentage of fibroblasts containing focal complexes compared to both Flii$^{+/-}$ and wild-type

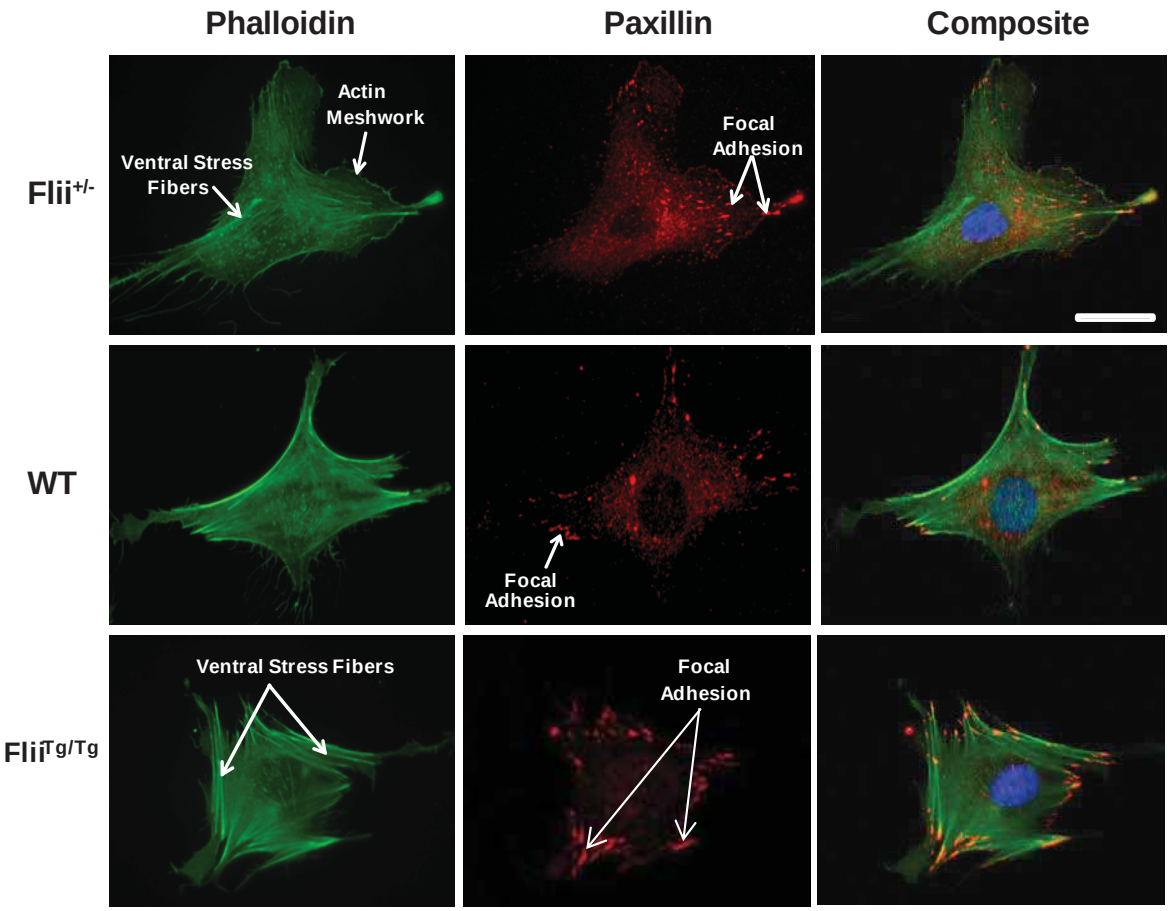

Figure 5.1: Flightless I over-expression stabilizes actin filaments and impairs focal adhesion turnover. Primary fibroblasts extracted from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice skin were plated on fibronectin and laminin substrates and sub-confluent cells, 90 min after seeding were fixed and coimmunostained with phalloidin (green) and paxillin (red) antibody. Phalloidin staining of filamentous actin fibers shows actin meshwork at the leading edges of Flii$^{+/-}$ and WT fibroblasts and increased ventral stress fibers in Flii$^{Tg/Tg}$ fibroblasts. Paxillin staining shows focal adhesions at the leading edge of cells associated with actin meshwork and increased peri-nuclear paxillin staining in both Flii$^{+/-}$ and WT fibroblasts. Paxillin stained Flii$^{Tg/Tg}$ fibroblasts had individual focal adhesions around the periphery of the cell. Magnification x40. Scale Bar=25μm refers to all images.

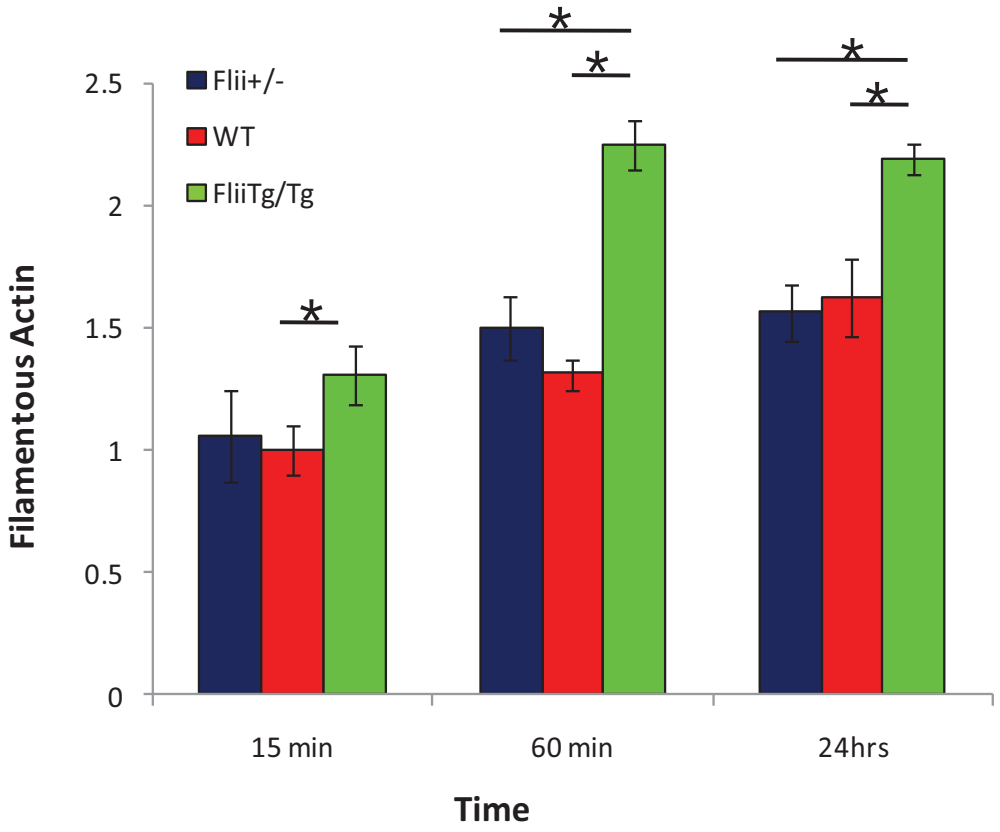

Figure 5.2: Flightless I over-expressing fibroblasts have increased expression of filamentous actin. Quantification of F-actin content in Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts adhering to fibronectin coated wells for 15 min, 60 min and 24 hrs. Cells were fixed, phalloidin stained and the amount of filamentous actin determined by quantifying the amount of bound phalloidin. The relative F-actin content was determined by dividing the amount of bound phalloidin in fibronectin adherent cells by the amount of bound phalloidin in fibronectin adhered WT cells at 15 min post seeding for all cell types and time-points. Flii$^{Tg/Tg}$ fibroblasts show significantly increased filamentous actin at 15 min compared to WT fibroblasts and at 60 min and 24 hrs compared to both Flii$^{+/-}$ and WT fibroblasts, suggesting that increased Flii expression may be involved in increased filamentous actin assembly to stress fibers in response to cell adherence to fibronectin. Mean +/- SEM from triplicate samples of different experiments. *P<0.05.

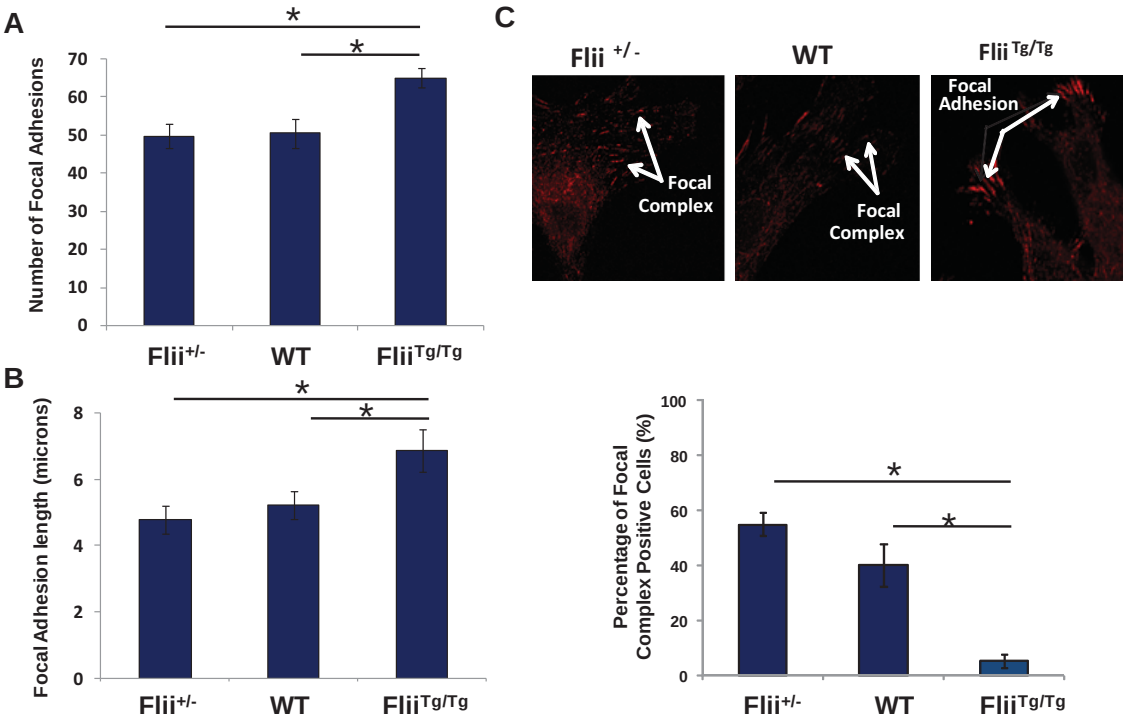

Figure 5.3: Flightless over-expressing fibroblasts have increased focal adhesions and reduced focal complex formation. Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts were fixed after 90 min of seeding on mixture of fibronectin and laminin substrates at 60% density and analysed for focal adhesion and focal complex formation. **A-B** Graphical representation of individual focal adhesion assessments represented as a mean of 10 different cells. Flii$^{Tg/Tg}$ fibroblasts have a significantly increased number of focal adhesions which are longer in length compared to both Flii$^{+/-}$ and WT fibroblasts. **C** Representative images of focal complexes at leading edges of Flii$^{+/-}$ and WT cells and focal adhesion at the edges of Flii$^{Tg/Tg}$ fibroblasts. Flii$^{+/-}$ and WT fibroblasts plated onto fibronectin and laminin substrates and stained with paxillin illustrate focal complex formation while Flii$^{Tg/Tg}$ fibroblasts have significantly reduced percentage of cells positive for focal complex formation compared to both Flii$^{+/-}$ and WT fibroblasts. n=100 cells measured. Focal adhesion and focal complexes were distinguished based on size, location and composition (see Figure 1.8). Magnification x100. Scale Bar=10μm refers to all images. Mean +/- SEM. *P<0.05.

cells (Fig 5.3C-D). Interestingly, Flii deficient cells showed no significant difference in focal adhesion or focal complex formation compared to wild-type fibroblasts but significantly more focal complex positive cells compared to Flii$^{Tg/Tg}$ fibroblasts (Fig 5.3A-D).

5.2.2 Reduced paxillin phosphorylation in Flightless over-expressing fibroblasts impairs focal adhesion turnover

Results described in Chapter 4 of this thesis have shown that Flii associates with paxillin in a cytoskeletal complex, suggesting a possible mechanism of Flii effect on integrin mediated cellular adhesion and migration. To explore the role of Flii in focal adhesion dynamics the phosphorylation of paxillin on tyrosine residue 118 was examined, a major site for downstream adhesion mediated signaling (Petit *et al*., 2000). Paxillin and p-paxillin immunostaining of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ fibroblasts plated on fibronectin and laminin extracellular matrix substrates revealed decreased expression of p-paxillin in Flii$^{Tg/Tg}$ fibroblasts both at cell periphery and under the cell body (Fig 5.4A). Semiquantitative calculation of the distribution of ratios of p-paxillin over total paxillin levels at individual focal adhesions showed significantly decreased levels of p-paxillin in Flii$^{Tg/Tg}$ fibroblasts compared to both Flii$^{+/-}$ and wild-type fibroblasts (Fig 5.4B).

Consistent with the immunohistochemical pattern of significantly decreased paxillin phosphorylation in Flii$^{Tg/Tg}$ fibroblasts compared to both Flii$^{+/-}$ and wild-type cells, p-paxillin protein levels, as measured by Western blotting, were significantly decreased in Flii$^{Tg/Tg}$ fibroblasts, suggesting impaired focal adhesion turnover (Fig 5.4C-D). While Flii$^{+/-}$ fibroblasts did show an increasing trend towards higher levels of p-paxillin this increase

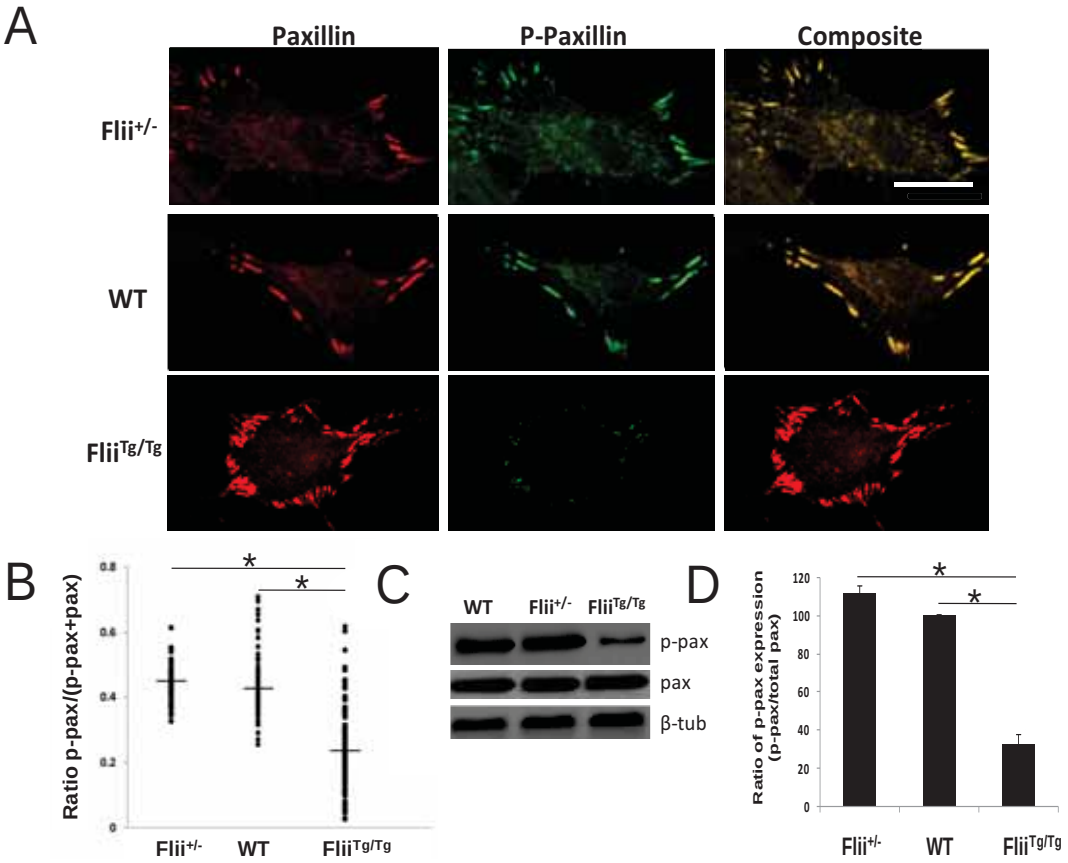

Figure 5.4: Flightless over-expressing fibroblasts have reduced paxillin phosphorylation. Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts were fixed after 90 min of seeding on mixture of fibronectin and laminin substrates at 60% density and analysed for paxillin phosphorylation. **A** Representative images of Paxillin (red) and P-Paxillin (green) immunostaining of fibroblasts plated on fibronectin and laminin coated coverslips, showing decreased expression of P-Paxillin in Flii$^{Tg/Tg}$ fibroblasts. Magnification x40. Scale Bar=25μm refers to all images. **B** Distribution of the ratios of P-Paxillin at individual focal adhesions (n=100 from >5 different cells). Horizontal bar indicates the mean of each population. p-pax=phosphopaxillin; pax=total paxillin. Flii$^{Tg/Tg}$ fibroblasts have significantly decreased p-paxillin compared to both Flii$^{+/-}$ and WT fibroblasts. **C-D** Western blot analysis of phosphorylated and total paxillin levels and β-tubulin to demonstrate equal loading. Image was quantified using GeneSnap analysis software program (Syngene, Maryland, USA). Flii$^{Tg/Tg}$ fibroblasts have significantly decreased levels of p-paxillin compared to both Flii$^{+/-}$ and WT fibroblasts suggesting impaired focal adhesion turnover. The histogram shows the ration of p-paxillin divided by paxillin. All values were first normalized to β-tubulin. The data show the mean of three independent experimental repeats. Mean +/- SEM. *P<0.05.

was not statistically significant when compared to wild-type counterparts (Fig 5.4A-D and Fig 5.5). We further investigated this result by assessing paxillin and p-paxillin fluorescence intensity at individual focal adhesions using line scan analysis in over 150 individual focal adhesions which also revealed significantly decreased p-paxillin levels in $Flii^{Tg/Tg}$ fibroblasts (Fig 5.5).

5.2.3 Flightless over-expressing fibroblasts have enhanced fibrillar adhesion formation

Increased staining of centrally located focal adhesions (Fig 5.1) and peri-nuclear p-paxillin expression (Fig 5.4A) in $Flii^{+/-}$ and wild-type fibroblasts suggested efficient turnover of cell adhesion structures required for directional cellular migration. To further explore the effect of Flii in mediating cellular adhesion and migration, immunohistochemical analysis of tensin and fibronectin staining was analysed in $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ fibroblasts plated on collagen and laminin extracellular matrix substrates. Fibrillar adhesions form in response to increased acto-myosin contractibility and are distinguished both by parallel staining of fibronectin to the actin stress fibers and colocalization between paxillin and tensin (Bach *et al*., 2009). In agreement with a pattern of decreased cellular spreading observed in Chapter 3 of this thesis, increased stress fiber formation (Fig 5.1) and decreased expression of phosphorylated paxillin (Fig 5.4) the data presented in Figures 5.6 and 5.7 shows that $Flii^{Tg/Tg}$ fibroblasts also exhibit enhanced fibrillar adhesion. $Flii^{Tg/Tg}$ fibroblasts have parallel staining of fibronectin with actin stress fibers and colocalization between paxillin and tensin indicative of enhanced fibrillar adhesions (Fig 5.6 and 5.7). In contrast, $Flii^{+/-}$ and wild-type fibroblasts appear to have increased peri-nuclear fibronectin expression and punctuate tensin staining suggesting that these cells have a highly dynamic turnover of adhesion structures.

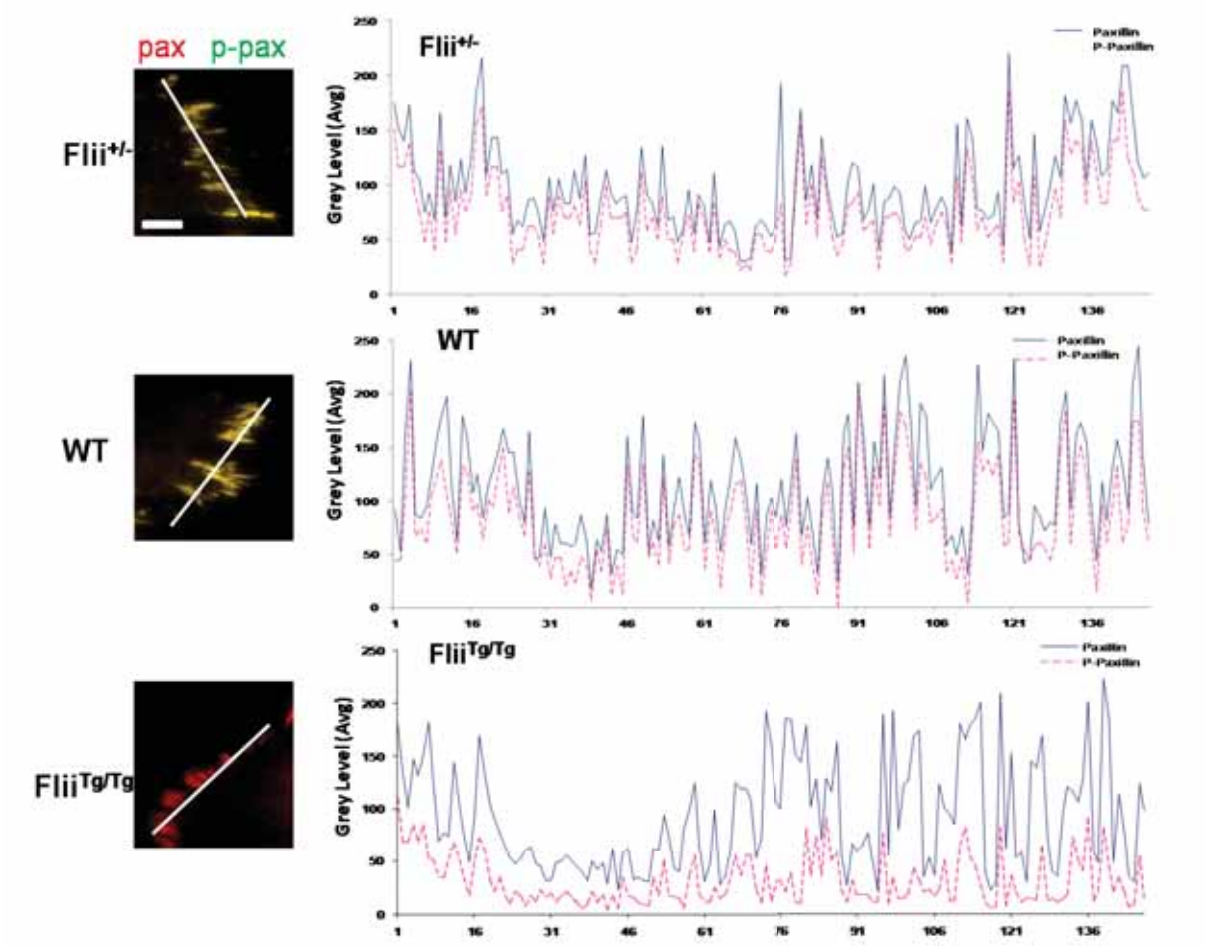

Figure 5.5: Reduced expression of phosphopaxillin in Flightless over-expressing fibroblasts impairs focal adhesion turnover. Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts were fixed after 90 min of seeding on mixture of fibronectin and laminin substrates at 60% density and analysed for paxillin phosphorylation using immunocytochemistry and pixel line scans. Line scans showing fluorescence intensities of individual focal adhesions. Representative composite images of Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblast focal adhesions stained for paxillin (red) and p-paxillin (green), composite = yellow. Fluorescence intensities expressed as Grey Level average are shown in solid blue line (paxillin), and dotted red line (p-paxillin). Flii$^{Tg/Tg}$ fibroblast focal adhesions have reduced expression of p-paxillin. Line scans of individual focal adhesions were performed on 5-pixel width lines using AnalySIS software package (Soft – Imaging System GmbH, Munster, Germany). n=150 individual focal adhesions. Scale bar=5μm refers to all images.

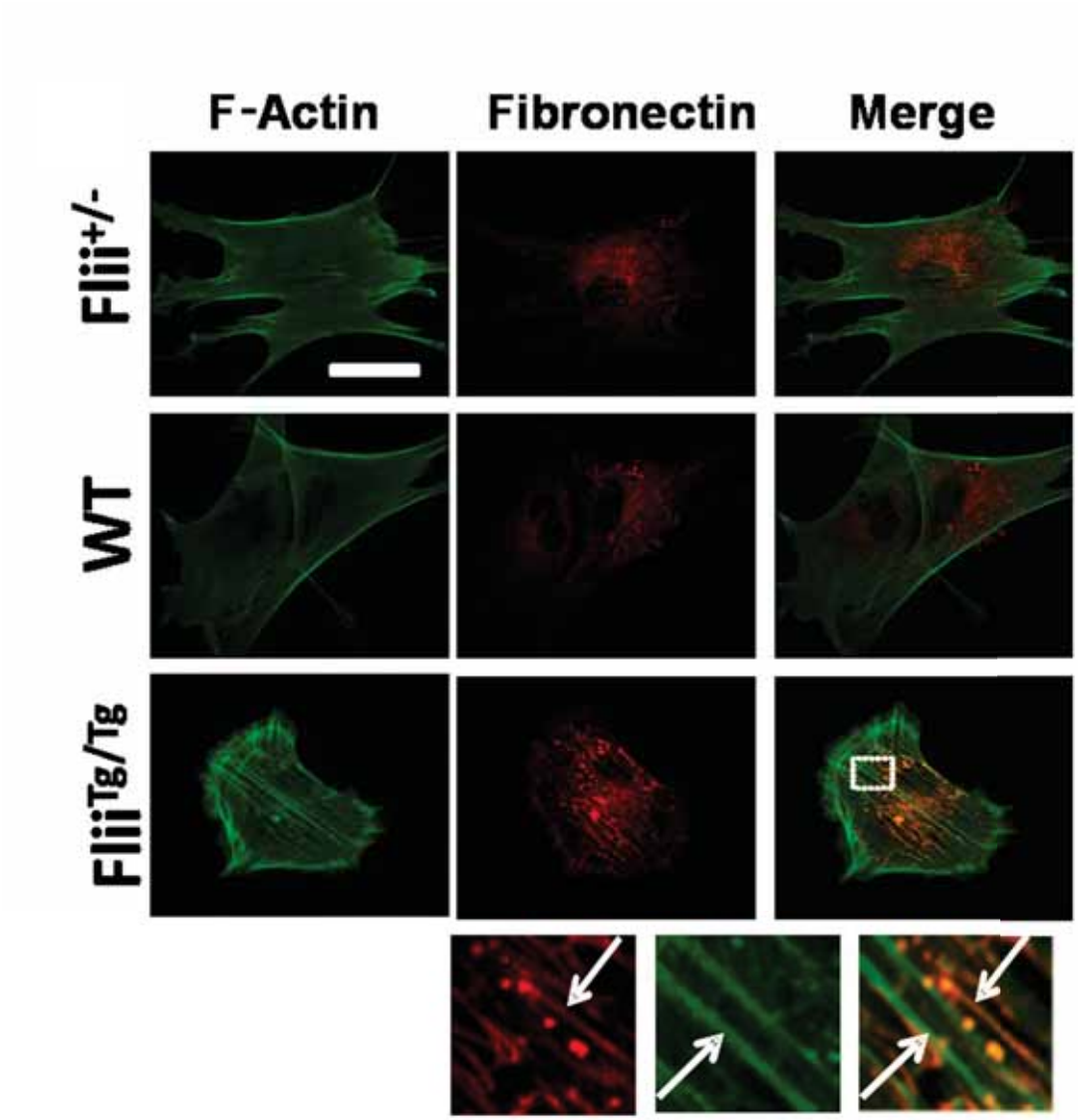

Figure 5.6: Enhanced fibrillar adhesion formation in Flightless over-expressing fibroblasts. Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts were fixed after 90 min of seeding on mixture of collagen and laminin substrates at 60% density and analysed for fibrillar adhesion formation using immunohistochemistry. Representative images of cells coimmunostained for F-actin (green) and fibronectin (red). Magnification x40. The boxed region in the Flii$^{Tg/Tg}$ merged cell images is magnified at higher magnification (x100) below. Arrows indicate F-actin (green) and fibronectin (red) in positive fibrillar adhesions. Flii$^{Tg/Tg}$ fibroblasts exhibit enhanced fibrillar adhesions with parallel staining of fibronectin and F-actin compared to both Flii$^{+/-}$ and WT fibroblasts. Flii$^{+/-}$ and WT fibroblasts do not exhibit parallel fibronectin and F-actin fibers. Scale Bar=25μm and refers to all images.

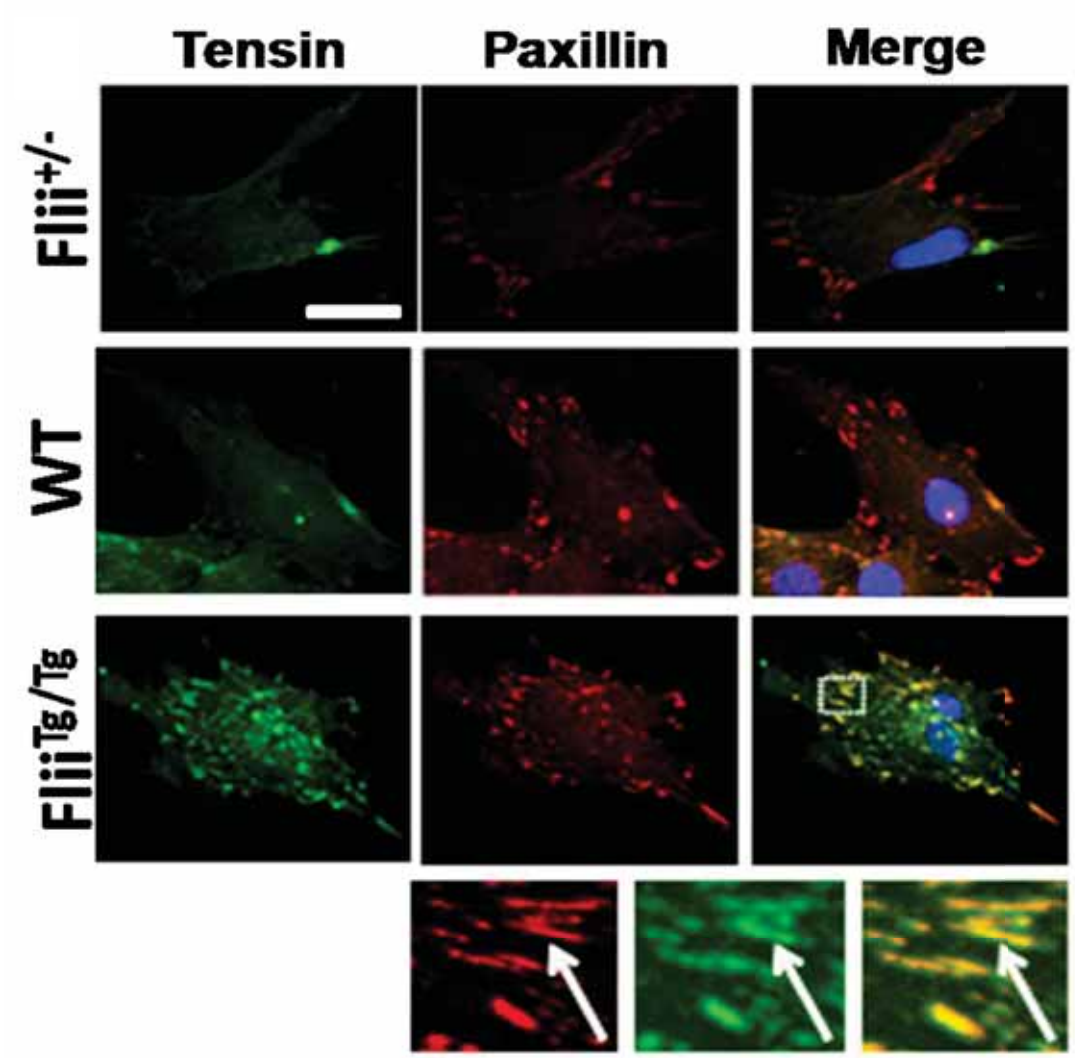

Figure 5.7: Enhanced fibrillar adhesion formation in Flightless over-expressing fibroblasts. Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts were fixed after 90 min of seeding on mixture of collagen and laminin substrates at 60% density and analysed for fibrillar adhesion formation using immunohistochemistry. Representative images of cells coimmunostained for tensin (green) and paxillin (red). Magnification x40. The boxed region in the Flii$^{Tg/Tg}$ merged cell images is magnified at higher magnification (x100) below. Arrows indicate tensin (green) and paxillin (red) colocalization in positive fibrillar adhesions. Flii$^{Tg/Tg}$ fibroblasts exhibit enhanced fibrillar adhesions with tensin and paxillin colocalization compared to both Flii$^{+/-}$ and WT fibroblasts. Flii$^{+/-}$ and WT fibroblasts do not exhibit tensin and paxillin colocalization. Scale Bar=25μm and refers to all images.

5.2.4 Over-expression of Flightless results in impaired cellular spreading and down regulation of adhesion signaling pathways

Chapter 3 of this thesis describes the association of Flii with talin and vinculin in the cytoskeletal complex. Flii association with talin was postulated to control the pool of talin available for integrin activation (Kligys and Jones, 2009, Kopecki *et al*., 2009). Using primary $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ fibroblasts, the differential effect of Flii gene expression was examined on a number of structural and signaling proteins involved in integrin-mediated cellular adhesion including talin, vinculin, α-actinin, Src tyrosine kinase and downstream target of adhesion signaling p130Cas. Additionally, we investigated the effect of Flii on activation of Rho family of GTPases including: RhoA, Rac1 and Cdc42. No significant difference was observed in the expression of talin and there was a trend towards elevated expression of vinculin in $Flii^{Tg/Tg}$ fibroblasts, however α-actinin was significantly increased in $Flii^{Tg/Tg}$ fibroblasts compared to both $Flii^{+/-}$ and wild-type counterparts (Fig 5.8A-B). Using immunohistochemistry it was demonstrated that $Flii^{+/-}$ and wild-type fibroblasts have mainly peri-nuclear α-actinin, with low levels of α-actinin at sites of focal adhesions (Fig 5.8C). Increased intracellular α-actinin expression is associated with decreased focal adhesion dynamics (Le Clainche and Carlier, 2008) and results presented here reveal that $Flii^{Tg/Tg}$ fibroblasts had increased expression of α-actinin at both peri-nuclear region of the cell and at cell periphery at sites of focal adhesions (Fig 5.8C). Over expression of α-actinin is a marker of focal adhesions which exhibit impaired turn-over (Webb *et al*., 2004). Addressing both cellular spreading and impaired turn-over of adhesion structures we examine the spreading of $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ fibroblasts on a mixture of extracellular substrates over a time course of 120 minutes, demonstrating impaired cellular spreading compared to both $Flii^{+/-}$ and wild-type counterparts (Fig 5.9A).

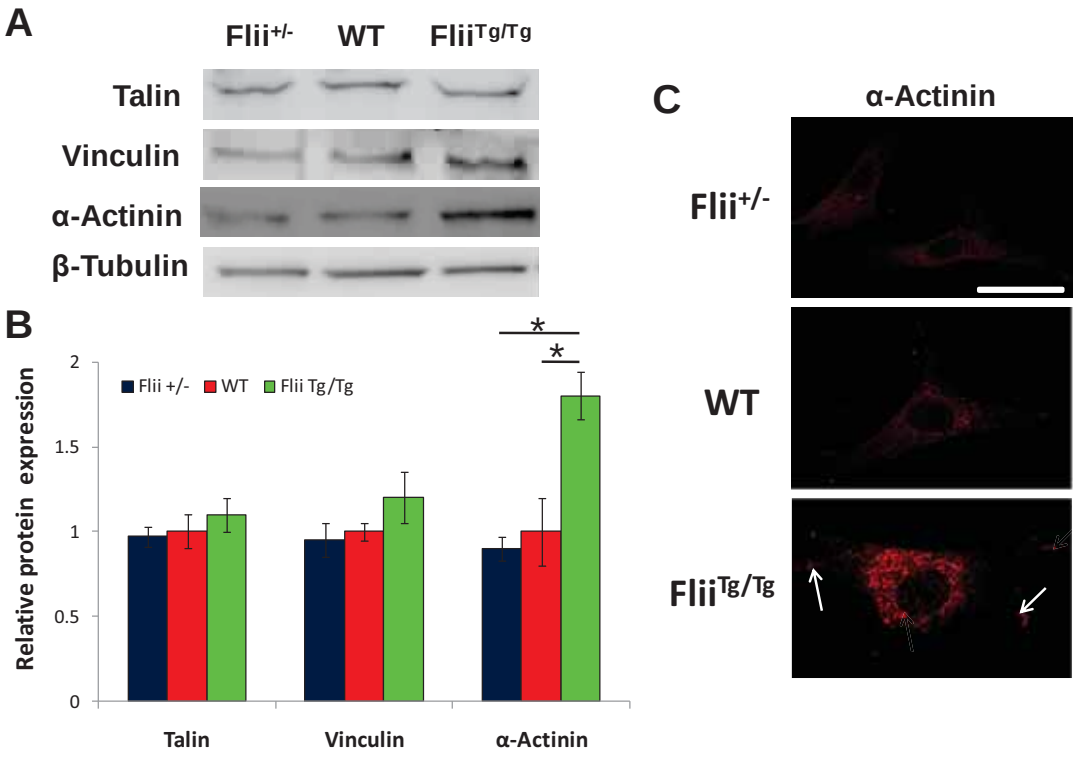

Figure 5.8: Effect of Flightless on expression of different cytoskeletal proteins involved in formation of adhesion structures. A-B Protein extracts from sub-confluent Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts were analysed for expression of focal adhesion proteins including: talin, vinculin and α-actinin levels and β-tubulin to demonstrate equal loading using Western blot analysis. The histogram shows the relative expression of talin, vinculin and α-actinin expression in Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts as determined by band densitometry of experimental replicates. All values were first normalized to β-tubulin. Flii$^{Tg/Tg}$ fibroblasts have increased levels of α-actinin, a marker of inactive focal adhesions, compared to both Flii$^{+/-}$ and WT fibroblasts. Mean +/- SEM. *P<0.05. **C** α-actinin staining of primary Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts plated on fibronectin and laminin substrates shows mainly peri-nuclear α-Actinin in Flii$^{+/-}$ and WT fibroblasts and increased expression in Flii$^{Tg/Tg}$ fibroblasts with α-actinin staining both around nucleus and at sites of focal adhesions (white arrows). Magnification x40. Scale Bar=25μm refers to all images.

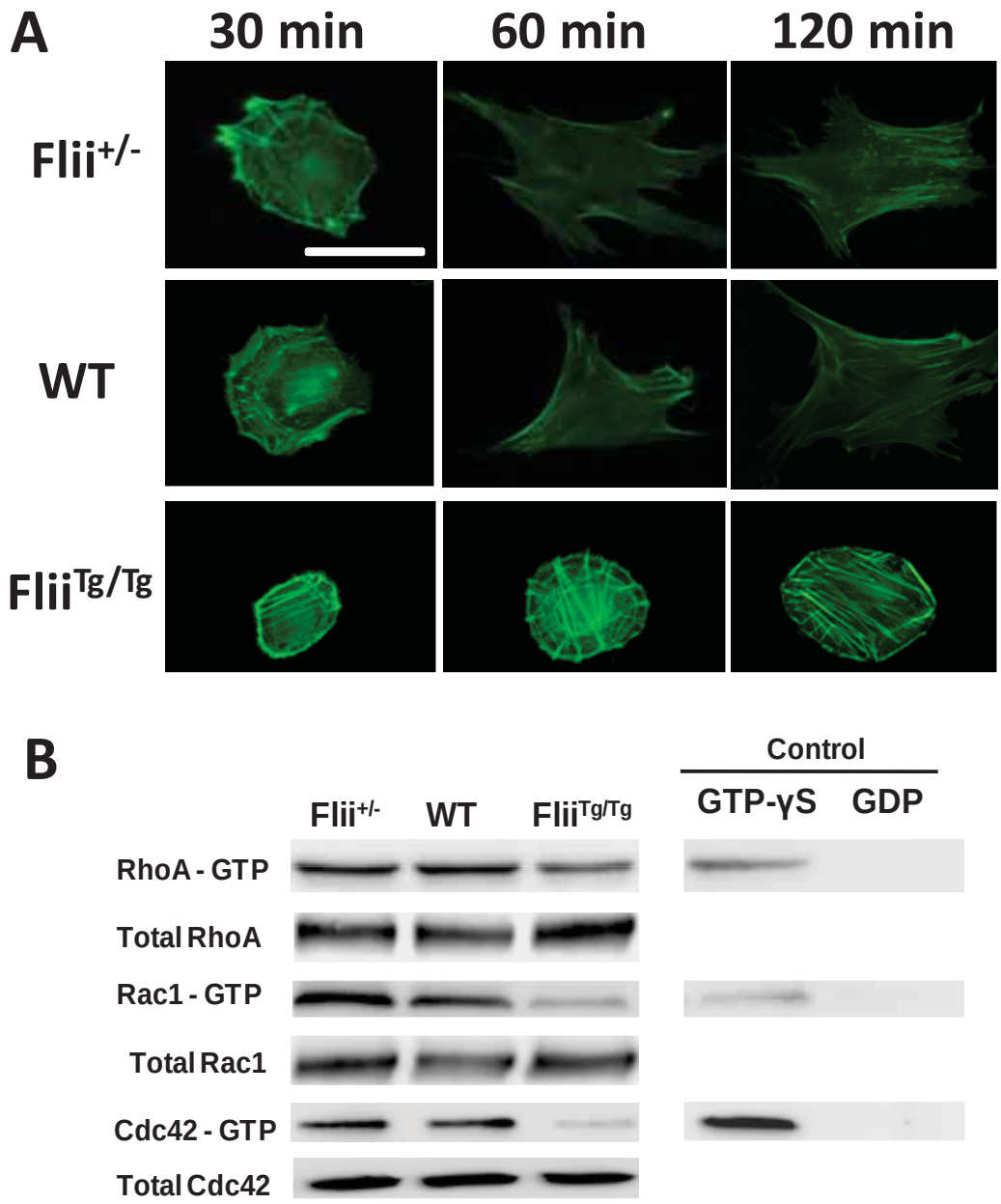

Figure 5.9: Effect of Flightless on cellular spreading and activation of Rho family of GTPase. A Spreading of Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts on a mixture of extracellular matrix proteins (laminin and collagen) over a time course of 120min post seeding. Images show representative FITC-Phalloidin stained cells highlighting filamentous actin during different stages of cell spreading. Flii$^{Tg/Tg}$ fibroblasts reveal delayed cellular spreading and increased formation of actin stress fibers. Magnification x40. Scale Bar = 20μm refers to all images. **B** Total and active levels of RhoA, Rac1 and Cdc42 in cell lysates of sub confluent Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts as determined by pull down assay and Western Blotting. Flii$^{Tg/Tg}$ fibroblasts have decreased activation of Rac1 and Cdc42 GTPases required for focal complex formation and turnover of adhesion structures. Image is a representative of three independent experimental replicates.

Dynamic regulation of Rho family of GTPases activity plays a major role in cell motility as both inhibition or constituent activation of these GTPases can alter cellular spreading and migration (Arthur *et al*., 2000). No significant difference was observed between levels of activated RhoA in Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ fibroblasts however Flii over-expression significantly reduced the activation of both Rac1 and Cdc42 (Fig 5.9B). Over-expression of Flii also had a profound effect on signaling components of focal adhesions namely Src tyrosine kinase and p130Cas (Fig 5.10). Using Western blotting and computer assisted densitometry of protein bands we found that Flii$^{Tg/Tg}$ had significantly increased levels of total p130Cas levels (p130Cas + pp130Cas) compared to Flii$^{+/-}$ fibroblasts but had significantly decreased ratio of activated pp130Cas (Fig 5.10A-C) and decreased expression of Src tyrosine kinase compared to both Flii$^{+/-}$ and wild-type fibroblasts (Fig 5.10D) suggesting that Flii over-expression down regulates signaling pathways required for cellular adhesion and migration.

5.2.5 Effect of Flii on paxillin activation and signalling in wounds in vivo

To determine if the *in vitro* cellular responses to differential Flii gene expression were also observed *in vivo,* an incisional wound model was used. Successful wound healing is reliant on the ability of cells to migrate and adhere to the extracellular matrix, hence the negative effect of Flii in wound healing may be due in part to its effects on adhesion formation and signaling. Over-expression of Flii resulted in larger, more gaping wounds at 3 days post-injury while Flii deficiency resulted in smaller wounds with improved wound appearance (Fig 5.11A-F and K). These findings were in agreement with previously published studies identifying Flii as a negative regulator of wound healing (Cowin *et al*., 2007). The wounds were then stained for p-paxillin, revealing increased p-paxillin in keratinocytes and fibroblasts adjacent to the wound in Flii$^{+/-}$ mice (Fig 5.11 G-

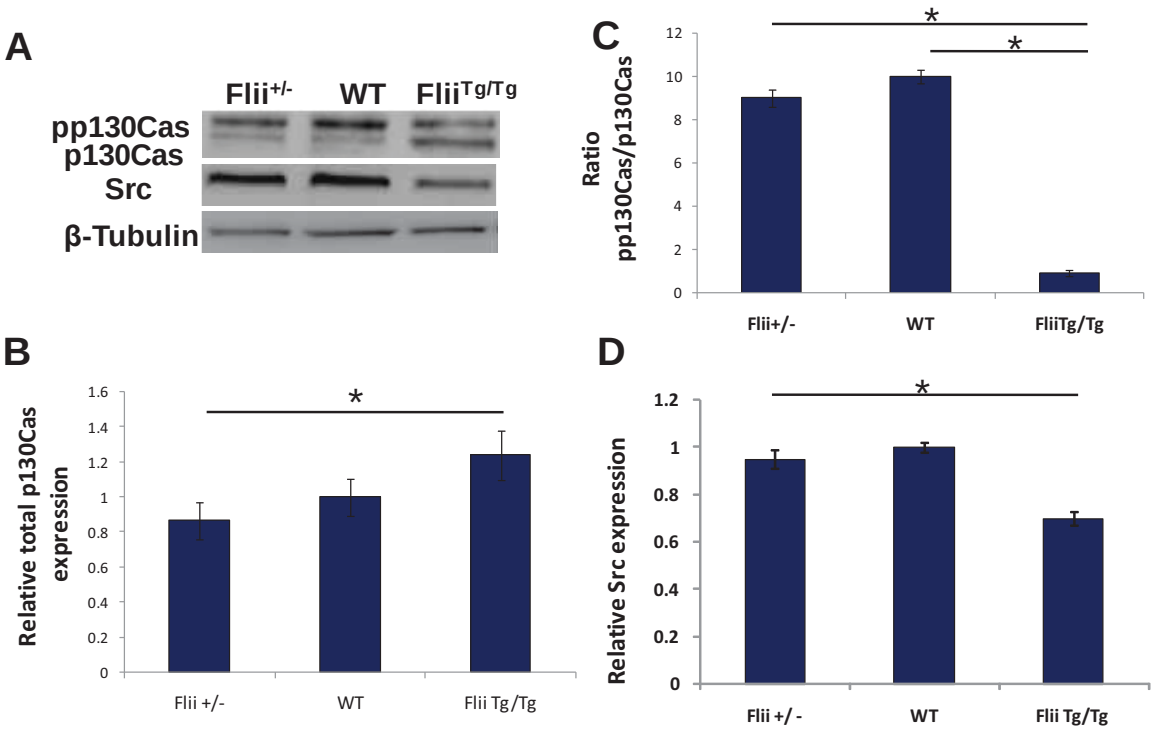

Figure 5.10: Over-expression of Flightless alters the expression of Src and p130Cas. A Protein extracts from sub-confluent Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ fibroblasts were analysed for expression of adhesion signaling proteins p130Cas, pp130Cas, Src and β-tubulin protein levels using Western Blot analysis. **B** Band densitometry illustrating total p130Cas (p130Cas + pp130Cas) expression and **C** the ration of pp130Cas and p130Cas protein levels. All values were first normalized to β-tubulin. Flii$^{Tg/Tg}$ fibroblasts show significantly increased total p130Cas expression compared to Flii$^{+/-}$ fibroblasts but decreased levels of active pp130Cas compared to both Flii$^{+/-}$ and WT fibroblasts. **D** Graphical analysis of Src protein expression determined by densitometry. All values were first normalized to β-tubulin. Flii$^{Tg/Tg}$ fibroblasts demonstrate decreased levels of Src proteins compared to both Flii$^{+/-}$ and WT fibroblasts. The data show the mean of three independent experimental repeats. Mean +/- SEM. *P<0.05.

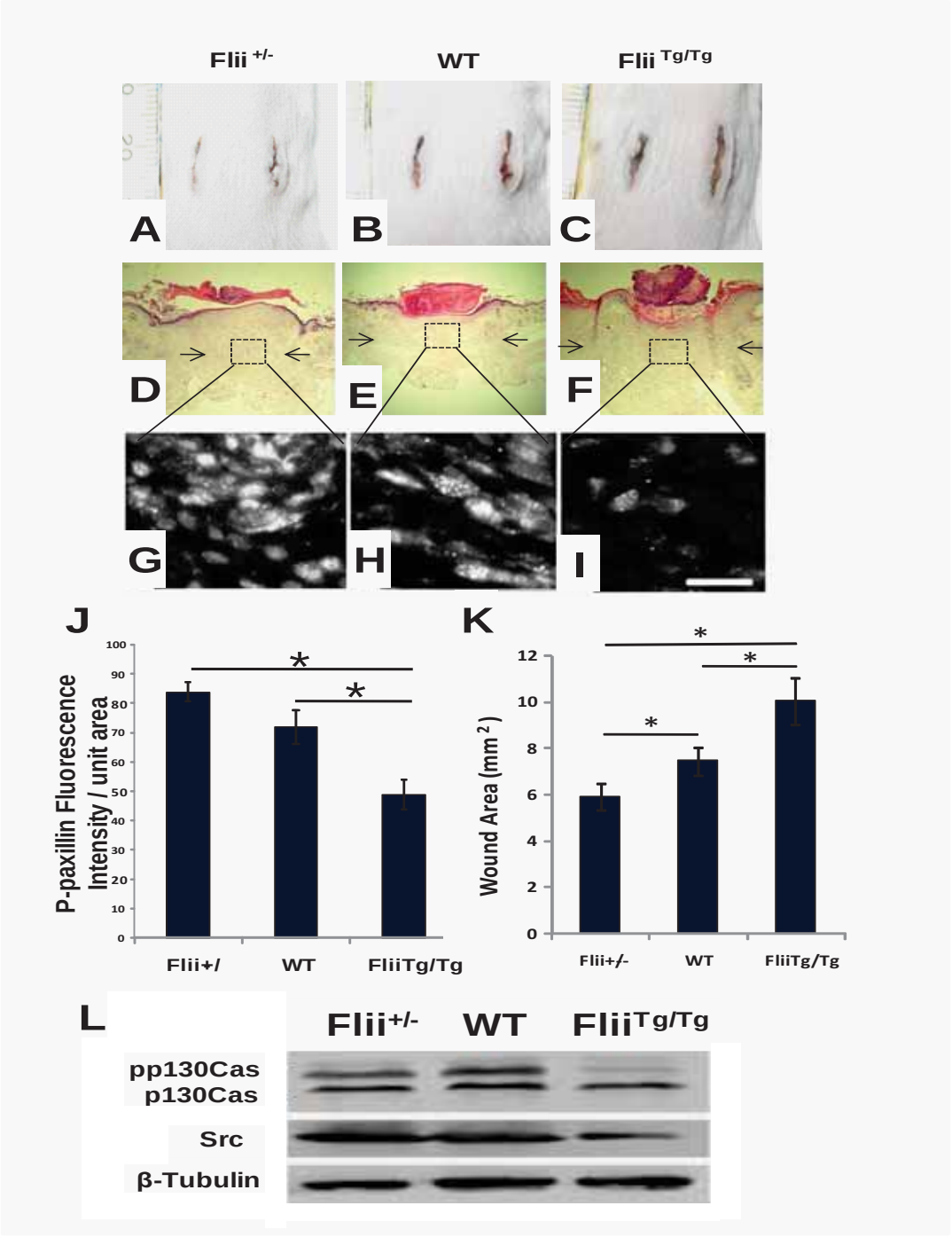

Figure 5.11: Effect of Flightless on paxillin activation and signalling in wounds in vivo. A-F and K Two, full-thickness incisions were made through the dorsal skin in Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice. Representative 3 day images of wounds are shown illustrating impaired wound healing in Flii$^{Tg/Tg}$ mice. **G-I** Vertical sections through the middle of the wounds was taken and stained for p-paxillin expression. Immunofluorescent staining of p-paxillin in fibroblasts of the wound bed and **J** Graph showing p-paxillin fluorescence intensity in 3 day wound sections of Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice. Flii$^{Tg/Tg}$ mice wounds show significantly reduced paxillin staining compared to Flii$^{+/-}$ and WT counterparts. n=6 wounds from 3 mice / group. Magnification x40. Scale Bar = 100μm and refers to all images. Mean +/- SEM. *P<0.05. **L** Flii$^{Tg/Tg}$ mice wounds have decreased expression of adhesion signalling molecules pp130Cas and Src in wounds in vivo, compared to Flii$^{+/-}$ and WT counterparts.

J). Flii over-expression resulted in decreased p-paxillin levels in wounds in vivo (Fig 5.11G-J). Similarly, decreased expression of pp130Cas and Src in wounds *in vivo* was observed by Western blot analysis comparing $Flii^{Tg/Tg}$ vs. $Flii^{+/-}$ and wild-type mice (Fig 5.11L).

To investigate whether Flii effects on actin stress fibers, paxillin activity and adhesion signaling affected collagen contraction, $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ primary fibroblasts were incorporated into a 3-D fibroblast populated floating collagen gel contraction assay *in vitro* (Fig 5.12A). Floating collagen matrices resemble the dermal architecture of stromal fibroblasts allowing assessment of the dermal contraction which would take place during wound healing. Despite observing increased stress fibers in $Flii^{Tg/Tg}$ fibroblasts *in vitro* (Fig 5.1) no differences were observed in the rate of collagen gel contraction compared to wild- type counterparts (Fig 5.12A-B) implying that the impaired wound healing observed in these mice wounds (Fig 5.11C) may be attributed more to deficient turnover of adhesion structures and impaired cellular migration and proliferation than an effect on cellular contraction. Conversely, decreased contraction of collagen gels was observed in $Flii^{+/-}$ fibroblasts and this response what maintained over 72 hr period (Fig 5.12B). Decreased contraction and remodelling of the collagen fibers alongside increased cellular migration would all be expected to lead to improved wound repair outcomes. This suggests that the improved healing response observed in $Flii^{+/-}$ mice (Fig 5.11A) may be attributed more to improved turn-over of adhesion structures resulting in improved cellular migration and proliferation rather than a direct effect on wound contraction.

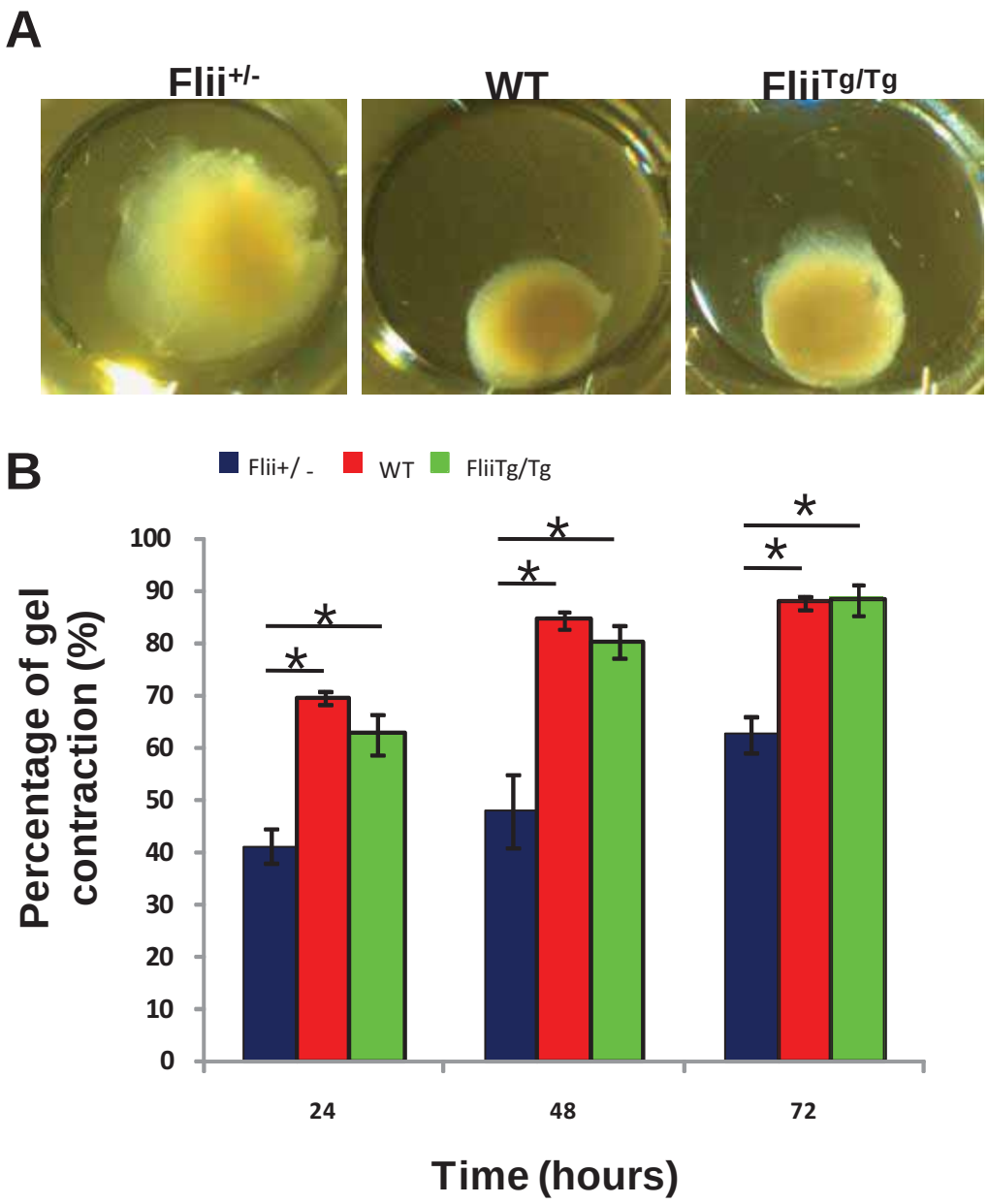

Figure 5.12: Effect of Flightless on collagen gel contraction *in vitro*. Contraction of fibroblast populated collagen gels was analysed based on gel size over time. Initially, gel size occupied the whole well. **A** Representative images of 3-D floating collagen gel contraction assay at 24hrs post plating. Magnification x6.5 **B** Flii deficient fibroblasts have significantly reduced collagen gel contraction compared to both WT and Flii$^{Tg/Tg}$ fibroblasts at 24, 48 and 72hrs post media addition respectively. n=5. Mean +/- SEM. *P<0.05.

5.3 Discussion

Flii mediates rapid remodelling of actin filaments and has been shown to associate with actin arcs and membrane ruffles, illustrating its role in actin dynamics, cellular adhesion and motility (Davy *et al*., 2000). Here we have demonstrated a novel role for Flii in the regulation of focal adhesions, affecting paxillin phosphorylation, downstream adhesion signaling and formation and stabilization of actin stress fibers. Flii, like gelsolin, mediates remodelling of actin filaments through severing of actin filaments, capping of growing ends of actin polymers and nucleating polymerization of actin filaments affecting actin dynamics and subsequent cellular responses during wound healing (Azuma *et al*., 1998, Davy et al., 2000).

Results presented in the previous chapter demonstrated Flii association with focal complex proteins, including paxillin. Results presented here now demonstrate that Flii may also play a role in formation and stabilization of actin stress fibers. Chapter 3 of this thesis described impaired spreading of Flii over-expressing cells on different extracellular matrices and here we now also show impaired spreading of $Flii^{Tg/Tg}$ fibroblasts on a mixture of extracellular matrices over a period of time suggesting that impaired spreading is independent of the extracellular matrix and might be linked to impaired turn-over of adhesion structures. While both $Flii^{+/-}$ and wild-type fibroblasts show active actin dynamics with actin meshwork of actin involved in cellular spreading and migration resulting in greater degree of cellular spread at same time-point post seeding, $Flii^{Tg/Tg}$ fibroblasts are smaller and display impaired spreading and increased expression of ventral stress fibers, linking the focal adhesions at the cell periphery. Chapter 3 of this thesis described the Flii interaction with paxillin and localisation at the site of focal adhesions. It is not surprising to observe an actin remodelling protein present at sites of focal adhesion and involved in

promotion of stress fiber formation. Indeed, gelsolin is also found at sites of stress fiber formation (Arora *et al*., 1999). Micro-injection of gelsolin into gelsolin depleted cells has resulted in the re-appearance of stress fiber formation illustrating a role for gelsolin in promoting stress fiber formation. In contrast, high gelsolin expression resulted in disrupted cell architecture (Arora *et al*., 1999). A recent study using NIH 3T3 fibroblasts suggested a decreased formation of stress fibers in response to decreasing Flii levels using siRNA (Higashi *et al*., 2010), however no difference in stress fiber formation between $Flii^{+/-}$ and wild-type fibroblasts was observed in this study which could be attributed to different cell types and experimental conditions used. Results presented in this chapter show a significantly increased expression of filamentous F-actin over a time course of 24hrs in $Flii^{Tg/Tg}$ cells, suggesting a maintained response to cellular adhesion on extracellular matrix substrate. This suggests a potential role for Flii in the dynamics of actin filament turnover and assembly to stress fibers in response to fibroblast adhesion to extracellular matrix. Gelsolin facilitates formation of stress fibers by shortening actin filaments and enabling their rotation into position that resulting in increased actin stabilization (Arora *et al*., 1999) and increased stress fiber formation observed in $Flii^{Tg/Tg}$ cells implies that Flii might be acting in similar fashion to gelsolin however this is yet to be investigated.

In agreement with increased stress fiber formation, $Flii^{Tg/Tg}$ fibroblasts also showed an increased number and larger size of focal adhesions around the periphery of the cell compared to $Flii^{+/-}$ and wild-type fibroblasts. The latter only expressed focal adhesions at leading edges of the cell. In addition, over-expression of Flii also resulted in a decreased percentage of cells positive for focal complex formation suggesting impaired turn-over of adhesion structures. Flii over-expressing fibroblasts have increased focal adhesion formation indicating that these cells are capable of forming focal complexes and

suggesting that Flii effects on focal adhesion turn-over might be more important for impaired cellular spreading observed in Flii over-expressing fibroblasts. Results presented in Chapter 4 have established a link between Flii and paxillin and novel findings presented here now suggest that the effect of Flii on paxillin function may be a key to better understanding focal adhesion turnover and subsequent effects on cell adhesion and motility.

Paxillin is an adaptor protein which does not possess enzymatic activity however it recruits multiple intermediate proteins of different signaling pathways to specific regions of the cell. This allows the cell to respond to changes in the external environment through multiple pathways, including modulation of the gene expression, extracellular matrix and cytoskeleton as well as cell shape, adhesion and motility (Turner, 1998, Zaidel-Bar *et al*., 2007). Like Flii, paxillin also contains leucine rich motifs, allowing association with many signaling and structural proteins. Dispersed throughout the paxillin molecule are multiple serine/threonine and tyrosine phosphorylation sites which are phosphorylated in response to cell adhesion and/or exposure to various growth factors (Brown and Turner, 2004, Haq and Trinkaus-Randall, 1998, Schaller, 2001). While serine/threonine phosphorylation sites are believed to be important in paxillin localization to focal adhesion sites, tyrosine phosphorylation generates docking sites for SH2 domain containing proteins like FAK and Src to facilitate downstream signaling and regulate cell migration and focal adhesion turnover (Brown and Turner, 2004). An experiment where all four phosphorylation sites of paxillin have been mutated, demonstrated slower cell adhesion disassembly and showed that phosphorylation of paxillin promotes adhesion disassembly (Webb *et al*., 2004).

Paxillin tyrosine phosphorylation is induced in response to cell stress stimuli such as tissue injury where a rapid response to tyrosine phosphorylation leads to dynamic turnover of focal adhesions and increased cellular motility of stromal fibroblasts (Haq and Trinkaus-Randall, 1998). Data presented in Chapter 4 showed a co-localization between Flii and paxillin and the results presented in this chapter suggest that the association between Flii and paxillin might be involved in regulating paxillin phosphorylation and subsequent cellular responses. Adhesions containing different ratios of phosphorylated to non-phosphorylated paxillin display marked differences in cell dynamics (Zaidel-Bar *et al*., 2007). Two distinct functions have been proposed to explain why paxillin associates with multiple actin binding proteins like Flii and vinculin. Firstly, strengthening the anchoring of the actin cytoskeleton to the focal adhesion complex and secondly forming the protein complex with the actin cytoskeleton so that actin based contractility causes further clustering of paxillin and associated proteins which may be an important mechanism for activation of signaling molecules (Schaller, 2001).

Examining the tyrosine phosphorylation of paxillin at tyrosine residue 118 in $Flii^{+/-}$ wild type and $Flii^{Tg/Tg}$ fibroblasts suggests an inhibitory role for Flii in paxillin activation. This site is phosphorylated by Src or FAK and is specifically activated at focal adhesions, facilitating paxillin function in adhesion turnover (Bach *et al*., 2009). $Flii^{Tg/Tg}$ fibroblasts have decreased levels of total paxillin and a significantly reduced ratio of p-paxillin levels compared to both $Flii^{+/-}$ and wild-type fibroblasts. This was surprising considering the increased number of focal adhesions in $Flii^{Tg/Tg}$ fibroblasts. However, compared to $Flii^{+/-}$ and wild-type fibroblasts, these cells had decreased cytoplasmic paxillin staining around the nucleus, suggesting that paxillin in $Flii^{Tg/Tg}$ fibroblasts predominantly associates with focal adhesions. Decreased paxillin tyrosine phosphorylation observed in $Flii^{Tg/Tg}$

fibroblasts suggests that over-expression of Flii and its association with paxillin may inhibit the interaction of paxillin with other signaling molecules like Src involved in paxillin phosphorylation, resulting in decreased paxillin activation and impaired focal adhesion turnover required for directional cellular movement. Indeed studies have shown that focal adhesions that remain stable in position and have impaired turnover contain low levels of phosphorylated paxillin (Zaidel-Bar *et al*., 2007). Cell spreading and migration are driven by focal complex transition into strong focal adhesions binding to the extracellular matrix, matrix remodelling by fibrillar adhesions and efficient disassembly and turnover of cell adhesion structures. The results presented here show increased phosphorylation of paxillin and focal complex formation in $Flii^{+/-}$ and wild-type vs. $Flii^{Tg/Tg}$ fibroblasts and previous studies have suggested that phosphorylation of paxillin enhances lamellipodial formation and stimulates formation of novel focal complexes enabling dynamic turnover of adhesion structures (Zaidel-Bar *et al*., 2007).

Results in this chapter demonstrate that Flii plays an important role in regulating focal adhesion formation and that over-expression of Flii results in stable focal adhesions with impaired turnover; this may explain the decreased cellular migration observed in $Flii^{Tg/Tg}$ fibroblasts. The effect of Flii on formation of fibrillar adhesions with the extracellular matrix was also investigated by examining the alignment of fibronectin and F-actin fibers and the colocalization of paxillin and tensin as markers of fibrillar adhesions. Src-induced phosphorylation of paxillin results in dissociation of talin with ligand bound integrin-β tails, allowing their interaction with tensin and conversion of focal adhesions into fibrillar adhesion structures (Sawada *et al*., 2006). Transition to fibrillar adhesions is dependent on both integrin interactions with the actin cytoskeleton and acto-myosin contractibility. Cells which have increased ventral stress fibers, low levels of

phosphorylated paxillin and enhanced acto-myosin contractibility have enhanced formation of fibrillar adhesions (Bach *et al*., 2009). Indeed, Flii$^{Tg/Tg}$ fibroblasts which have increased stress fiber formation and decreased paxillin phosphorylation also showed parallel staining of fibronectin and F-actin fibers and colocalization between paxillin and tensin, indicative of enhanced fibrillar adhesions. This result was somewhat surprising as the *in vitro* adhesion assay used on primary keratinocytes and fibroblasts in Chapter 3 of this thesis suggested decreased cellular adhesion in response to Flii over-expression. This assay, analysed through the colorimetric reaction, was based on production of hexosaminidase enzyme and cleavage of the 4-Nitrophenoyl-N-acetyl-β-D-glucosaminide (PNAG) substrate in response to cellular adhesion and efficient turnover of adhesion structures. Cells which have impaired adhesion turnover would have low levels of enzyme production that would suggest impaired cellular adhesion using this assay.

Examining cellular adhesion using microscopy and expression of molecular proteins involved in mediating cellular adhesion, suggests that Flii over-expressing cells may have enhanced fibrillar adhesion but impaired adhesion structure turnover. Impaired turnover of adhesion structures would result in decreased cellular spreading and migration (as illustrated in Chapter 3) and detachment from the extracellular matrix substrate. Flii$^{+/-}$ and wild-type fibroblasts, which have been shown to have increased cell motility compared to Flii$^{Tg/Tg}$ fibroblasts (Cowin *et al*., 2007), have significantly increased tyrosine phosphorylation of paxillin and no evidence of ventral stress fiber formation or fibrillar adhesions, suggesting a high turnover of adhesion structures required for efficient cell migration. These results suggest that Flii may also affect different structural and signaling proteins involved in actin dynamics and adhesion signaling pathways.

Chapter 4 of this thesis described the association of Flii with components of focal adhesions, including talin, paxillin and vinculin but not α-actinin. This chapter further examined the effect of differential Flii gene expression on levels of these structural proteins. Talin is the essential mediator of integrin activation, where binding of talin to the integrin cytoplasmic tail regulates integrin affinity for particular ligand (Kopecki *et al*., 2009, Moser *et al*., 2009). Studies have suggested that paxillin is incorporated into focal complexes in the early stages together with talin, where it functions to link talin to the integrin α-cytoplasmic tail to enhance the matrix-actin cytoskeleton bond and increase resistance to mechanical stress (Alon *et al.*, 2005). Mutational studies have shown that talin is required for integrin clustering into focal adhesions (Alon *et al*., 2005). It has been hypothesized that Flii over-expression results in talin sequestration, leading to decreased integrin activation and impaired wound healing (Kligys and Jones, 2009). However, results presented in this chapter now indicate an increased number of focal adhesions in $Flii^{Tg/Tg}$ fibroblasts but no significant difference in talin or vinculin expression between $Flii^{+/}$, wild-type or $Flii^{Tg/Tg}$ fibroblasts. This suggests that a role of Flii in focal adhesion regulation through paxillin phosphorylation may be more important in integrin-mediated cellular adhesion and migration.

Flii might play more of a regulatory than a structural role in focal adhesion and act similarly to vinculin. Over-expression of vinculin in fibroblasts stabilizes focal adhesions, increases the number and size of focal adhesions and reduces cell motility, whereas decreased levels of vinculin lead to increased cell motility with smaller and fewer focal adhesions (Critchley, 2000). While dynamic focal adhesions are characterized by paxillin phosphorylation, adhesions with impaired turnover are characterized by increased presence of α-actinin. Here, we show that $Flii^{Tg/Tg}$ fibroblasts have significantly increased expression

of intracellular α-actinin, both around the nucleus and at sites of focal adhesions at cell periphery. Reduced α-actinin intracellular levels are associated with increased cellular motility while over expression of α-actinin is a marker of focal adhesions which exhibit decreased dynamics and impaired turnover (Webb *et al.*, 2004). Together with our observations of inhibited paxillin phosphorylation and enhanced formation of fibrillar adhesions we now suggest that Flii over expression not only regulates focal adhesion formation but may also impair focal adhesion turnover.

The mechanism behind impaired turn-over of focal adhesion structures in Flii over-expressing fibroblasts was examined by analyzing the activation of the Rho family of GTPases. Members of the Rho family of small GTPases, namely RhoA, and Cdc42 are implicated in cytoskeletal control and were found to colocolize with Flii on the actin arc and the actin-rich leading edge of motile Swiss 3T3 fibroblasts (Davy *et al*., 2000), hence suggesting a common functional pathway in Flii mediated cytoskeletal regulation. It is currently unclear whether this interaction is direct as these proteins can lie downstream or upstream of Flii and can link to Flii via intermediary proteins like talin, paxillin or vinculin.

Transformation of focal complexes into focal adhesions requires the activation of RhoA which activates Daam1 and participates in the Src-regulated tyrosine phosphorylation of multiple substrates including paxillin (Tominaga *et al*., 2000). Recent study had suggested that Flii promotes the GTP-bound active RhoA mediated relief of the autoinhibition of Daam1 hence modulating the actin assembly activity (Higashi *et al*., 2010). Examining the effect of Flii on activation of RhoA we found no significant

difference in activation of RhoA in Flii$^{+/-}$, wild-type or Flii$^{Tg/Tg}$ fibroblasts. This was not surprising as Flii$^{Tg/Tg}$ fibroblasts show effective focal adhesion formation but decreased formation of novel focal complexes, RhoA activates Rho-kinase and dominant active Rho-kinase induces the stress fiber formation in Swiss 3T3 fibroblasts while dominant negative Rho-kinase inhibits the wound-induced cell migration (Kaibuchi *et al*., 1999). Consequently, a tight control of Rho family of GTPases is instrumental in cellular adhesion, spreading and motility. We now show that Flii$^{Tg/Tg}$ fibroblasts have reduced active Rac1 and Cdc42 both of which are required for formation of focal complexes, cell spreading and formation of lamellipodia and filopodia respectively (Geiger and Bershadsky, 2001, Lunn and Rozengurt, 2004). Consequently, Flii effect on activation of Rho family of GTPases might contribute to the impaired turn-over of adhesion structures and delayed cellular spreading observed in Flii over-expressing fibroblasts.

The effect of Flii on adhesion signaling pathways, at sites of focal adhesions and further downstream, was investigated by examining the expression of Src kinase and p130Cas in Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ fibroblasts. Tyrosine kinase activity of Src is required for effective disassembly of adhesion structures and focal adhesion turnover during cell motility (Petit and Thiery, 2000). Experiments using Src$^{-/-}$ fibroblasts have shown that Src is not essential for the formation of focal adhesions but is required for integrin mediated adhesion and spreading, focal adhesion disassembly and turnover (Brown and Turner, 2004, Cary *et al*., 2002, Sastry and Burridge, 2000, Schaller, 2001). Formation of focal complexes creates a signal leading to the activation of Rac and suppression of RhoA via Src-dependent mechanisms which contribute to the disassembly of focal adhesions at the rear of the motile cells and delay the development of contraction until the cell is successfully spread (Geiger and Bershadsky, 2001). Signaling of small Rho

GTPases has previously been linked to Src kinase activity where Src activation of p-130Cas leads to activation of Ras-MAP kinase siganling pathway (Ben-Ze'ev, 1997). We observed reduced Src kinase expression in Flii$^{Tg/Tg}$ fibroblasts which may contribute to the decreased paxillin phosphorylation and reduced suppression of RhoA resulting in impaired focal adhesion turn-over and increased formation of stress fibres observed in these cells. Similarly to Flii$^{Tg/Tg}$ cells, Src and FAK$^{-/-}$ cells have an increase in number and size of focal adhesions which may occur due to inhibited focal adhesion turnover (Cary *et al*., 2002, Defilippi *et al.*, 1999, Webb *et al.*, 2004). Src-FAK complex regulates cellular migration by phosphorylation and dephosphorylation of key adaptor molecules like paxillin and p130Cas (Webb *et al*., 2004). Tyrosine phosphorylation of integrins and cytoskeletal proteins including paxillin, FAK and p130Cas regulates adhesion signaling and affects cellular migration (Panetti, 2002). Src tyrosine kinase phosphorylates downstream effectors of adhesion signaling pathways, of which p130Cas is a primary substrate, in an integrin dependent fashion affecting cellular spreading and migration (Cowell *et al*., 2006). Cells with decreased activated p130Cas display impaired actin filament assembly and significantly decreased rates of migration and spreading (Bach *et al*., 2009, Webb *et al*., 2004). Interactions between paxillin, Src, FAK and p130Cas following integrin mediated adhesion to extracellular matrix substrate are essential in regulating adhesion dependent focal adhesion formation and turnover as well as actin cytoskeleton organization into tensile stress fibers and subsequent coordinated assembly/disassembly of adhesion structures during cellular spreading and migration (Defilippi *et al*., 1999).

Reduced Src kinase expression in Flii$^{Tg/Tg}$ fibroblasts in vitro and in vivo was observed and this may lead to the decreased paxillin phosphorylation and impaired focal adhesion turnover observed in these cells and associated impaired wound healing in Flii

over-expressing mice. Compared to Flii$^{+/-}$ fibroblasts, Flii$^{Tg/Tg}$ fibroblasts have increased total levels of p130Cas (p130Cas + pp130Cas), a major Src substrate, however the ratio of activated pp130Cas to total p130Cas is significantly decreased in these cells, compared to both Flii$^{+/-}$ and wild-type counterparts. Similar findings were also observed using protein extracts from whole skin wounds in vivo with increased active pp130Cas levels in wounds of Flii$^{+/-}$ and wild-type mice compared to Flii$^{Tg/Tg}$ mice wounds, suggesting a functional effect of Flii on cellular adhesion and signaling during wound repair in vivo where Flii over-expression down regulates adhesion signaling pathways. Phosphorylation of p130Cas initiates Rac1 activation and increased membrane ruffling and lamellipodial extensions (Sharma and Mayer, 2008) consequently, decreased activated p130Cas in Flii$^{Tg/Tg}$ fibroblasts may lead to impaired cell spreading observed in these cells.

Flii is up-regulated in response to wounding and results presented in this chapter now demonstrate that the observed responses to Flii over-expression on the adhesion signaling pathways may also have a direct functional effect during wound healing. Flii over-expression results in decreased levels of activated paxillin and down regulation of downstream signaling proteins Src and p130Cas in wounds *in vivo*. Contrary to Flii over-expression, reduction of Flii results in active actin dynamics and increased focal complex formation, regular turnover of focal adhesion structures and decreased contractibility, illustrating a more controlled response to cellular adhesion during wound repair. Indeed, a previous study has described slower more controlled production of Collagen I in wounds of Flii deficient mice (Cowin et al., 2007) suggesting that the improved wound healing observed in Flii$^{+/-}$ mice is also attributed to the more organized and controlled cellular response during wound repair.

Post translational modification and phosphorylation of adaptor molecules like paxillin and p130Cas regulates their interactions with other adhesion components and signaling molecules. This results in recruitment of molecules which favour adhesion or allow disassembly of adhesion structures, affecting the integrin-cytoskeleton linkage. Consequently, proteins like Flii that have the ability to associate with paxillin and modulate its phosphorylation or interaction with other signaling proteins are important regulators of cellular responses. Our results now suggest that Flii over-expression may inhibit phosphorylation of paxillin on tyrosine residue 118 by associating with paxillin and inhibiting binding of Src kinase and modulating the activity of Rho family of GTPases, hence resulting in decreased paxillin phosphorylation and impaired focal adhesion turnover. Decreased paxillin phosphorylation and expression of adhesion signalling molecules observed in $Flii^{Tg/Tg}$ fibroblasts may account for decreased cellular migration and spreading of these cells and in the context of tissue repair can lead to impaired wound outcomes. These findings suggest that the role of Flii in focal adhesion formation and turnover through its association with paxillin might be similar to its inhibitory function in the "switch on – switch off" mechanism proposed for induction and repression of Toll-like receptor (TLR) signaling where Flii interaction with signaling proteins negatively mediates the mechanism of TLR signaling in a time course dependent cooperative manner (Dai *et al*., 2009, Lee and Stallcup, 2006, Wang *et al*., 2006). Further studies should examine the precise signaling pathways involved with Flii association with paxillin and how the signals from multiple paxillin binding partners result in a coordinated physiological response during cell adhesion, spreading and migration.

Chapter 6

FLIGHTLESS IS A NEGATIVE CONTRIBUTING FACTOR TO SKIN BLISTERING IN EPIDERMOLYSIS BULLOSA

Parts of this chapter have been published in following journals:

Kopecki Z, Murrell DF, Cowin AJ. Raising the roof on epidermolysis bullosa (EB): a focus on new therapies. *Wound Practice and Research.* 2009;17(2):76-82.

Kopecki Z, Arkell R, Ludwig R, Zilikens D, Has C, Bruckner-Tuderman L, Murrell DF and Cowin AJ. Increased Flii expression correlates with decreased Collagen VII in Epidermolysis Bullosa blistered skin. *Wound Repair and Regeneration.* 2010 July-August: 18 (4). **IF 2.230 RIF 10/39.**

Kopecki Z, Arkell R, Strudwick XL, Hirose M, Ludwig RJ, Kern JS, Bruckner-Tuderman L, Zilikens D, Murrell DF and Cowin AJ. Overexpression of the Flii gene increases dermal-epidermal blistering in an autoimmune ColVII mouse model of Epidermolysis Bullosa Acquisita. Under review at *J Pathol.* Feb 2011. **IF 6.4. RIF 1/66**.

6.1 Introduction

The dermal-epidermal basement membrane zone is an important epithelial and stromal interface where a number of different intracellular, transmembrane and extracellular matrix proteins are involved in the maintenance of the stable adhesion of skin layers (Kho *et al*. 2010). Altered expression of different proteins involved in maintaining stable adhesion of skin layers results in skin blistering diseases called EB. EB comprises a group of inherited skin fragility disorders characterized by blistering of the skin and mucosal surfaces following mild mechanical trauma (Ferrari *et al*., 2005).

Wound healing in patients suffering from EB poses a major challenge to their survival. Altered structure of hemidesmosome adhesion units, ColVII anchoring fibrils and expression of integrin surface receptors and proteins involved in mediating skin adhesion and cellular migration results in constant blistering and epidermal detachment in EB patients (Aumailley *et al*., 2006). At present there is no cure for EB and therapy is focused on improved wound management, however EB is associated with multiple underlying symptoms and the quality of life of patients is impaired. Moreover, patients suffering from recessive DEB have 90% chance of developing aggressive SCC and 78% chance of subsequent death from aggressive SCC by the age of 55 (South and O'Toole, 2010) illustrating the need for the development of novel therapies aimed at improved wound healing.

Thirteen genes coding for the proteins present at the dermal-epidermal interface have been identified to be involved in the EB phenotype to date, however classification of different EB subtypes is largely based on mode of inheritance, clinical, laboratory and

epidemiological studies (Natsuga *et al*. 2010). Four main types of EB include: EB: EBS, characterized by fragility of basal keratinocytes; JEB, characterized by tissue separation within the lamina lucida; DEB, characterized by blister formation at the level of ColVII anchoring fibrils below the lamina densa; and Kindler Syndrome, characterized by acral trauma induced blistering (Uitto and Pulkkinen, 2000). EBA is a chronic acquired, immune based blistering disease of the skin and mucous membrane classified by sub-epidermal blistering and tissue bound and circulating autoantibodies at the dermal-epidermal junction against the ColVII anchoring fibrils (Lehman *et al*., 2009). As EBA is not a genetic but an auto-immune disease, it is not classified as one of the main EB types but its clinical presentations mimic those observed in DEB patients, hence allowing researchers to use more accessible and robust animal models of EBA to investigate the effect of different proteins on skin blistering (Sitaru *et al*., 2007).

Advances in the understanding of the pathogenesis of EB in the last ten years have led to the identification of several new candidate genes and proteins as important mediators of wound repair and possible targets for future therapy developments (Kopecki *et al*., 2009b, Solovan *et al*., 2005). Results presented in previous chapters, have illustrated an important role for Flii in regulating wound healing via its affects on hemidesmosome formation, integrin-mediated cellular adhesion and migration and adhesion-signaling pathways. Over-expression of Flii results in significantly thinner and more fragile skin, suggestive of impairments in adhesion of skin layers (Kopecki *et al*., 2009a) (see Chapter 3), features typically observed in patients with EB. These findings suggest that Flii may also play an important role in wound healing of EB patients. Indeed, involvement of Flii in wound healing of different wound pathologies would not be surprising as previous studies have shown that Flii affects cellular motility and matrix production and is a contributing

factor to poor wound healing in both incisional wounds, partial thickness burn wounds and elderly skin (Adams *et al*., 2009, Adams *et al*., 2008, Cowin *et al*., 2007). Interestingly, Flii is also a secreted protein (Cowin *et al*., 2007) and while exact mechanisms of Flii action during wound repair are yet to be elucidated, the role of this negative regulator of wound repair in different wound pathologies identify it as a potential target for development of future therapy aimed at improved wound healing. The results presented in this chapter describe the role of Flii in wound healing of blister lesions using both human samples and two animal models of EB.

6.2 Results

6.2.1 Flightless expression is significantly increased in blister skin of EB patients

Human skin biopsies of both blister and non-blister lesions of patients suffering from EBS, JEB and DEB were analyzed for both Flii and gelsolin expression. Flii but not gelsolin was elevated in blistered skin of patients suffering from all three main EB types compared to both non-blistered patient skin and biopsy samples from healthy control individuals (Fig 6.1). Flii staining was evident both at the dermal epidermal junction and at the sites of blister formation suggesting an involvement of Flii in blister lesions. Flii expression was evident in both the epidermis and dermis of patients blister skin lesions and this was most pronounced in patients suffering from DEB (Fig 6.1).

Patient samples were categorized into three main EB types based on clinical diagnosis, namely EBS (Fig 6.2A), JEB (Fig 6.2B) and DEB (Fig 6.2C) and Flii optical density expressed as fluorescence was analysed statistically. Flii expression was increased in the majority of different EB sub-types compared to both non-blistered patient's skin and

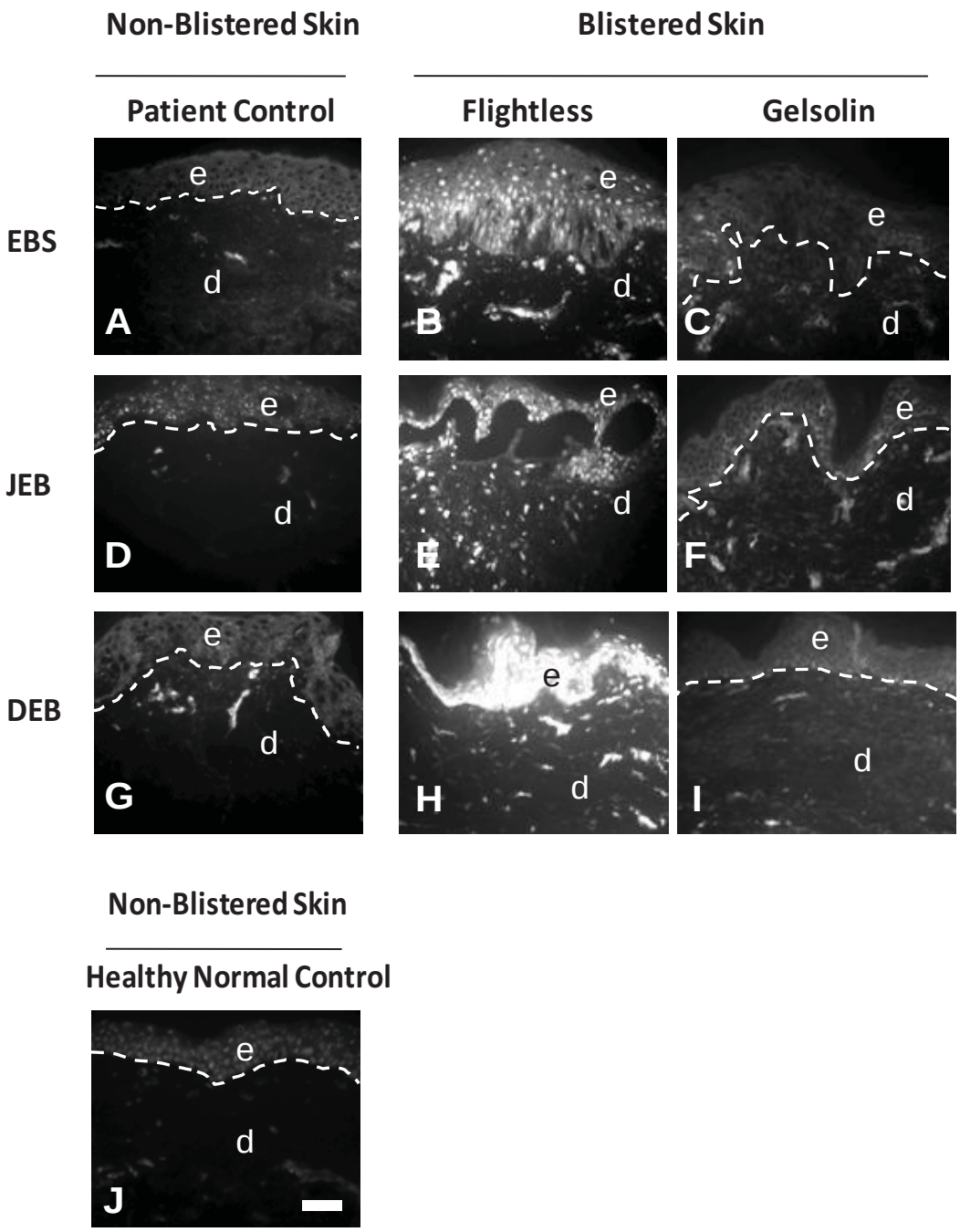

Figure 6.1: Flightless but not gelsolin is significantly increased in response to blistering in human wounds of different EB types. Representative images of Flii expression detected as white fluorescence in human samples of blister and non-blister skin from patients with different EB types (n=30) and control non-blister skin samples from healthy individuals (n=4). Flii activity is specifically increased in response to skin blistering in EBS **C**, JEB **F** and DEB **I** patients both at the dermal-epidermal junction and at the blister site compared to non-blister patients skin **B, E, H** or skin from healthy individuals **A**. Gelsolin expression is not increased in response to blistering, suggesting a Flii specific role in wound healing of blister lesions. Dermal-epidermal junction = white dashed line. Dermis = d. Epidermis = e. Scale bar in A = 200μm and refers to all images.

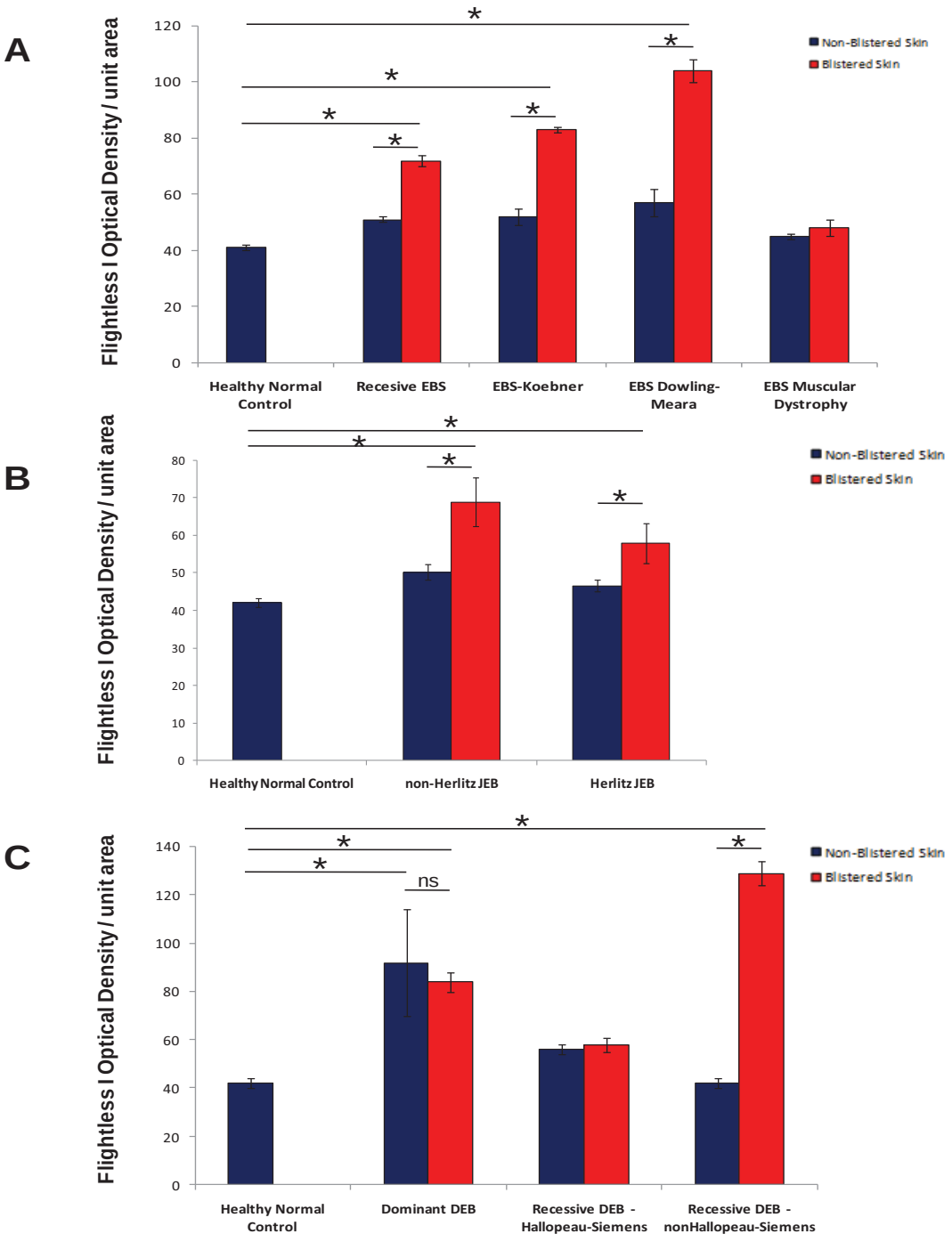

Figure 6.2: Graphical representation of increased Flightless expression in response to blistering in human wounds of different EB subtypes. Flii expression in blister wounds of different EB types and healthy normal control showed in Figure 6.1 was quantified statistically using AnalySIS software package. Flii activity is elevated in response to blistering in Recessive EBS, EBS-Koebner, EBS Dowling-Meara **A**; both non-Herlitz and Herlitz JEB **B**; and Recessive DEB nonHallopeau-Simmens **C** compared to patients non-blister skin and healthy normal control skin. Flii expression is also increased in both non-blister and blister skin of Dominant DEB. Mean +/- SEM. n= 1 for EBS Muscular Dystrophy. n=3 for EBS-Koebner, Dominant DEB and Recessive DEB Hallopeau-Siemens. n=4 for healthy normal control, Recessive EBS, EBS Dowling Meara, non-Herlitz JEB, Herlitz JEB and Recessive nonHallopeau-Siemens. n=30 EB patients in total. *=P<0.05.

control sections from healthy individuals. The highest expression of Flii was observed in the blistered skin of patients with recessive DEB nonHallopeau-Simmens. These patients have the most severe clinical symptoms associated with EB (Bruckner-Tuderman, 2009). Patients with Dominant DEB also had elevated Flii activity in both non-blistered and blistered patient skin compared to control sections (Fig 6.2C).

To further determine the role of Flii in EB we analysed Flii expression in blistered skin of patients suffering from the fourth recently added main type of EB, namely Kindler Syndrome. Similar to the observations of Flii activity observed in EBS, JEB and DEB, Flii expression was increased in response to skin blistering in Kindler Syndrome patients (Fig 6.3A). Based on cell location and morphology Flii staining appears to be observed specifically in epidermal keratinocytes, dermal fibroblasts and inflammatory cells present in the blister wounds as well as around the blister site, and Flii expression was significantly elevated when compared to non-blistered skin of healthy individual (Fig 6.3). While Flii expression was not as strong as observed in blistered skin of DEB patients, these results demonstrate that skin blistering is associated with an increase in Flii activity in all four main types of EB.

6.2.2 An autoimmune model of EBA

An autoimmune model of EBA which has phenotypic features of human DEB was used to investigate the role of Flii in EB. EBA was induced in wild-type mice following methods described previously to establish the model in our laboratory (Sitaru *et al*., 2005). Multiple injections of ColVII antibody over a period of 16 days led to an increasing immune response to ColVII, resulting in skin disease with high numbers of blisters and

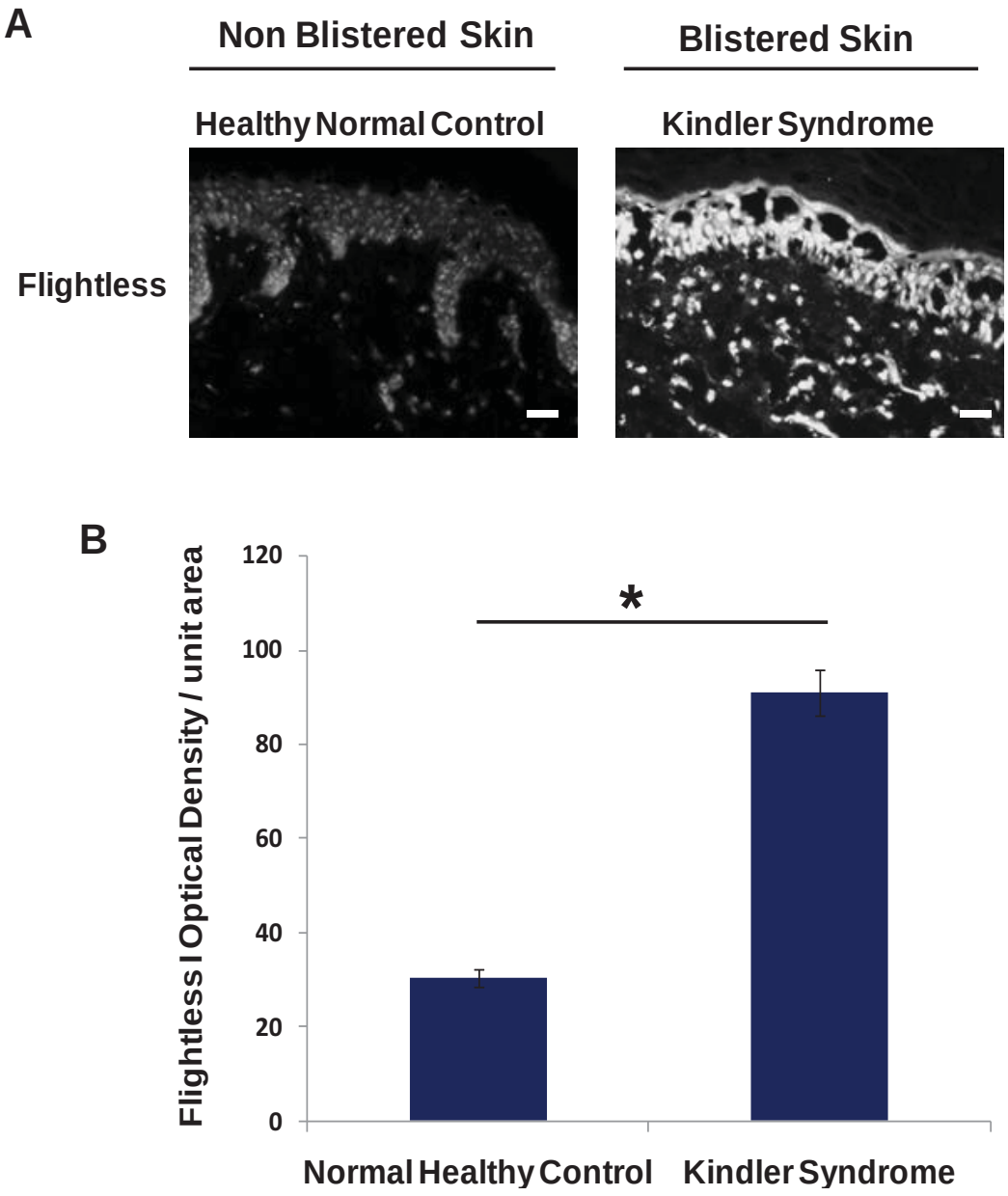

Figure 6.3: Flightless expression is significantly increased in response to blistering in Kindler Syndrome patient's skin. A Representative images of Flii expression detected as white fluorescence in human samples of blister skin from patients suffering from Kindler Syndrome and control non-blister skin samples from healthy individuals. Flii activity is specifically increased in response to skin blistering in Kindler Syndrome patient's skin, both at the blister site and in epidermal and dermal cells of blister wounds. n = 4. Magnification x40. Scale Bar = 100μm. **B** Graphical analysis of Flii optical density in response to blistering in Kindler Syndrome patient's skin. Flii activity is significantly increased in response to blister wounds of Kindler Syndrome patient's compared to non-blister skin of normal healthy individuals. Mean +/- SEM. n = 4 for normal healthy control and Kindler syndrome samples. *=P<0.05.

erosive lesions and crusts (Fig 6.4A-B). Compared to IgG control animals which did not develop an immune response, mice injected with ColVII antibody acquired extensive generalized blistering on >20% of skin surface with lesions on the trunk, back, face, ears, paws and tail base, many of which became lanced (Fig 6.4B). Using histology and electron microscopy we were able to demonstrate that the EBA mouse model reproduced the human EBA disease and mimics human DEB phenotypical features at the clinical, histological and electron microscopical level, where sub-basal blistering was evident in ColVII treated mice below the intact hemidesmosome adhesion units at the level of ColVII anchoring fibrils (Fig 6.5A-C).

6.2.3 Flightless over-expressing mice exhibit severe blister formation

Mice with different levels of *Flii* gene expression ($Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ mice) were injected with rabbit anti-mouse ColVII antibody which leads to varying degrees of blister formation. While all ColVII treated mice developed EBA with >20% of skin surface acquiring blister lesions, macroscopically $Flii^{+/-}$ and wild-type EBA induced mice had less extensive blistering and a decreased number of lesions compared to $Flii^{Tg/Tg}$ EBA mice (Fig 6.6). Electron microscopy was used to examine the blistered skin in $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ EBA mice to study the separation of skin layers and the effect on anchoring fibrils arrangements. All blistered skin lesions from $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ EBA mice exhibited separation of the skin layers, however Flii over-expression resulted in a greater degree of separation at the dermal epidermal junction compared to $Flii^{+/-}$ and wild-type EBA counterparts (Fig 6.7A-C). Examining the arrangement of anchoring fibrils around blister sites and at the edges of dermal-epidermal separation revealed impaired and diffused anchoring fibril arrangement in Flii over-expressing EBA mice (Fig 6.7F). In contrast, $Flii^{+/-}$ and wild-type EBA mice blister skin revealed a lesser

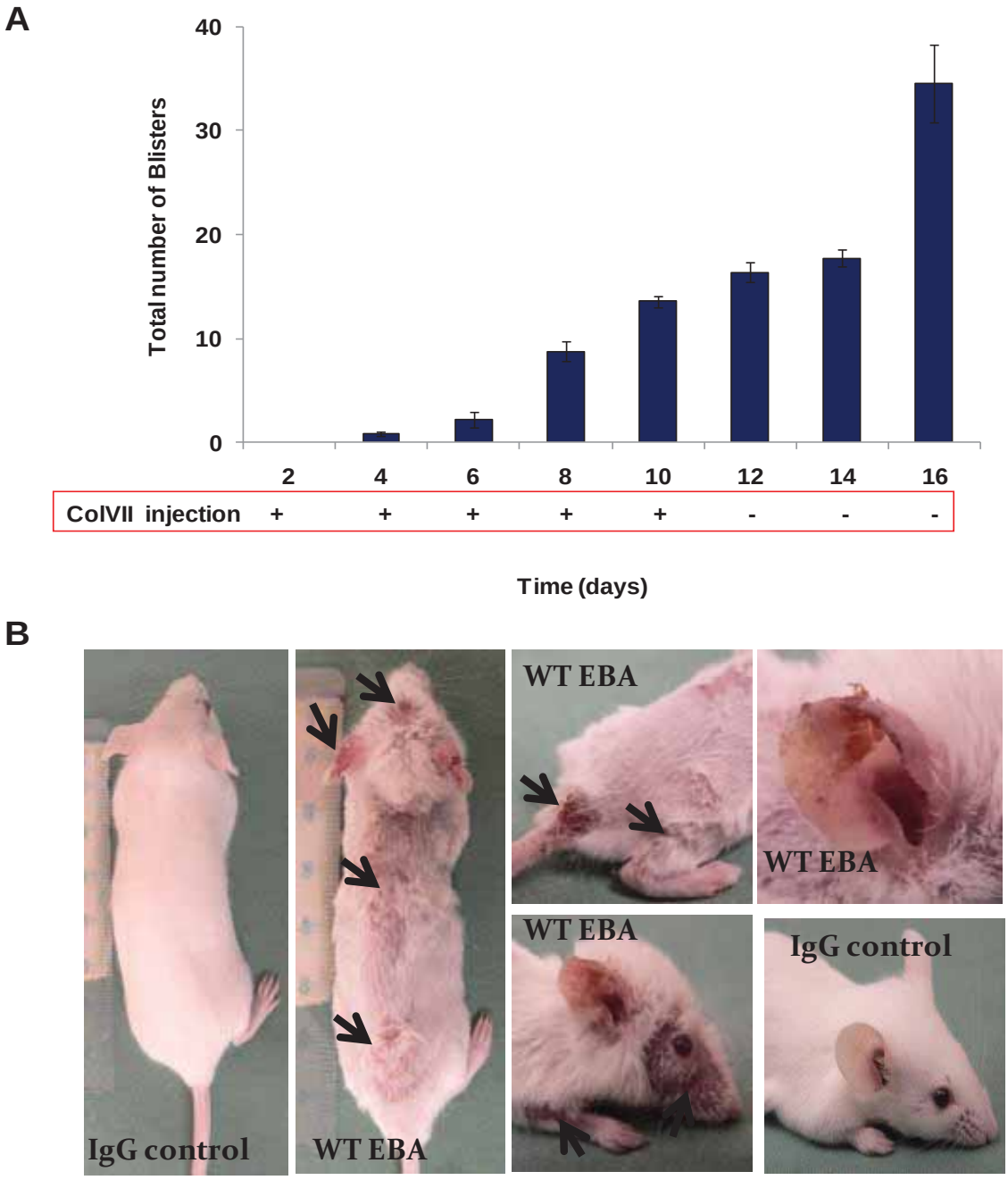

Figure 6.4: Induction of the EBA mouse model. A Three week old female WT mice were injected with rabbit anti-mouse ColVII antibody (0.3µg/g body weight) at days 0, 2, 4, 6, 8 and 10. Total number of blisters increased with time peaking at day 16 post initial injection when mice were euthanised. Mean +/- SEM. n=4. **B** Compared to normal IgG controls, mice injected with rabbit anti-mouse ColVII antibody developed extensive blistering on the trunk, ear, back, tail, face and paws (black arrows). Autoimmune EBA was induced in Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice. n=4. Representative images of wild-type EBA mice and IgG control mice at day 16 of experiment are shown.

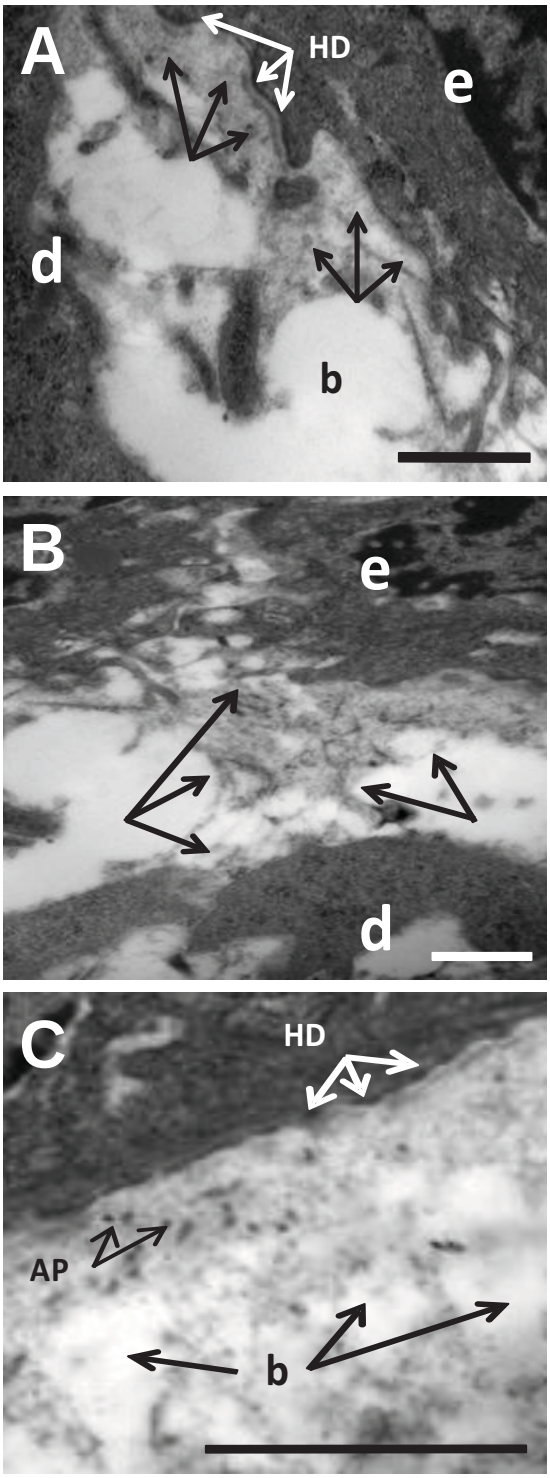

Figure 6.5: Sub-basal blistering in the EBA mouse model. Using transmission electron microscopy, sub-basal blistering was confirmed in WT EBA induced mice, validating the EBA model in our laboratory. In this EBA model, mice developed blisters (b) below the intact hemidesmosome adhesion (HD) units at the level of ColVII anchoring fibrils (black arrows) **A**. Sub-basal blistering with disrupted ColVII anchoring fibrils (black arrow) was observed in EBA blister mouse skin **B**. Occasional anchoring plaque with fried ends (AP) was observed along large deep dermal blisters (b) **C**. e = epidermis. d = dermis. Scale bar = 1μm applies to all images. Magnification range x 7900 – 64000. n=2.

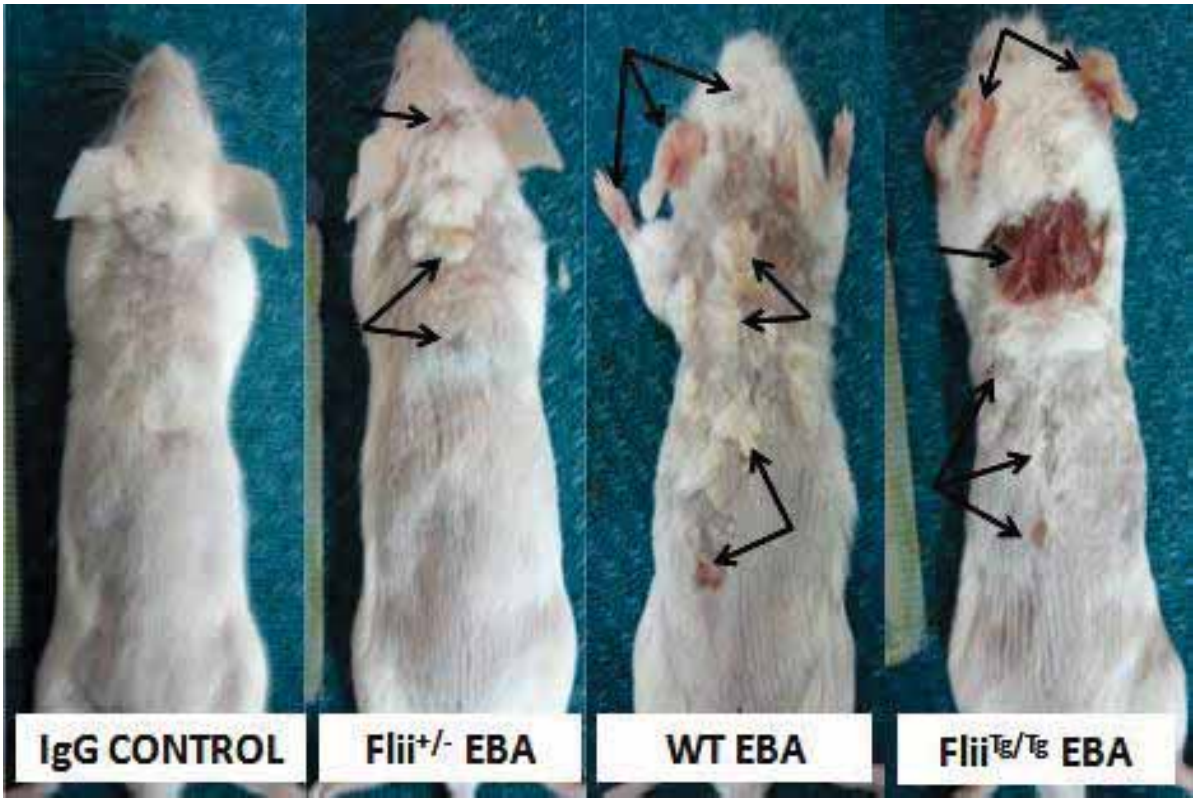

Figure 6.6: Effect of differential *Flightless* gene expression on blister formation. EBA was induced in three week old female Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice with injections of rabbit anti-mouse ColVII. Control mice received injections of control IgG and show no skin blistering. Representative images of skin blistering in Flii$^{+/-}$, WT, Flii$^{Tg/Tg}$ EBA and IgG treated control mice at day 10 of the experiment are shown, highlighting lanced blisters, crusts and erosions on mice back, ears and paws (black arrows). Flii$^{Tg/Tg}$ EBA mice develop more severe blistering quicker that Flii$^{+/-}$ and WT EBA mice counterparts. Quantification of skin blistering in these mice is shown in figures 6.7 and 6.8. n = 4 per mice group/treatment.

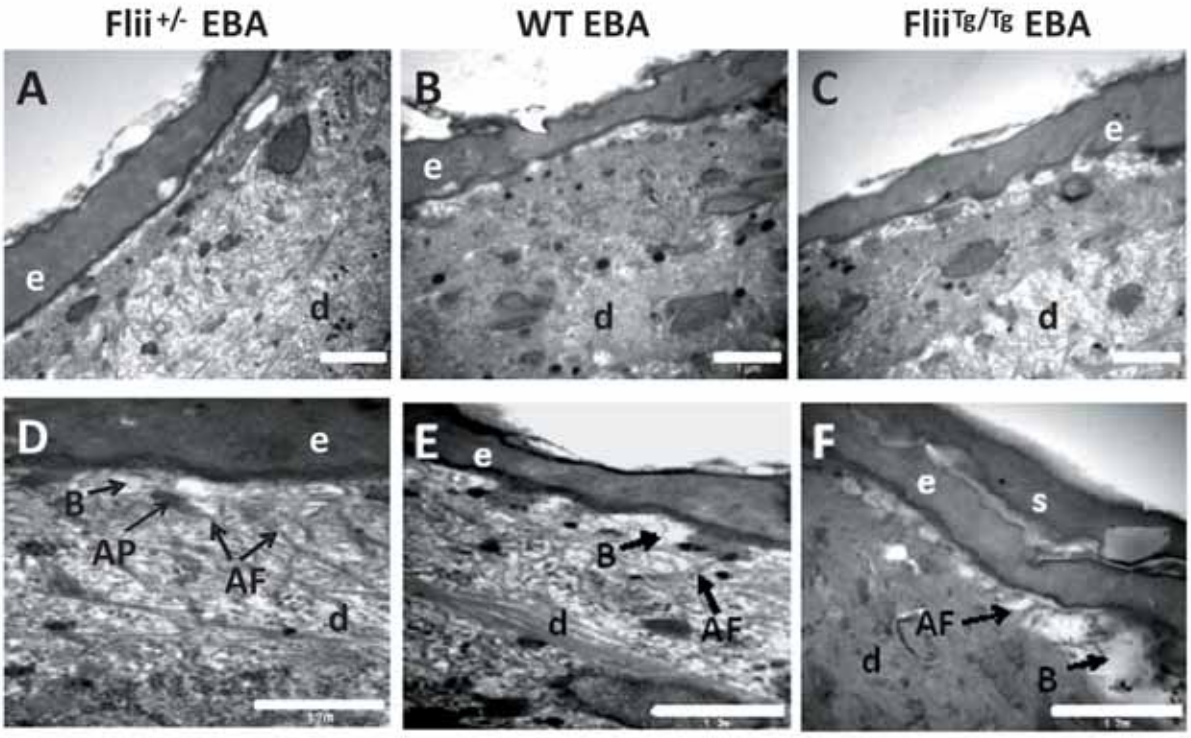

Figure 6.7: Flightless over-expression EBA mice have a greater degree of dermal-epidermal separation and diffuse arrangement of Collagen VII anchoring fibrils. EB was induced in three week old female Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice. **A-C** Using transmission electron microscopy dermal-epidermal separation was confirmed in blister skin of Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ EBA mice. Flii over-expressing EBA mice blister skin showed a greater degree of dermal-epidermal separation compared to Flii$^{+/-}$ and WT counterparts. e = epidermis. d = dermis. Magnification x13500. Scale bar = 1μm. n=2. **D-F** Flii$^{Tg/Tg}$ EBA mice blister skin shows diffuse arrangement of anchoring fibrils (AF) at site of blister formation (b) while Flii$^{+/-}$ and WT EBA mice blister skin reveals anchoring fibrils arranged into anchoring plaques (AP) suggesting stronger dermal-epidermal adhesion of skin layers. e = epidermis. d = dermis. AF = anchoring fibrils. B = blister. AP = anchoring plaque. Magnification = x25 000. Scale bar = 1μm. n=2.

degree of separation at the dermal epidermal junction, anchoring fibrils arranged into thick anchoring fibers and occasional anchoring plaque (Fig 6.7D-E).

Examining the blister lesions using light microscopy revealed more disrupted dermal architecture in Flii$^{Tg/Tg}$ EBA mice compared to both Flii$^{+/-}$ and wild-type EBA mice skin (Fig 6.8A). Over-expression of Flii in Flii$^{Tg/Tg}$ EBA mice lead to disrupted dermal architecture, large blisters below the basement membrane and a significantly higher histological blister score compared to both Flii$^{+/-}$ and wild-type EBA mice (Fig 6.8A-B) suggesting that therapy aimed at reducing the expression of Flii might improve wound repair of blister lesions in patients suffering from EBA.

6.2.4 Flightless expression is increased in blistered skin of mice

Flii expression was examined in skin sections of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ EBA induced mice. Increased Flii expression was observed in both blister and non-blister sections of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ EBA mice compared to IgG controls (Fig 6.9A). Flii$^{+/-}$ EBA mice had significantly reduced Flii expression in blister skin compared to wild-type and Flii$^{Tg/Tg}$ EBA mice, while Flii$^{Tg/Tg}$ EBA mice had significantly elevated Flii expression compared to wild-type mice (Fig 6.9B). Western blotting confirmed the immunofluorescence data with Flii expression being increased in response to blistering in both wild-type (2 fold increase) and Flii$^{Tg/Tg}$ (3 fold increase) EBA mice skin (Fig 6.9C).

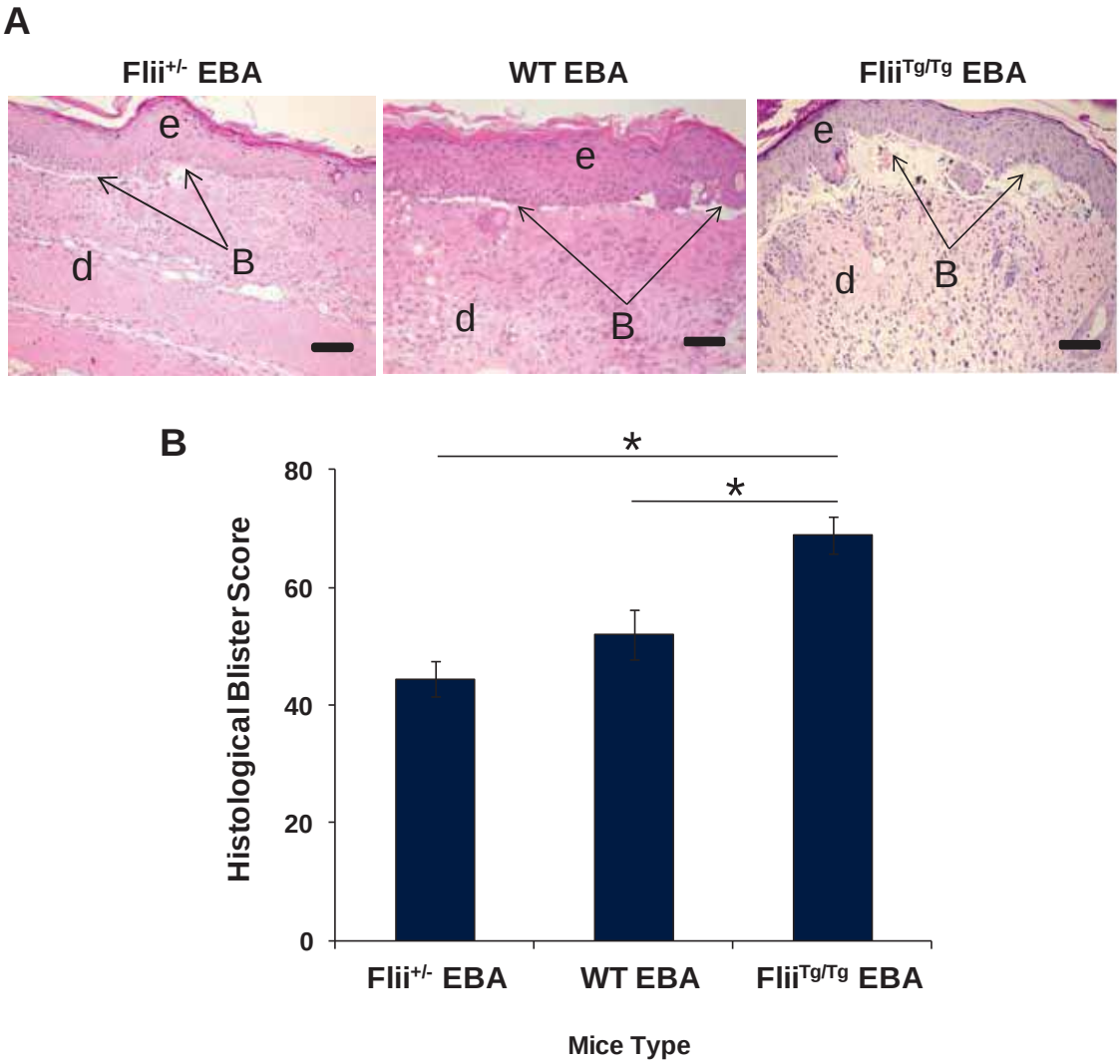

Figure 6.8: Flightless over-expressing EBA mice have a significantly higher blister score. EB was induced in three week old female Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice and skin blistering analysed using light microscopy. **A** Representative images of H&E stained blister wounds used to measure the histological blister score based on the length of the blister regions. Flii$^{Tg/Tg}$ EBA mice have larger blister areas and less organised dermal wounds compared to their counterparts. B =blister, e=epidermis, d=dermis. Magnification x10, Scale Bar = 500µm. **B** Graphical representation of significantly higher histological blister score in Flii$^{Tg/Tg}$ EBA mice compared to both Flii$^{+/-}$ and WT EBA counterparts. n=4. Mean +/- SEM. *=P<0.05.

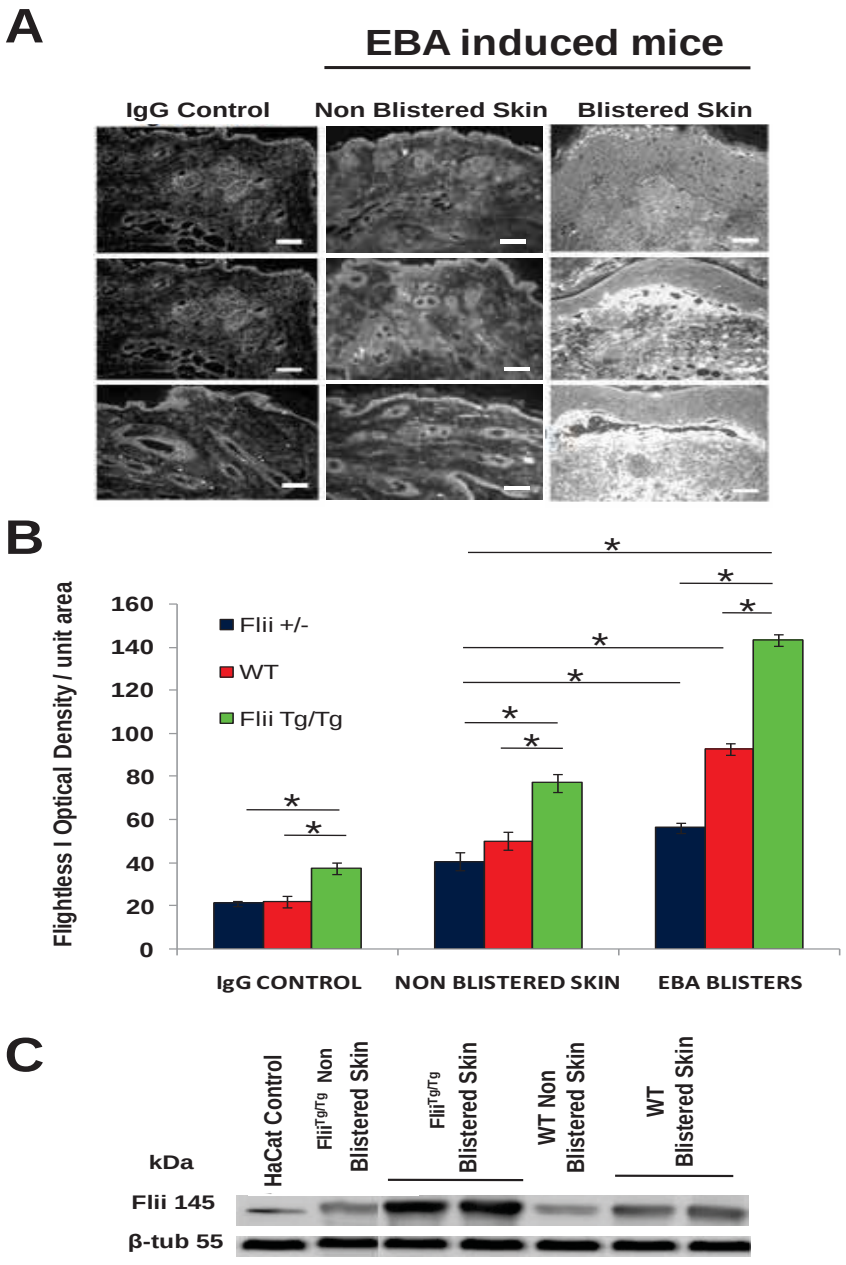

Figure 6.9: Increase of Flightless activity in response to skin blistering in EBA mice skin. EB was induced in three week old female Flii+/-, WT and FliiTg/Tg mice and Flii expression examined at day 16 when severity of blistering is at peak. **A-B** Flii expression is increased in response to blistering in both non-blister and blister skin of EBA mice. Flii$^{+/-}$ EBA mice have significantly reduced Flii expression in blister skin compared to both WT and Flii$^{Tg/Tg}$ EBA mice while Flii$^{Tg/Tg}$ EBA mice have significantly increased Flii expression in response to blistering compared to their counterparts. Scale bar in A = 500µm refers to all images. Mean +/- SEM. n=4 *=P<0.05. **C** Western Blotting of Flii expression in WT and Flii$^{Tg/Tg}$ EBA mice. Flii is increased in response to blistering in both WT (2 fold increase) and Flii$^{Tg/Tg}$ (3 fold increase) EBA mice skin. Data are representative of two independent repeats. Fold increase was analysed based on band intensity and normalized to β-Tubulin control.

6.2.5 Integrin expression is reduced in Flii$^{Tg/Tg}$ EBA blister skin

Integrins are important cell surface adhesion receptors which mediate cell-matrix adhesion and communication and promote attachment of cells to the extracellular matrix during wound healing (Berrier and Yamada, 2007). Chapter 4 of this thesis has described an effect of Flii on integrin mediated cellular adhesion and migration. To determine if Flii affects the integrin receptor expression during wound healing of blister lesions, the expression of integrin α3 and integrin β1 subunits was examined in non-blister skin of wild-type IgG control mice and blister skin of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ EBA mice. Compared to non-blister skin of wild-type IgG control mice, blistered skin of both Flii$^{+/-}$ and wild-type EBA mice wounds showed strong expression of integrin α3 and β1, with staining throughout the epidermis. In contrast, Flii$^{Tg/Tg}$ EBA mouse blisters failed to up-regulate integrin expression in response to skin blistering and demonstrated weak integrin staining mainly concentrated on the apical part of the epidermis (Fig 6.10). Quantifying integrin α3 and integrin β1 optical density revealed significantly increased expression in response to skin blistering in both Flii$^{+/-}$ and wild-type EBA wounds compared to non-blistered skin and IgG controls (Fig 6.10 and 6.11). Increased integrin β1 expression in Flii$^{+/-}$ and wild-type EBA mice blisters was also confirmed by Western blotting (6.10I). Furthermore, the up-regulation of integrin expression that occurs in blistered skin of wild-type and Flii$^{+/-}$ mice does not occur in Flii$^{Tg/Tg}$ blister lesions (Fig 6.11).

6.2.6 Fibroblasts but not keratinocytes adhesion is affected by Flightless gene expression in EBA-induced mice

Examining the cellular adhesion properties of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ primary fibroblasts and keratinocytes cultured from EBA-induced and IgG control mice skin showed improved cellular adhesion of Flii$^{+/-}$ EBA fibroblasts and keratinocytes compared

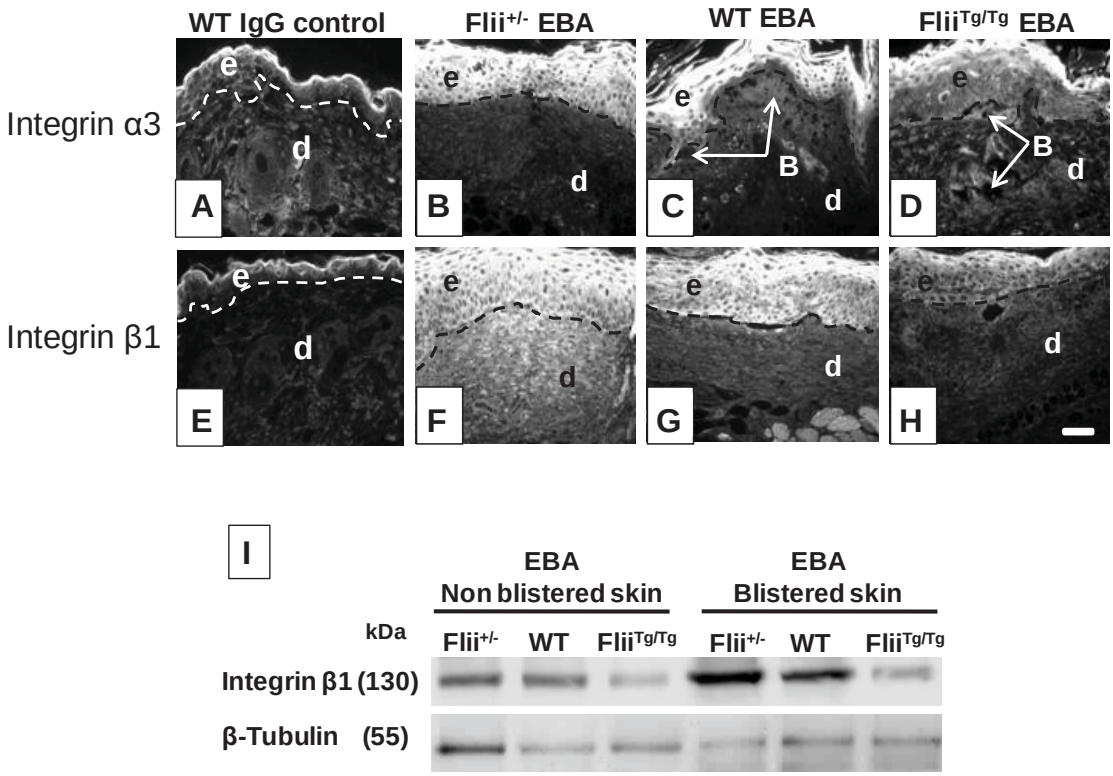

Figure 6.10: Effect of Flightless on integrin α3 and integrin β1 expression in response to skin blistering. A-H Representative images of integrin α3 and integrin β1 expression detected as white fluorescence in blister skin of Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ EBA mice and non blister skin of WT IgG control mice. Flii$^{Tg/Tg}$ EBA mice have reduced expression of integrin subunits α3 and β1 both in epidermis, dermis and around blister site when compared to Flii$^{+/-}$ and WT EBA counterparts. B = blister. e = epidermis. d = dermis. Dotted line = dermal-epidermal junction. Magnification x40. Scale bar in A = 100μm and refers to all images. **I** Representative Western Blot of integrin β1 in Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ EBA mice showing increased integrin β1 expression in response to skin blistering. Unlike in Flii$^{+/-}$ and WT mice skin, integrin β1 is not up-regulated in skin of Flii$^{Tg/Tg}$ mice in response to blistering. Data are representative of two independent repeats; equal loading is demonstrated with the β-Tubulin control.

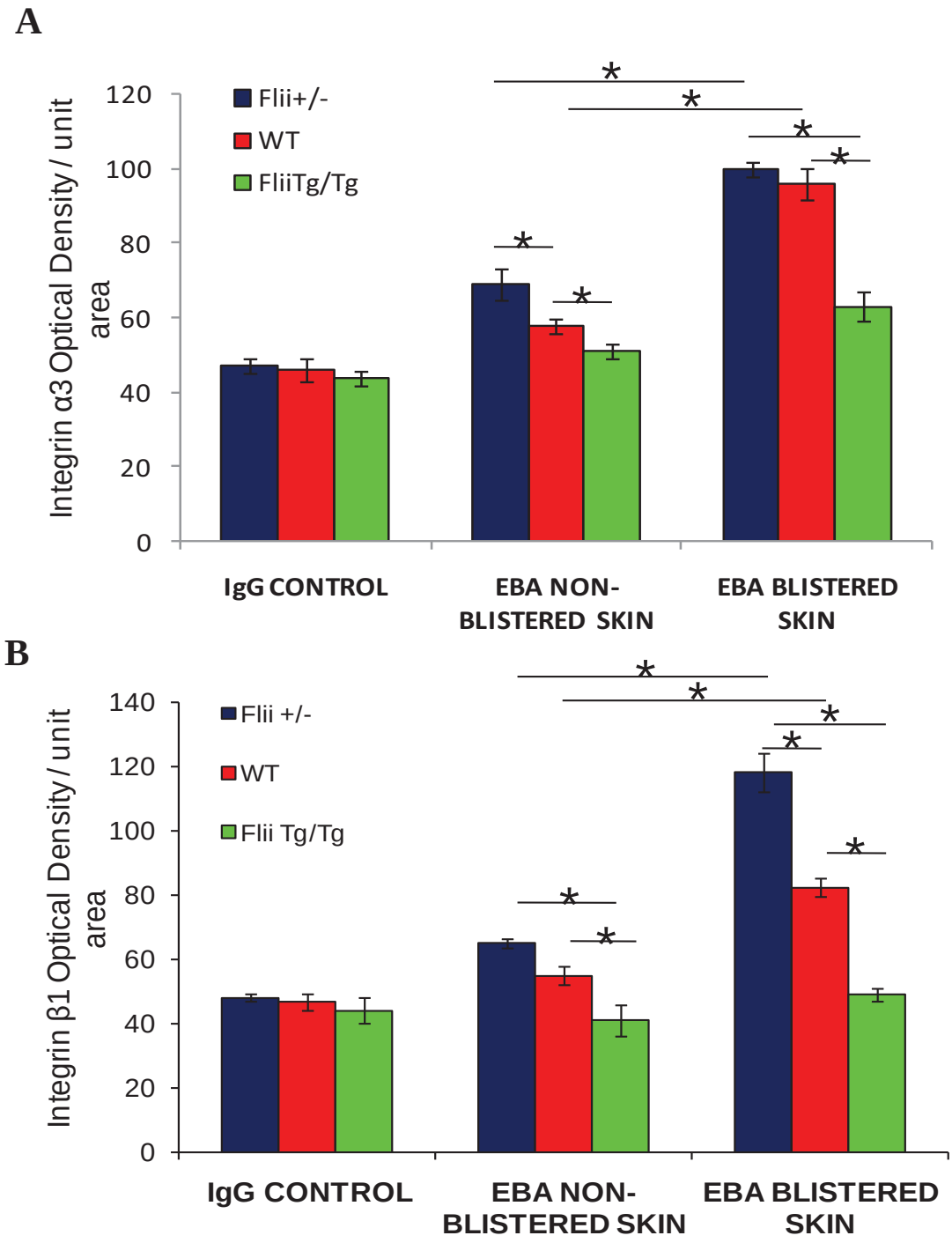

Figure 6.11: Flightless over-expression impairs integrin up-regulation. Graphical analysis of integrin α3 **A** and integrin β1 **B** expression in Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ EBA mice blister skin and IgG treated controls. Flii$^{Tg/Tg}$ EBA mice have significantly decreased integrin α3 and integrin β1 expression in non-blister skin compared to Flii$^{+/-}$ and WT EBA counterparts, and fail to up-regulate integrin expression in response to blistering. Mean +/- SEM. n=4. *P<0.05.

to wild-type EBA counterparts (Fig 6.12). In contrast, Flii$^{Tg/Tg}$ EBA fibroblasts and keratinocytes had impaired cellular adhesion on all three different extracellular matrix substrates. No significant difference was observed in the adhesion of keratinocytes cultured from EBA-induced or IgG control Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice (Fig 6.12A). However, adhesion of fibroblasts on different extracellular matrix substrates showed significantly improved adhesion of IgG control treated mouse fibroblasts compared to the EBA- induced mouse fibroblasts (Fig 6.12B). Flii$^{+/-}$ EBA mice had improved fibroblast adhesion compared to wild-type mice, while Flii over-expression led to significantly decreased adhesion of fibroblasts from both EBA-induced and IgG control mice. No significant difference in adhesion was observed with any of the extracellular matrix substrates investigated, suggesting that the effect on adhesion is independent of matrix substrate (Fig 6.12).

To further investigate whether reducing Flii expression would result in improved cellular responses, fibroblasts from wild-type EBA-induced and IgG non-induced control mice were treated with Flightless neutralizing antibody (FnAb) or isotype control and cellular proliferation and adhesion responses on collagen I extracellular matrix were analyzed *in vitro*. Fibroblasts from EBA-induced mice had significantly reduced adhesion and proliferation compared to fibroblasts extracted from IgG non-induced control mice (Fig 6.13) showing the effect of EBA on cellular responses. Treatment of cells with FnAb significantly enhanced adhesion and proliferation of non-induced control fibroblasts and fibroblasts from EBA-induced mice. Importantly, treating fibroblasts from EBA-induced mice with FnAb rescued the cell phenotype, with levels of cellular adhesion and proliferation similar to those of fibroblasts from non-induced mice and treated with isotype control antibody (Fig 6.13).

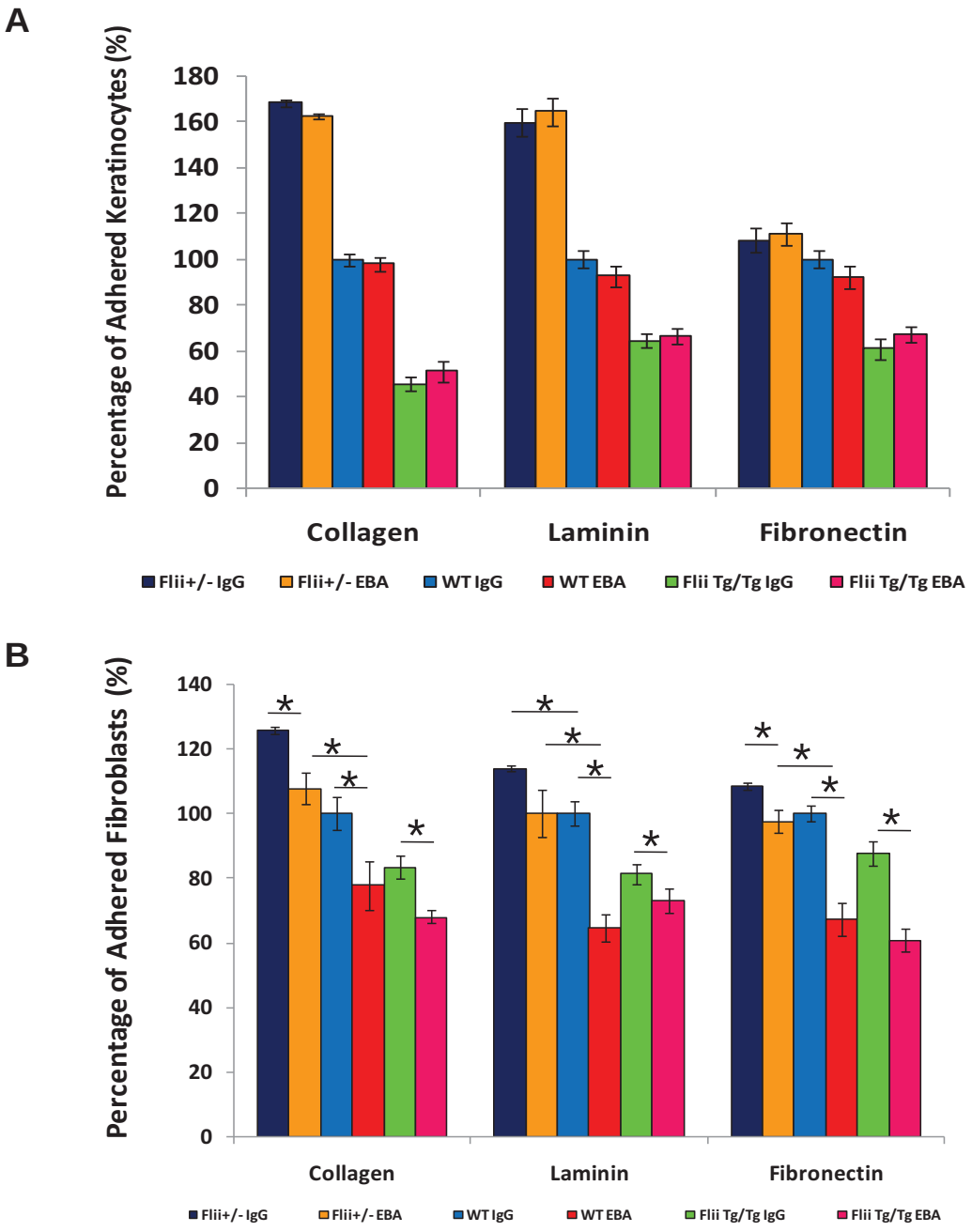

Figure 6.12: Effect of Flightless on EBA cellular adhesion. Adhesion properties of primary keratinocytes **A** and fibroblasts **B** cultured from Flii$^{+/-}$, WT, Flii$^{Tg/Tg}$ EBA mice and IgG treated counterparts were analysed on different extracellular matrix substrates. Cells from Flii$^{+/-}$ mice have improved cellular adhesion compared to WT controls while Flii$^{Tg/Tg}$ cells have significantly impaired cellular adhesion. No significant difference is observed between keratinocytes from EBA-induced mice or IgG treated Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ control mice. Reducing Flii expression improved the adhesion of EBA derived fibroblasts comparable with adhesion of fibroblasts extracted from WT IgG control mice. Mean +/- SEM. Experiment repeated in triplicates. n=2. *=P<0.05

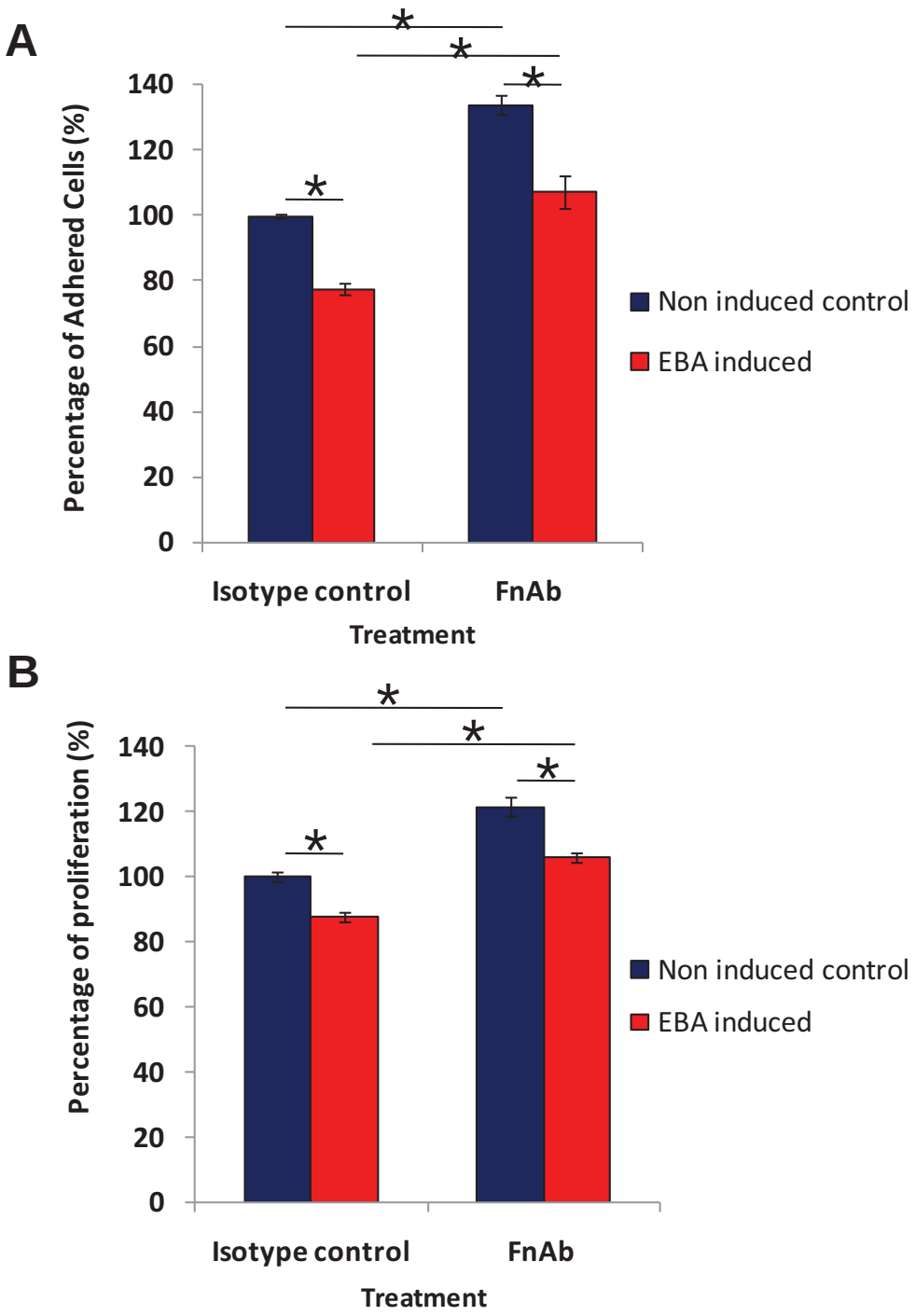

Figure 6.13: Application of FnAb improves the cellular adhesion and proliferation of fibroblasts cultured from EBA-induced mice. A-B Adhesion and proliferation properties of primary WT fibroblasts cultured from EBA-induced mice and IgG treated non-induced control counterparts were analysed on collagen I extracellular matrix substrate. Cells from EBA-induced mice have significantly decreased adhesion and proliferation properties. Treatment of cells with FnAb significantly improves cellular adhesion and proliferation and rescues the cell phenotype observed in cells from EBA-induced mice with adhesion and proliferation properties similar to cells from control non-induced mice treated with isotype control antibody. Experiment repeated in triplicates. Mean +/- SEM. n=6. *=P<0.05.

6.2.7 Flightless affects ColVII expression in unwounded skin in vivo but not the secretion of total collagen by keratinocytes and fibroblasts from EBA-induced mice in vitro

The effect of Flii on ColVII expression *in vivo* was examined using the unwounded skin of $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ mice. Skin of $Flii^{Tg/Tg}$ mice showed areas of significantly decreased ColVII staining while Flii deficient mice had improved ColVII production (Fig 6.14). Collagen secretion was examined from keratinocytes and fibroblasts cultured from wild-type and $Flii^{Tg/Tg}$ EBA-induced and IgG control mice (Fig 6.15A-B). Over expression of Flii or induction of EBA did not affect the secretion of collagen in conditioned cell culture media by primary keratinocytes or fibroblasts. While fibroblasts from $Flii^{Tg/Tg}$ EBA and IgG control mice appear to have a trend towards increased secretion of collagen *in vitro* compared to wild-type counterparts this was not statistically significant (Fig 6.15B).

6.2.8 Flightless is increased in response to blistering in ColVII hypomorphic mice

The genetically modified immunocompetent ColVII hypomorphic mice have 10% of normal ColVII levels and most closely present the severe phenotypical features charcteristic of human Recessive DEB, including mucocutaneous blistering, growth retardation and pseudosyndactyly (Fig 6.16). The ColVII hypomorphic mice develop blistering on the back in response to mechanical trauma, high inflammation in the back paws and extensive blistering and pseudosyndactyly in the front paws (Fritsch et al., 2008). Flii expression was increased in the epidermis, dermis and around the blister site in the front paws of ColVII hypomorphic mice compared to wild-type counterparts (Fig 6.17A-B). Increased Flii expression was observed in the paws of ColVII hypomorphic mice but no no significant difference in expression was observed in the back skin (Fig 6.17A-B).

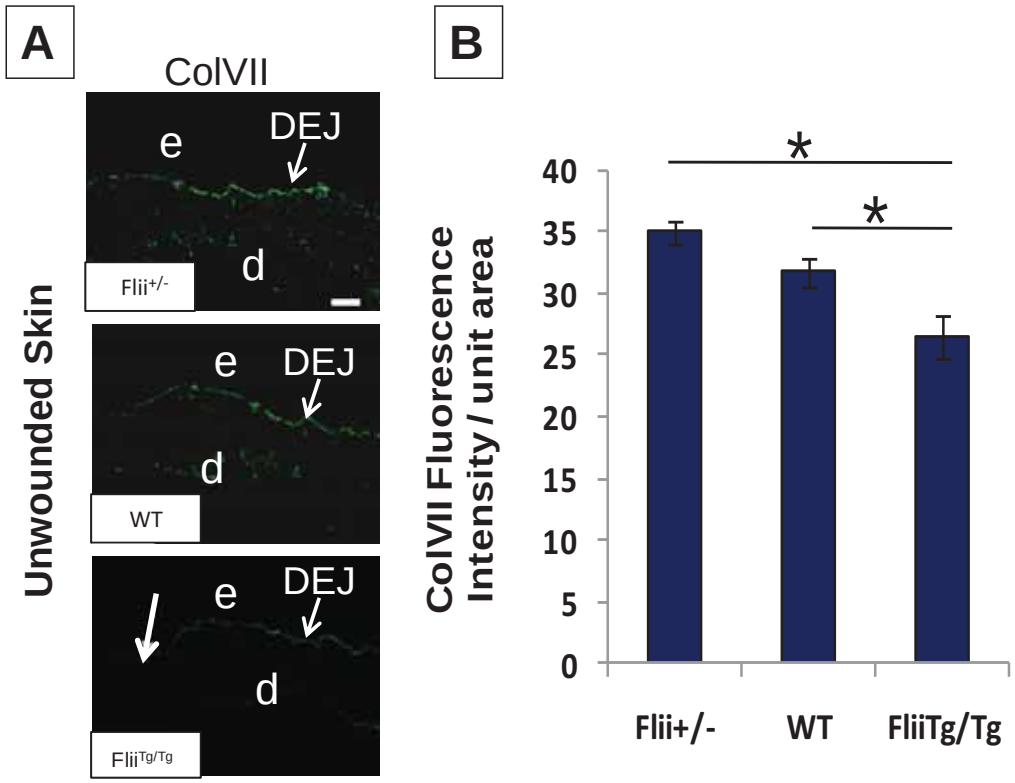

Figure 6.14: Thin and fragile skin of Flii$^{Tg/Tg}$ mice has reduced ColVII expression. A Immunofluorescence for ColVII in Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ unwounded skin shows areas of reduced ColVII (white arrow) at the dermal-epidermal junction. Magnification x40. Scale Bar = 100μm. DEJ = dermal-epidermal junction. **B** Graphical representation of ColVII expression in unwounded skin of unwounded Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice skin. Flii$^{Tg/Tg}$ mice skin has significantly reduced ColVII expression compared to Flii$^{+/-}$ and WT counterparts while Flii deficient mice skin has increased production of ColVII. Mean +/- SEM. n=6. *=P<0.05.

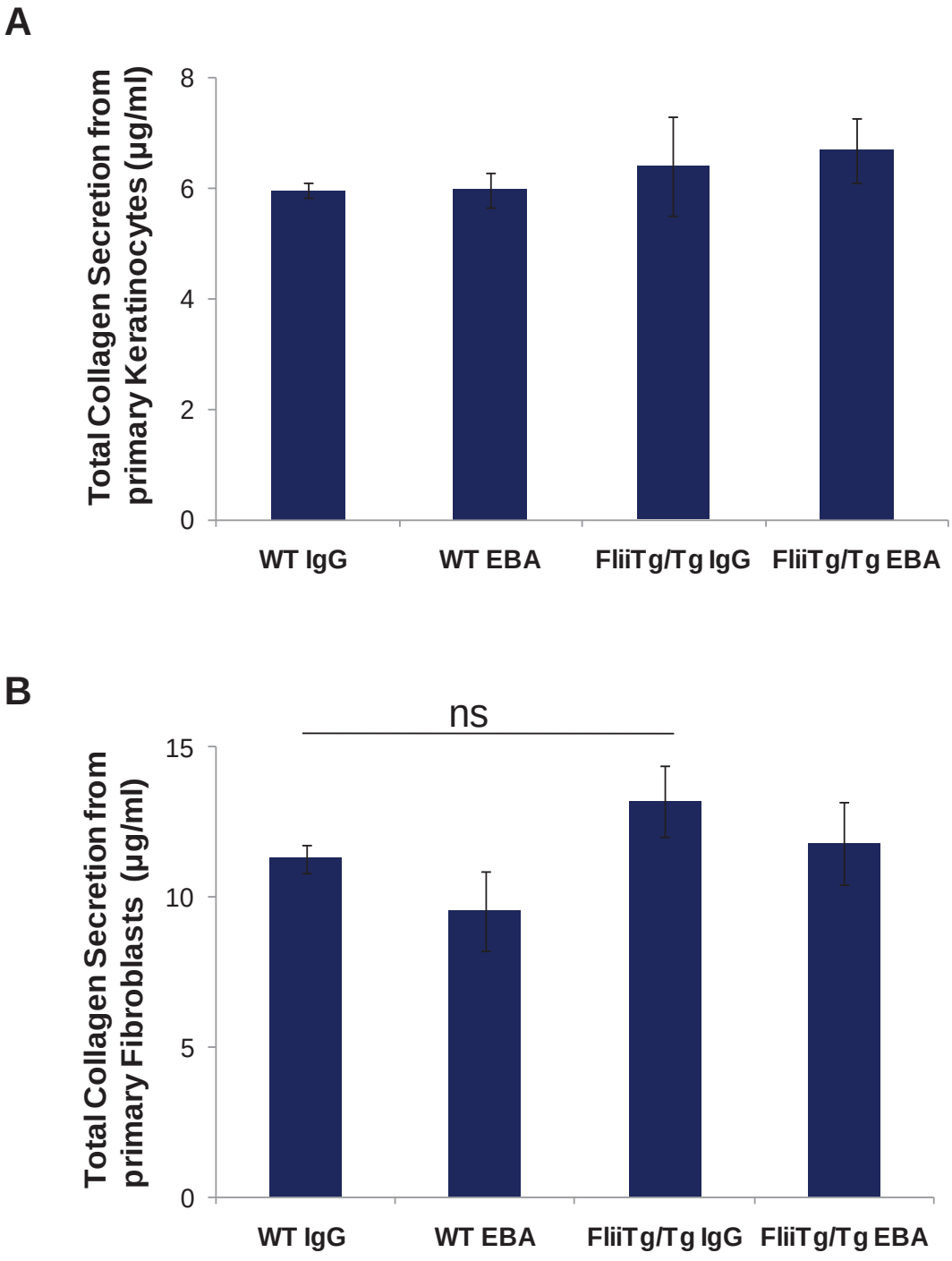

Figure 6.15: Effect of Flightless over-expression on collagen secretion. Collagen secretion from confluent primary keratinocytes and fibroblasts from EBA-induced or IgG control mice was analysed using the Sircol Dye. Total collagen secretion was analysed from cell media in triplicates using the Sircol Collagen Assay as per manufacturer's recommendations. Graphical analysis of total collagen secretion *in vitro* from primary keratinocytes **A** and fibroblasts **B** showed no significant difference in total collagen secretion *in vitro* from EBA-induced or IgG-treated cells in response to Flii over-expression. n = 2. Mean +/- SEM.

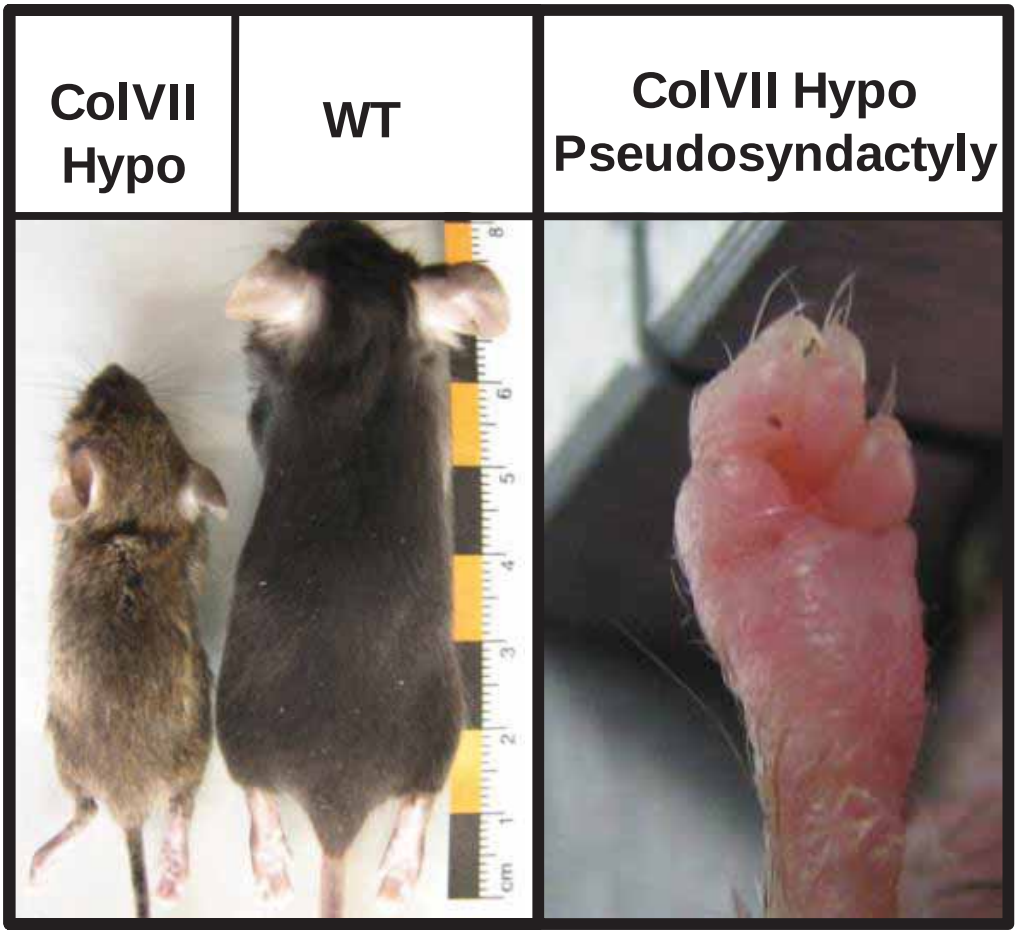

Figure 6.16: ColVII Hypomorphic mouse model. Representative images of the clinical features observed in ColVII Hypomorphic (Hypo) mice. Hypomorphic ColVII mice were generated in the laboratory of Prof L Bruckner-Tuderman. $COL7A1^{WT/WT}$, $COL7A1^{flNeo/WT}$, and $COL7A1^{flNeo/flNeo}$ animals were born in a normal Mendelian ratio. Hypomorphic ColVII mice have retarded growth compared to wild-type control mice and develop haemorrhagic blisters and pseudosyndactyly on front paws, resembling features observed in patients with Dystrophic EB. The strain was maintained by interbreeding and $COL7A1^{flNeo/flNeo}$ mice were obtained by mating homozygous siblings. Genotyping was performed to confirm the conditional inactivation of ColVII expression.

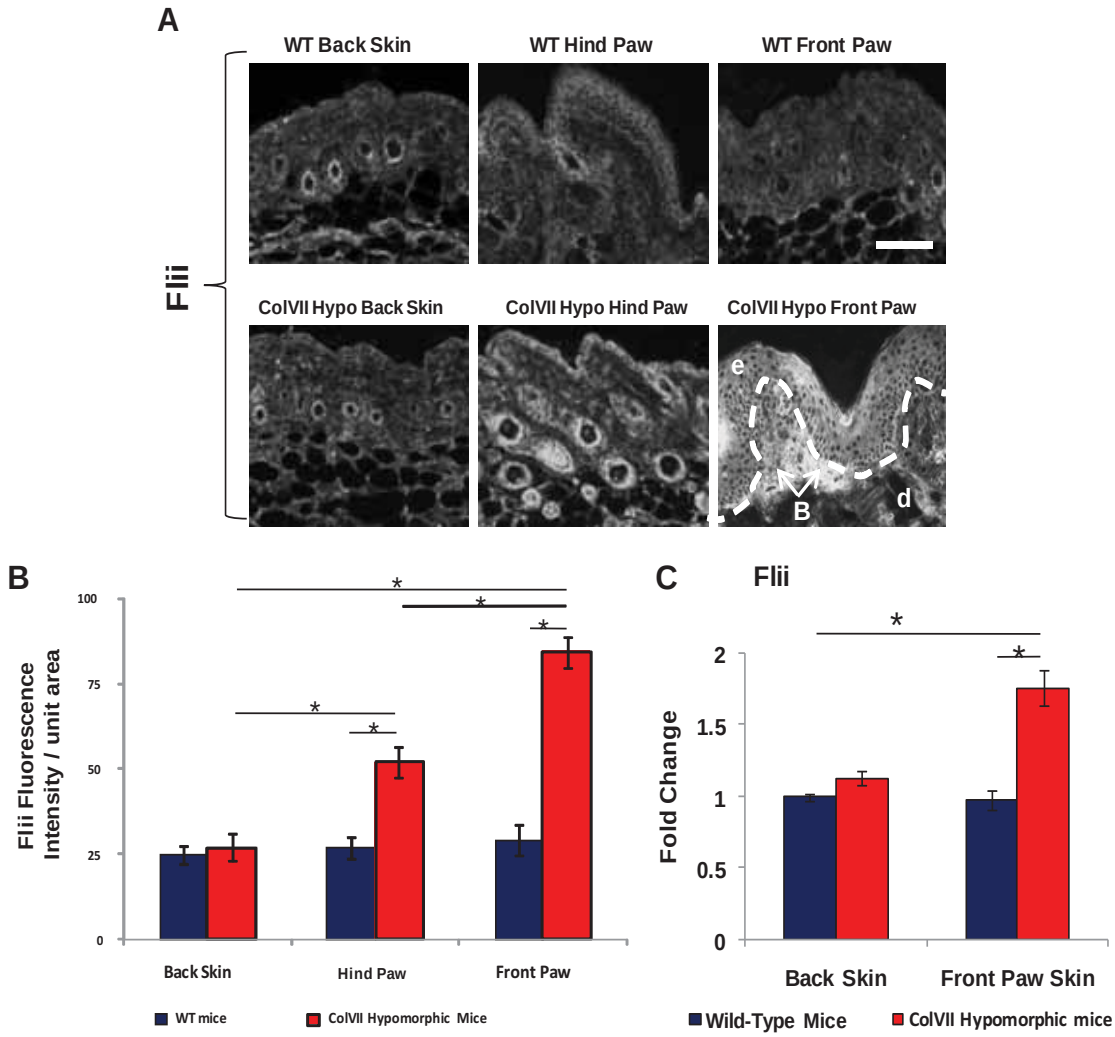

Figure 6.17: Flightless expression is increased in inflamed and blistered skin of ColVII Hypomorphic mice. A-B Representative images and graphical analysis of Flii expression in response to blistering in ColVII Hypomorphic mice. Flii expression is increased in both epidermis and dermis and around the blister site in skin from front paws and inflamed back paws when compared to non-blistered back skin of ColVII Hypomorphic mice and WT counterparts. Blistering in the front paw skin resulted in significantly increased Flii expression compared to non-blistered back paw skin of ColVII Hypomorphic mice. Magnification x40. e = Epidermis. d = Dermis. b = Blister. Dotted line = dermal-epidermal junction. Scale bar = 250µm refers to all images. **C** RT-qPCR detection of Flii mRNA levels in non-blistered back skin and blistered paw skin of ColVII Hypomorphic mice and non-blistered skin of WT counterparts collected from the same anatomical sites. *Flii* gene expression is significantly elevated in response to skin blistering in front paw skin compared to non-blistered back skin of ColVII hypomorphic mice and compared to WT counterparts. Mean +/- SEM. n = 4. *P<0.05.

Flii gene expression was significantly up-regulated in blister front paws of ColVII hypomorphic mice compared to non-blister front paws of wild-type mice (Fig 6.17C).

6.2.9 Flightless requires TGF-β1 for fibroblast differentiation and presence of TGF-β1 affects Flightless association with ColVII

TGF-β1 is an important contributor to excessive fibrosis and scarring and is considered the major growth factor promoting fibroblast differentiation into myofibroblasts. Repeated blistering observed in EB patients leads to excessive induction of tissue repair and up-regulation of TGF-β1 resulting in contractile fibrosis generated by myofibroblasts and pseudosyndactyly in the extremities (Fine *et al*., 2005). To determine if Flii modulation of blister lesions involves TGF-β1 we examined both TGF-β1 and α-SMA expression in $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ EBA-induced mice and wild-type IgG control mice. Flii over-expressing EBA-induced mice had significantly increased TGF-β1 expression in both non-blistered, dermis of the blistered skin and at the site of blister formation compared to $Flii^{+/-}$ and wild-type EBA counterparts (Fig 6.18). Conversely, $Flii^{+/-}$ EBA-induced blistered mice wounds had significantly reduced TGF-β1 and α-SMA expression *in vivo* compared to wild-type and $Flii^{Tg/Tg}$ EBA-induced mice (Fig 6.18). Incorporation of α-SMA into the actin cytoskeleton allows fibroblasts to exert enhanced contractile activity (Meyer-Ter-Vehn *et al*., 2006). Reduced Flii expression in $Flii^{+/-}$ fibroblasts extracted from EBA-induced mice resulted in weak expression and decreased incorporation of α-SMA into stress fibers (Fig 6.19A). Addition of TGF-β1 to $Flii^{+/-}$ EBA fibroblasts resulted in increased α-SMA indicating that $Flii^{+/-}$ EBA fibroblasts are still capable of differentiating into myofibroblasts given the appropriate stimulation (Fig 6.19A).

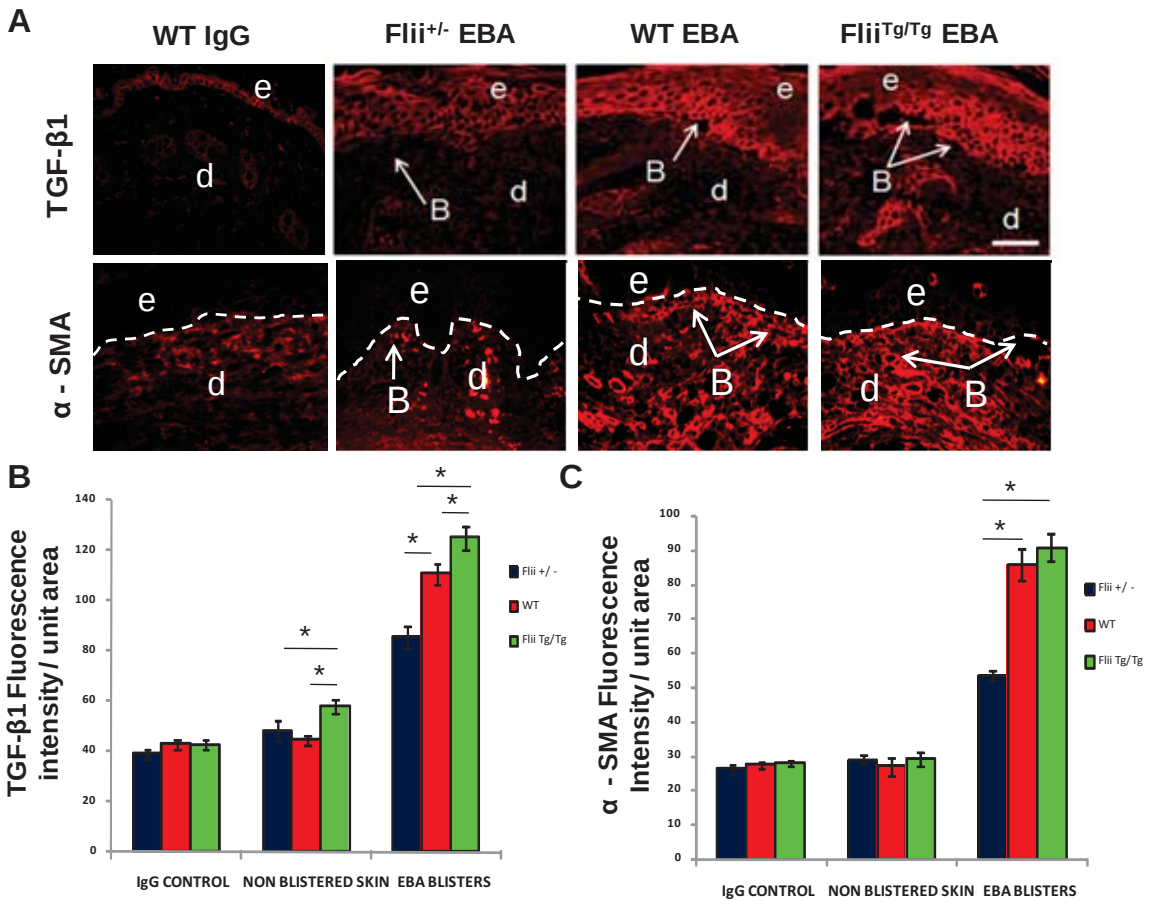

Figure 6.18: Flightless deficiency decreases TGF-β1 and α-SMA expression in EBA-induced mice wounds *in vivo*. A-C EB was induced in three week old female Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ mice and expression of TGF-β1 and α-SMA investigated in blistered skin of EB-induced mice. Flii$^{+/-}$ have significantly decreased TGF-β1 and α-SMA expression in blister skin compared to WT and Flii$^{Tg/Tg}$ EBA mice. Magnification x40. e = Epidermis. d = Dermis. B = Blister. Dotted line = dermal-epidermal junction. Scale Bar in A = 250μm. n = 4. Mean +/- SEM. *=P<0.05.

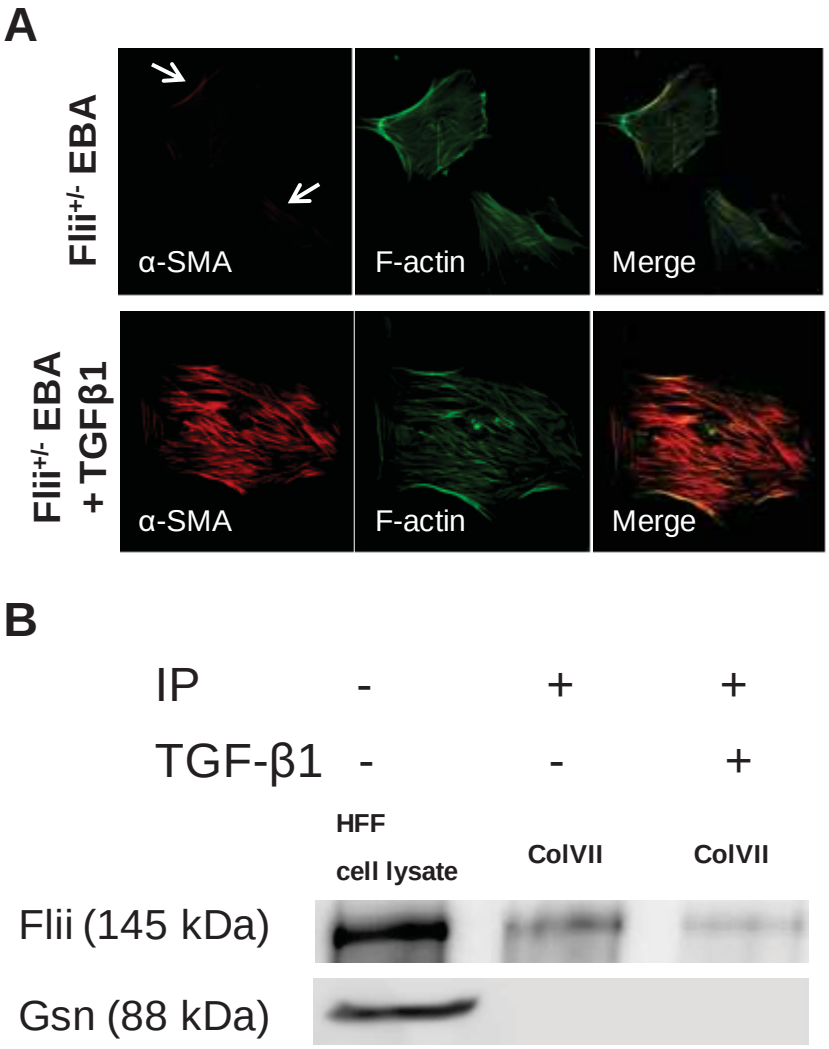

Figure 6.19: Flii$^{+/-}$ EBA fibroblasts are capable of differentiation upon stimulation with TGF-β1. A Fibroblasts cultured from Flii deficient EBA-induced mice with or without 48hr TGF-β1 stimulation were fixed and examined for development of α-SMA positive stress fibers. Flii$^{+/-}$ EBA fibroblasts are capable of differentiation and development of α-SMA positive stress fibers upon TGF-β1 stimulation. Arrow shows α-SMA in non treated control Flii$^{+/-}$ EBA fibroblasts. **B** The effect of TGF-β1 on Flii association with ColVII was investigated using immunoprecipitates (IP) from wounded HFF with or without TGF-β1. Rabbit anti-mouse ColVII antibody raised against NC-1 domain of ColVII used for *in vivo* mouse trials for induction of EBA in this study was also used in preparing the immunoprecipitates and these were immunoblotted with Flii or gelsolin (positive control) antibodies. Flii colocolizes with ColVII and this interaction is modulated in presence of TGF-β1. Data are representative of three independent experiments.

TGF-β ligand induces ColVII synthesis in dermal fibroblast cell culture (Konig and Bruckner-Tuderman, 1994) and previous studies have shown that Flii modulated TGF-β signaling (Adams et al., 2008) suggesting a possible mechanistic link between Flii and ColVII. To explore possible relationship behind Flii effect on diffuse arrangement of ColVII anchoring fibrils resulting in decreased ColVII and more thinner and fragile skin in $Flii^{Tg/Tg}$ mice, we investigated the association between Flii and ColVII proteins. Immunoprecipitates and cell lysate from wounded Human foreskin fibroblasts (HFF) were analysed using Western Blotting. Flii but not gelsolin associated with ColVII (Fig 6.19B). Additionally, the addition of TGF-β1 reduced the Flii association with ColVII suggesting that TGF-β1 might be involved in Flii interactions with matrix proteins, however exact mechanism is yet to be elucidated (Fig 6.19B).

To determine the ability of $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ fibroblasts extracted from EBA-induced mice to contract collagen, a fibroblast-populated collagen gel contraction assay was used. Wild-type and $Flii^{Tg/Tg}$ fibroblasts extracted from EBA-induced mice were able to contract the collagen gel unlike the Flii-deficient fibroblasts extracted from EBA-induced mice which had reduced contractile ability (Fig 6.20A). Exogenous addition of TGF-β1 ligand to the Flii-deficient fibroblasts extracted from EBA-induced mice in the collagen gel restored contractile ability of the $Flii^{+/-}$ fibroblasts to a level similar to wild-type fibroblasts extracted from EBA-induced mice (Fig 6.20A-B). Conversely when Flii neutralizing antibodies were added to collagen gels populated with $Flii^{Tg/Tg}$ fibroblasts extracted from EBA-induced mice, collagen contraction was similar to that observed in $Flii^{+/-}$ fibroblasts extracted from EBA-induced mice (Fig 6.20A-B).

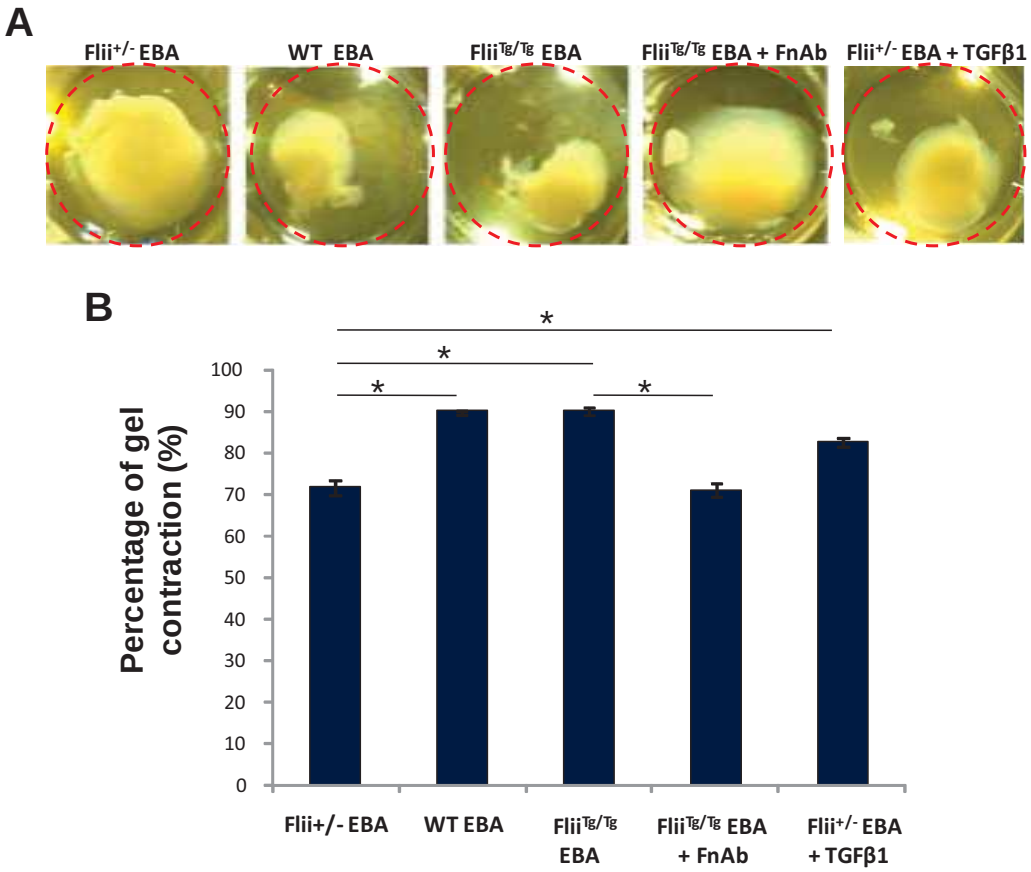

Figure 6.20: TGF-β1 mediates Flightless effects on collagen contraction. A-B Fibroblasts cultured from Flii$^{+/-}$, WT and Flii$^{Tg/Tg}$ EBA-induced mice were used in a 3D floating collagen gel contraction assay. Contraction and remodelling of collagen gel by Flii$^{Tg/Tg}$ fibroblasts extracted from EBA-induced mice in response to Flii neutralizing antibody (FnAb) and by Flii$^{+/-}$ fibroblasts extracted from EBA-induced mice in response to TGF-β1 was also examined. The degree of collagen remodelling and contraction at 72hr was determined from floating gel images using Image Pro-Plus software. Treatment of Flii$^{Tg/Tg}$ EBA fibroblasts with FnAb significantly reduces collagen gel contraction comparable to Flii$^{+/-}$ EBA mice fibroblasts while treatment of Flii$^{+/-}$ fibroblasts with TGF-β1 increases the collagen gel contraction. n=6. Experiment repeated in triplicates. Mean +/- SEM. *=P<0.05.

6.3 Discussion

EB is a complex group of genetic disorders producing various degrees of recurrent skin and mucous membrane blistering and epidermal detachment from the basement membrane. Wound healing is a major challenge for patients suffering from different EB subtypes (Remington *et al*., 2009). Due to ongoing blistering, persistent inflammation, microbial colonization with frequent infections, poor nutrient uptake and decreased oxygen supply, most blister EB lesions develop into chronic non-healing ulcers (Mellerio *et al*., 2007). Several therapeutic approaches for different EB types are currently being investigated, including gene based therapy, cell and protein based therapy and systemic therapy based on bone marrow transplantation. While some of these strategies have shown limited success all are still at the investigative stage and face numerous technical challenges (Aumailley *et al*., 2006, Bruckner-Tuderman, 2002).

Current available therapeutic approaches for inherited skin blistering disorders are only symptomatic and aimed at minimizing factors which induce blistering and complicate wound healing (Kern *et al*., 2009, Kopecki *et al*., 2009b). Patients suffering from DEB are at highly increased risk of developing SCC and while current treatments for DEB-SCC involve surgery, radiotherapy or chemotherapy preventative measures they often result in amputation of the limbs or death from aggressive metastatic SCC (Bruckner-Tuderman, 2009). This highlights the requirement for development of novel therapies aimed at improved wound healing in EB patients. Recent developments in animal models which precisely mimic the human features of EB have led to identification of novel genes and proteins involved in the molecular aspects of EB (Natsuga *et al*. 2010). Using both human samples from all four main EB types and two different animal models of EB, this study is the first to present the involvement of cytoskeletal protein Flii, in EB and EBA.

Over-expression of Flii in mice leads to altered arrangement of ColVII anchoring fibrils and significantly thinner and more fragile skin (Kopecki *et al*., 2009a) (see Chapter 3) and these features are common to human patients suffering from EB. This led to investigation of a possible role for Flii in skin blistering and EB. The results presented in this chapter show that Flii expression is specifically increased around the blister sites of patients suffering from all four main types of EB. Wound healing is significantly impaired in EB and therefore Flii may affect either the pathology or wound repair of blister lesions. Flii activity was increased in response to blistering in the majority of different EB types, including EBS, JEB, DEB and Kindler Syndrome. The most pronounced Flii activity was observed in blister skin of patients suffering from recessive DEB, correlating with the clinical severity of blistering in these patients. Considering the involvement of different proteins in different types of EB and skin blistering at different levels of the epidermal-dermal interface, this suggests that Flii may be up-regulated as a secondary consequence of tissue damage. However, Flii activity was also increased in non-blister skin of patients suffering from dominant DEB, suggesting a possible role for Flii not only in formation of blister lesions but also in the underlying pathology of DEB. The exact mechanism of Flii action in EB is yet to be investigated; however the results presented in this chapter reveal a role for Flii in blister severity and cellular responses required for wound healing of blister lesions. A better understanding of the cellular and molecular events of wound healing are essential to designing the novel therapies and better wound management associated with different EB subtypes (Uitto and Pulkkinen, 2000).

In an attempt to elucidate the role of Flii in blister formation two different mouse models of EB were utilized, each resulting in decreased expression of ColVII. Firstly, an autoimmune model of EBA was utilized where rabbit anti-mouse ColVII antibodies

developed against the NC-1 domain of ColVII were used to induce EBA in mice as previously described (Sitaru *et al*., 2005). Secondly, a recently developed ColVII hypomorphic mouse model with 10% of normal ColVII levels and resembling human features of severe recessive DEB was utilized (Fritsch *et al*., 2008, Kern *et al*., 2009). These models are different in nature; EBA being an immune-mediated inflammation-dependent model and the ColVII hypomorphic model being a genetic model of DEB. However, both result in a significant reduction of ColVII expression and skin blistering at the level of ColVII anchoring fibrils. While DEB patients have reduced or absent anchoring fibrils, EBA patients have a decrease in normally functioning anchoring fibrils, secondary to the production of ColVII autoantibodies (Remington *et al*., 2008, Sitaru *et al*., 2006, Woodley *et al*., 2006).

In agreement with previous studies (Fritsch et al., 2008, Kern et al., 2009, Sitaru *et al*., 2005) the results presented in this chapter demonstrate that antibody-mediated reduction of ColVII in an EBA mouse model results in a severe skin blistering and subsequent widespread lesions and erosions. The genetic reduction of ColVII in the ColVII hypomorphic mouse model results in mucocutaneous blistering, TGF-β1 contractive fibrosis, pseudosyndactyly and retarded growth. Both resemble phenotypical features of severe human DEB (Bruckner-Tuderman, 2009, Sitaru *et al*., 2007). Using these two different models of EB, we show a specific increase in Flii protein and gene expression in response to skin blistering, suggesting a major role for Flii in formation of blister lesions.

In agreement with increased Flii activity in human EB wounds, we found that Flii expression was increased at the site of blister formation in both EBA blister skin and

ColVII hypomorphic mice front paws which exhibit TGF-β1 mediated contractive fibrosis and pseudosyndactyly. The back paws of ColVII hypomorphic mice, which were previously shown to have high inflammation and less severe skin blistering compared to front paws (Fritsch *et al*., 2008) also showed increased Flii expression compared to the non-blistered back skin. Moreover, skin blistering in EBA mice showed a 2 fold increase in Flii expression in wild-type EBA mice and a 3 fold increase in Flii expression in $Flii^{Tg/Tg}$ EBA mice skin. This increase in Flii expression correlated with increased severity of blister formation. Conversely, reducing *Flii* gene expression by 50% in the heterozygous knockout lead to reduced incidence of blister formation with decreased blister severity scores indicating that down-regulation of this protein could lead to improved outcomes for EB patients.

Using $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ mice the effect of differential Flii gene expression on the development of skin blisters and lesions was investigated using the EBA mouse model. All mice developed EBA, with over 20% of the skin surface covered in blisters and erosions by day 16. However, $Flii^{Tg/Tg}$ mice developed more severe skin blistering that wild-type and $Flii^{+/-}$ mice and had increased number of more severe larger blisters. In contrast, $Flii^{+/-}$ EBA mice exhibited decreased blister severity compared to both wild-type and $Flii^{Tg/Tg}$ EBA mice up to D12 of the experiment. Additionally, examination of the mice skin microscopically at D16 of the experiment and assessment of dermal-epidermal separation, anchoring fibril arrangement and histological blister formation, revealed significantly greater blister formation in Flii over-expressing mice. $Flii^{Tg/Tg}$ EBA mice blister skin showed greater dermal-epidermal separation, diffuse arrangement of Collagen VII anchoring fibrils and a significantly higher blister score with larger blisters and less organized dermal tissue compared to wild-type and $Flii^{+/-}$ counterparts. In contrast,

Flii$^{+/-}$ EBA mice blister skin showed anchoring fibril arrangement into anchoring plaques and improved wound healing with well re-epithelialised wounds. These findings are in agreement with previous studies which showed that delayed wound reepithelialisation and inhibited wound repair occurred in mice with increased Flii activity and improved wound healing in Flii deficient mice (Adams *et al*., 2009, Cowin *et al*., 2007). While the exact mechanism of Flii activity during wound repair is yet to be elucidated, these results identify Flii as a potential target for development of a novel therapy for improved wound healing in EB patients.

The results presented in Chapter 3 suggested one possible mechanism behind Flii regulation of wound healing is through modulation of integrin-mediated cellular adhesion. These findings indicated that Flii associates with talin and alters the pool of talin available for binding and activation of integrin receptors hence leading to changes in cellular adhesion and migration (Kligys and Jones, 2009, Kopecki *et al*., 2009a). Using both non-blistered and blister skin from Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ EBA mice, expression of integrin α3 and β1 were examined. The effect of Flii on integrin α3 and β1 expression was evident during wound repair of blister skin. In agreement with results described in Chapter 4 of this thesis, Flii over-expression inhibited the up-regulation of integrin α3 and β1 in EBA mice. Both Flii$^{+/-}$ and wild-type EBA wounds showed significantly elevated levels of integrin α3 and β1 throughout the epidermis allowing basal keratinocytes to utilize these receptors for cellular adhesion and migration over the wound bed. In contrast, Flii$^{Tg/Tg}$ EBA wounds had non-specific integrin α3 and β1 staining only in the apical part of the epidermis failing to up-regulate the integrin receptors in basal keratinocytes. Different integrin receptor expression and affinity for extracellular matrix ligands could adversely affect cellular adhesion, migration and fibroblast-mediated wound contraction. These

results suggest that Flii effect on integrin receptors may alter the wound healing of blister lesions by affecting cellular responses including adhesion, migration and wound contraction.

Examining the adhesion properties of primary keratinocytes and fibroblasts derived from Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ EBA-induced and IgG control mice revealed that fibroblasts retain a cell specific effect of Flii on cell adhesion as induction of EB in mice with varying expressions of Flii did not affect adhesion of keratinocytes compared to control non-induced mice with same expressions of Flii. Over-expression of Flii led to an expected decrease in keratinocyte and fibroblast cell adhesion, and induction of EBA further decreased cell adhesion properties. Fibroblasts cultured from Flii deficient EBA mice had similar adhesion to fibroblasts cultured from wild-type IgG control mice, suggesting that specific reduction of Flii expression in EB mice can rescue the fibroblast cell phenotype and improve cellular adhesion properties to similar levels of wild-type control cells hence indicating that the presence of Flii could be a negative contributing factor in pathology of the EBA or may contribute to the clinical symptoms observed.

In support of Flii being a negative contributing factor in EBA, *in vitro* assays of cellular adhesion and proliferation showed that application of FnAb significantly improved fibroblast adhesion and proliferation. Importantly, application of FnAb to fibroblasts cultured from EBA-induced mice rescued the cell phenotype with adhesion and proliferation levels similar to cells from control mice. Indeed, recent studies have identified fibroblasts as significant source of ColVII anchoring fibrils contributing to skin stability *in viv*o (Fritsch *et al*., 2009) and have demonstrated the therapeutic potential of fibroblasts in

EB (Fritsch *et al*., 2008, Kern *et al*., 2009, Wong *et al*., 2008, Woodley *et al*., 2003). Reducing *Flii* gene or protein expression rescued the EBA induced fibroblast cell phenotype and improved cellular adhesion and proliferation properties suggesting that the presence of Flii is a negative contributing factor in EBA. These data are the first to indicate that the development of novel treatment methods aimed at reducing Flii expression in blister lesions might improve the wound healing of EBA patients by improving cellular adhesion, migration and proliferation properties. In agreement with previous study establishing the effectiveness of FnAb on cellular migration and proliferation and subsequent improved wound healing (Cowin *et al*., 2007) these results also suggest that application of FnAb may be beneficial to improved wound healing of patients suffering from EBA.

Type VII collagen (ColVII) is a major component of anchoring fibrils, providing stability to the dermal-epidermal interface (Chung and Uitto, 2010). TGF-β1 is an important regulator of mesenchymal-epithelial interactions and regulates ColVII deposition through Smad dependant binding to the COL7A1 gene promoter and interaction with AP1 transcription factor (Konig and Bruckner-Tuderman, 1994, Naso *et al*., 2003, Ryynanen *et al*., 1991, Vindevoghel *et al*., 1998). In Chapter 3 we have shown that Flii over-expression results in diffuse arrangement of ColVII anchoring fibrils (Kopecki *et al*., 2009a). An increase in Flii expression in response to blister formation may therefore have an inhibitory effect on ColVII fibrilogenesis. Examining the unwounded skin of $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ mice showed significantly decreased ColVII expression in Flii over-expressing mice compared to both $Flii^{+/-}$ and wild-type mice while Flii deficiency improved ColVII expression. Investigating the potential mechanisms of Flii effect on ColVII indicated no significant difference between total collagen secretion in wild-type or Flii over-expressing

cells cultured from EBA-induced and IgG control mice. These results suggest that Flii might not affect the synthesis and secretion of pro-ColVII polypeptides but may interfere with their assembly into functional anchoring fibrils extracellularly, as Flii is also a secreted protein (Cowin *et al*., 2007), however this is yet to be investigated. This potential mechanism is supported by results presented in this chapter whereby using antibodies raised against the NC-1 domain of murine ColVII we confirmed that Flii associates with the NC-1 domain of ColVII. The association between Flii and ColVII is reduced in the presence of TGF-β1, suggesting that TGF-β1 may negatively influence Flii association with ColVII. These findings, in conjunction with previous studies showing increased numbers of hemidesmosomes in Flii$^{+/-}$ mice skin (Kopecki *et al*., 2009a) (see Chapter 3), suggest that Flii may have a role in modulating dermal-epidermal adhesion. Interestingly, although Flii$^{Tg/Tg}$ mice do have thinner, more fragile skin (Kopecki *et al*., 2009a) (see Chapter 3) they do not have an overt blistering phenotype. However, only 35% of normal ColVII expression is required for skin to maintain its stability and the barrier function (Fritsch *et al*., 2009). Moreover, a recently developed ColVII hypomorphic mouse model showed that the presence of only 10% of normal ColVII expression confers sufficient stability to the dermal-epidermal junction for long-term survival albeit with subepidermal blistering and development of symptoms resembling recessive DEB (Bruckner-Tuderman *et al*., 2010, Fritsch *et al*., 2008).

Previous studies have suggested that TGF-β1 is a key growth factor involved in fibrosis and scarring in recessive DEB (Fritsch *et al*., 2008, Konig *et al*., 1992, Ryynanen *et al*., 1991, Vindevoghel *et al*., 1998). TGF-β binds to the COL7A1 promoter and therefore modulators of TGF-β may be valuable in the prevention of excessive fibrosis in EB patients (Vindevoghel *et al*., 1998). Flii has previously been show to affect TGF-β gene

expression in response to burn injury and reducing *Flii* gene expression or protein levels using FnAb decreases pro-fibrotic TGF-β1 and increases anti-fibrotic TGFβ3 expression (Adams *et al*., 2009). In this study blisters of Flii deficient EBA-induced mice had decreased levels of pro-fibrotic TGF-β1 and reduced numbers of α-SMA positive myofibroblasts compared to both wild-type and $Flii^{Tg/Tg}$ EBA-induced mice lesions suggesting that Flii may modulate blister severity via its affects on TGF-β1. $Flii^{+/-}$ fibroblasts from EBA-induced mice, with decreased TGF-β1 levels, had reduced contractile ability which in a fibrotic condition would expect to result in reduced contracture. Flii deficient fibroblasts from EBA-induced mice showed reduced assembly of α-SMA positive stress fibers and addition of exogenous TGF-β1 enhanced the contractile activity with increased assembly of α-SMA positive stress fibers and increased collagen gel contraction *in vitro*. Similarly, $Flii^{Tg/Tg}$ fibroblasts extracted from EBA-induced mice and treated with FnAb were less able to contract collagen. While collagen contraction is an important prerequisite of a healing wound, excessive contraction and fibrosis contribute to hypertrophic scarring, esophageal stricturing and pseudosyndactyly, all of which are major problems for RDEB sufferers and in EBA. These results demonstrate the therapeutic potential of FnAb for treatment of EBA.

In conclusion, Flii is elevated in the blisters of EB patients and upregulation of Flii increases the severity of blistering in mouse models of RDEB and EBA. Reducing Flii expression either genetically or using neutralizing antibodies reduces the incidence and severity of the blisters, improves cellular adhesion and proliferation and reduces collagen contraction. Exogenous addition of TGF-β1 to Flii deficient fibroblasts from EBA induced mice restores the contractile phenotype of these cells, suggesting that TGF-β1 may be involved in mediating Flii function in EB. Reducing Flii levels within the blisters of

patients with EB and EBA may be a novel approach for reducing the severity of the blisters and for improving wound healing and decreasing scarring, thus improving the quality of life for these patients.

Chapter 7

GENERAL DISCUSSION AND FUTURE DIRECTION

7.1 Discussion

Current advances in wound research have led to improved patient wound care over the last few decades however impaired healing of human skin wounds still is a silent epidemic threatening public health and economy. In excess of US$25 billion is spent annually for treatment of chronic wounds in the USA alone and this figure is expected to rise in the future (Sen, 2009). Western society is rapidly aging with increasing health care costs and a sharp rise in obesity. These factors will lead to an increasing need for better wound care therapies in the future. In developed countries like Australia, it has been estimated that 2% of population will experience a chronic non-healing wound during their life-time. This percentage is much higher in patients suffering from genetic skin blistering conditions like EB who are faced with this challenge every day (Mellerio *et al*., 2007, Sen, 2009). EB patients experience large non-healing wounds and spontaneous skin blistering affecting over 70% of their bodies. With over a thousand EB patients in Australia this will significantly contribute to the public health system and the economy (Kho *et al*., 2010).

Skin fragility and constant blistering seen in EB patients is associated with chronic pain and functional limitations, resulting in a severely decreased quality of life (Frew and Murrell., 2010). Blister wounds are often “stuck” in the inflammatory phase of wound healing and exhibit impaired wound healing similar to that of chronic wounds. Additionally, they have a high potential for malignant transformation, resulting in high mortality rates (South and O'Toole, 2010). Consequently, genetic skin blistering conditions like EB highlight the need for further research and development of therapies for improved wound healing. The work described in this thesis has focused on understanding the role of

Flightless (Flii) protein on skin architecture, cellular responses during wound healing and skin blistering associated with EB.

7.2 The effect of Flightless on skin architecture and cellular responses during wound healing

Since 1997 when the human homologue of the Flii gene was identified (Campbell *et al*., 1997) the main research focus evolved around its role in development and its functions as a hormone-activated nuclear receptor co-activator (Archer *et al*., 2004, Campbell *et al*., 2002, Davy *et al*., 2000). Flii role in wound healing was not identified until a decade later when our laboratory showed that Flii was a negative regulator of wound repair (Cowin *et al*., 2007). Flii deficient mice had improved wound healing, with increased epithelial migration and improved cellular proliferation whereas Flii over-expression impaired wound healing (Cowin *et al*., 2007). These findings suggested that Flii is an important regulator of wound healing, however, prior to the present study described in this thesis little was known about its effects on skin architecture or cellular spreading and adhesion which may underpin the mechanism by which Flii affects cellular migration during wound healing.

The results described in this thesis have identified the effect of differential Flii expression on unwounded skin architecture. Flii over-expressing mice had significantly thinner dermal tissue and more fragile skin compared to wild-type and Flii deficient counterparts. These results suggest that Flii may affect the adhesion of skin layers, the

composition of the skin extracellular matrix proteins and may also be involved in skin atrophy. Although Flii expression modulates collagen I levels during incisional wound healing and burn injury repair (Adams *et al*., 2009, Cowin *et al*., 2007), no differences have been reported for its effects on other extracellular matrix proteins. Results in Chapter 4 and 6 show decreased expression of laminin protein in wounds of Flii over-expressing mice and decreased ColVII expression in unwounded skin of Flii over-expressing mice both of which may contribute to decreased cellular signaling and migration as well as increased skin fragility. Healed wounds attain approximately 80% tensile strength of unwounded skin, consequently it is important to distinguish the differences in expression of extracellular matrix proteins in unwounded vs. wounded skin as modulation of different extracellular proteins can allow us to improve skin strength post wound reepithealisation or prevent fragile skin blistering as seen in patients with EB. One protein which is crucial for the maintenance of skin integrity is collagen VII. Patients suffering from DEB have a genetic mutation in collagen VII and decreased or absent collagen VII production resulting in fragile skin and constant blistering following mild trauma (Bruckner-Tuderman, 2009).

Over-expression of Flii causes decreased skin tensile strength whereas decreased Flii expression causes increased strength and we have confirmed that Flii over-expression decreases the number and alters the structure of hemidesmosomes providing a potential explanation for increase in skin fragility in these mice. These data suggest a possible role for Flii in maintenance of stable adhesion at the dermal-epidermal junction. Additionally, over-expression of Flii results in an altered arrangement of keratin tonofilaments and collagen VII anchoring fibrils, suggesting that Flii has the ability to interact with various proteins involved in maintaining strong dermal-epidermal adhesions.

The functional effect of Flii on the cell responses results presented in Chapter 3, showed that over-expression of Flii impairs cell spreading, adhesion and migration of primary murine cells on different extracellular matrices (Kopecki *et al*., 2009a). While it may appear controversial that over-expression of Flii inhibits cell adhesion in unwounded skin and impedes cell migration during wound healing it is not unusual for a multifunctional protein to have different roles in response to skin wounding. Gelsolin, the founding member of the gelsolin family of actin remodelling proteins is involved in actin remodelling through severing, capping and nucleating actin fibers and yet in response to wounding it is also secreted in circulation of burn patients to scavenge actin released in response to tissue injury and may play a part in inflammation against components of the bacterial cell wall (Bucki *et al*., 2008, Silacci *et al*., 2004).

The results presented herein suggest that Flii may play a role in modulating the expression of integrin receptors responsible for adhesion to different extracellular matrix proteins and have an effect on different adhesion signaling pathways. Different proteins present at the dermal-epidermal junction have multiple roles and are involved in both the maintenance of structural integrity of the skin and signal transduction, including tetraspanin CD151, talin, kindlin-1, paxillin and integrins. These proteins link the extracellular environment to the actin cytoskeleton and mediate different cellular responses during wound healing (Geary *et al*., 2008, Moser *et al*., 2009, Ussar *et al*., 2008). Our results show that Flii has the ability to regulate the expression of different adhesion integrins during wound healing through associations with proteins present at the dermal-epidermal junction and to modulate the cellular responses in spreading, adhesion and migration. Furthermore, Flii over-expression alters skin architecture and impairs cell

adhesion and migration in wound repair. This study is the first to show that Flii indirectly affects hemidesmosome adhesion components, including CD151, laminin and laminin binding integrins through association with talin, paxillin and vinculin.

The effect of Flii on paxillin phosphorylation suggests a link between Flii and formation and turn-over of focal adhesion structures. Using a multidimensional approach of both *in vitro* and *in vivo* methodologies results presented in this thesis identify a potential function of Flii in focal adhesion formation and demonstrate the direct correlation between the *in vitro* cell based assays and *in vivo* observations in tissues. Flii over-expression results in larger focal adhesions and a decreased turnover of focal adhesion structures and down-regulation of adhesion signaling proteins. In addition to its role in cellular adhesion, spreading, migration and proliferation, Flii also affected cellular contraction and collagen lattice remodelling confirming another potential role for Flii in cellular responses. It was notable that decreased Flii expression resulted in a decreased contraction of collagen fibers. Decreased expression of gelsolin has also been shown to result in decreased contraction of collagen fibers (Arora *et al*., 1999) and a recent study in NIH 3T3 cells demonstrated that decreasing Flii expression using siRNA results in decreased stress fiber formation (Higashi *et al*., 2010). These findings suggest that in addition to modulating collagen I secretion (Cowin *et al*., 2007), Flii effect on collagen fiber contraction and lattice remodelling may also contribute to better physiological outcomes post wounding as slower more controlled laying of collagen results in decreased skin fibrosis. These findings suggest that Flii is important in maintenance of skin integrity and is a multifunctional protein which plays an important role in skin homeostasis; however its over-expression results in impaired wound healing. Flii may also be involved

in numerous signaling pathways involved in mediating cellular responses during wound repair. In both mice and humans Flii expression increases with age and amelioration of Flii activity represents a possible therapeutic strategy for improved wound healing in humans. Consequently, potential therapies aimed at decreasing Flii expression and improving wound healing should be targeted to the affected wound area as systemic reduction in Flii expression may cause unwanted side effects to both skin and other tissue sites where Flii functions are yet to be identified.

7.3 Mechanistic insight into the role of Flightless in cell signaling during wound repair

The process of wound repair largely depends on different complex signaling pathways that dictate cell responses by integrating extracellular and intracellular signals. Results presented in Chapters 4 and 5 have investigated the possible involvement of Flii in mediating wound repair through its associations with integrins and downstream adhesion signaling proteins like paxillin and p130Cas. However it is important to acknowledge that there are number of different signaling pathways mediating cellular responses during wound healing through which Flii may also function.

Current research has largely focused on the role of the TGF-β signaling pathway during wound healing as this pathway is a central mediator in regulating a wide range of pro-fibrotic events, including chemotaxis, extracellular matrix synthesis and autocrine activation of growth factors (Pannu *et al*., 2007, Wilkes *et al*., 2005). TGF-β, through its

downstream signalling of Smad proteins is a regulator of protease inhibitor expression and extracellular matrix deposition. These functions make TGF-β a powerful effector in wound healing. However it can act as a pathogenic growth factor when continuous TGF-β signaling leads to tissue fibrosis (Mauviel, 2009). A study by Denton and colleagues showed that TGF-β signaling in fibroblasts was important not only for production of extracellular matrix components and their differentiation into contractile myofibroblasts but also for promoting keratinocyte function during wound healing (Denton *et al*., 2009, Mauviel, 2009). Indeed, research has shown that TGF-β is a potent activator of ColVII expression and cell secretion and is vital for formation of anchoring fibrils post wound re-epithelialisation and an important factor in wound healing of patients suffering from EB (Naso *et al*., 2003). Research to date indicates that Flii is also involved in modulating the TGF-β/Smad signaling. Studies in our laboratory have shown that Flii improves wound repair through modulation of TGF-β1 and TGF-β3 expression in wounds *in vivo*. Results presented in this thesis have further established a link between Flii and TGF-β1 with TGF-β1 mediating Flii effects on fibroblasts differentiation and collagen contraction; and affecting Flii association with ColVII. These results will be further discussed in the EB section of this discussion.

Cell surface receptors do not directly regulate gene expression and rely on signal transduction to be mediated through intracellular signaling pathways to provide a link between extracellular matrix, actin cytoskeleton and transcriptional/translational activation. A number of studies have shown a cross-talk between pathways involved in cytoskeletal remodelling, cellular adhesion and migration during wound repair, including TGF-β signaling and members of the MAP kinase family, Ras, RhoA, MAPK-ERK kinase and

ERK1/2 (Begum *et al*., 2004, Bhowmick *et al*., 2001, Hall and Nobes, 2000, Wang *et al*., 2005). Flii co-localizes with different molecules involved in regulating cytoskeleton re-organisation, including Ras and Cdc42, and has both cytoskeletal and nuclear regulatory functions which suggest that it is likely to be involved in numerous different pathways (Campbell *et al*., 2002, Davy *et al*., 2000, Lee *et al*., 2004). The ability of Flii to bind Ras and Cdc42 suggests its involvement in both PI3K and MAPK signaling pathways as both Ras and Cdc42 are known effectors of these pathways which regulate numerous cellular responses during wound repair. The PI3K/Akt signaling pathways is known to regulate numerous fundamental cellular functions during wound repair, including cell growth, proliferation, motility and survival and is activated by a variety of extracellular signals (Wilkes *et al*., 2005). This signaling pathway is known to negatively regulate TGF-β1 activity due to inhibitory binding of Akt to Smad3 (Tian *et al*., 2002). Moreover, studies by Davy *et al*. (2001) have shown that inhibitors of the PI3K prevent the translocation of Flii to actin structures, implying Flii is involved in the PI3K signaling pathway (Davy *et al*., 2001). In support of Flii involvement in the PI3K signaling pathway, results presented in this thesis describe an effect of Flii on modulation of small GTPases, including Rac1, RhoA and Cdc42. Consequently, Flii could be involved in mediating different signaling pathways through both direct and indirect interactions with various signaling and adaptor proteins. Better understanding of these pathways and their role in regulating keratinocyte and fibroblast cell adhesion, proliferation and migration as well as Flii direct/indirect effect on these pathways and outcome of dermal fibrosis may provide new understanding about wound repair and reducing skin fibrosis.

How can a single protein, like Flii, be involved in multiple signaling pathways co-ordinating numerous cellular responses during a complex process of wound repair? Compared to the other members of the gelsolin family of actin remodelling proteins, Flii has a unique structure composed of gelsolin related domains and a LRR domain. Using transgenic animals and multiple *in vivo* and *in vitro* approaches in our laboratory, we have shown that Flii effects on wound healing are Flii specific (Cowin *et al*., 2007). We have developed a panel of neutralising antibodies against different domain regions of Flii and have demonstrated that intradermal application of neutralising antibodies raised against the LRR domain of Flii to the wounds significantly improved wound healing (Cowin *et al*., 2007) suggesting that Flii effects on wound healing are attributed to the LRR domain of Flii. However, Flii function through binding of the Flii gelsolin domain can still result in a Flii specific event. A recent study has identified Flii as a regulator of Rho induced linear actin assembly mediated by Diaphanous-related formins, Daam1 and mDia1. Flii but not gelsolin associates with formins and this binding is mediated specifically by the G4-6 gelsolin motif of Flii protein which has a 41% homology and 18% identity to the corresponding G4-6 region of gelsolin (Higashi *et al*., 2010). These results indicate that a conserved protein like Flii which has a unique structure can act as multifunctional protein in skin with specific Flii events on different cellular responses.

Results in this thesis show that Flii does not interact with integrins directly however it does associate with proteins present in the cytoskeletal complex, notably talin and paxillin. Association of Flii with these proteins can regulate both integrin activation and downstream signaling pathways. This implicates Flii in both the regulation of integrin receptors and downstream adhesion signaling pathways (Kopecki *et al*., 2009a). Further

research is required to precisely elucidate how Flii involvement in adhesion signaling pathways affects both the expression of integrin receptors and signaling proteins as well as dynamic actin changes required in response to cellular adhesion and directional migration during wound healing. Based on data from this thesis, a schematic diagram of Flii involvement with a potential cytoskeletal complex regulating cellular adhesion and migration responses during wound healing is presented in Figure 7.1 highlighting the association of Flii with structural and signaling cytoplasmic proteins.

To help understand the role of Flii in cellular adhesion dynamics, results described in this thesis show that Flii over-expressing fibroblasts have a significantly reduced number of focal complexes, decreased paxillin activation and increased number of actin stress fibers. These results suggest that Flii inhibition of paxillin phosphorylation at Y118 site inhibits paxillin-mediated focal adhesion disassembly. In agreement with our data, studies by Webb and colleagues (2004) have shown that mutations of Y118 to non-phosphorylatable amino acid impairs the disassembly of adhesions at the leading edge of migrating cells. Moreover, phosphorylation of paxillin at site Y118 indirectly actives Rac1, inhibits RhoA and enhances the binding of FAK to paxillin, all of which are required for adhesion and leading edge protrusion during cell migration (Zaidel-Bar *et al*., 2007). The precise mechanisms by which paxillin influences adhesion disassembly is currently unclear, but potentially involves interactions of paxillin with ERK, and Src-FAK to regulate the kinase dependant contractibility and protease activity (Ishibe *et al*., 2003, Zaidel-Bar *et al*., 2007). Flii is up-regulated in response to wounding and here we identify that over-expression of Flii inhibits paxillin activity and reduces adhesion signaling by modulating the activity of the Rho family GTPases. Flii over-expressing fibroblasts have

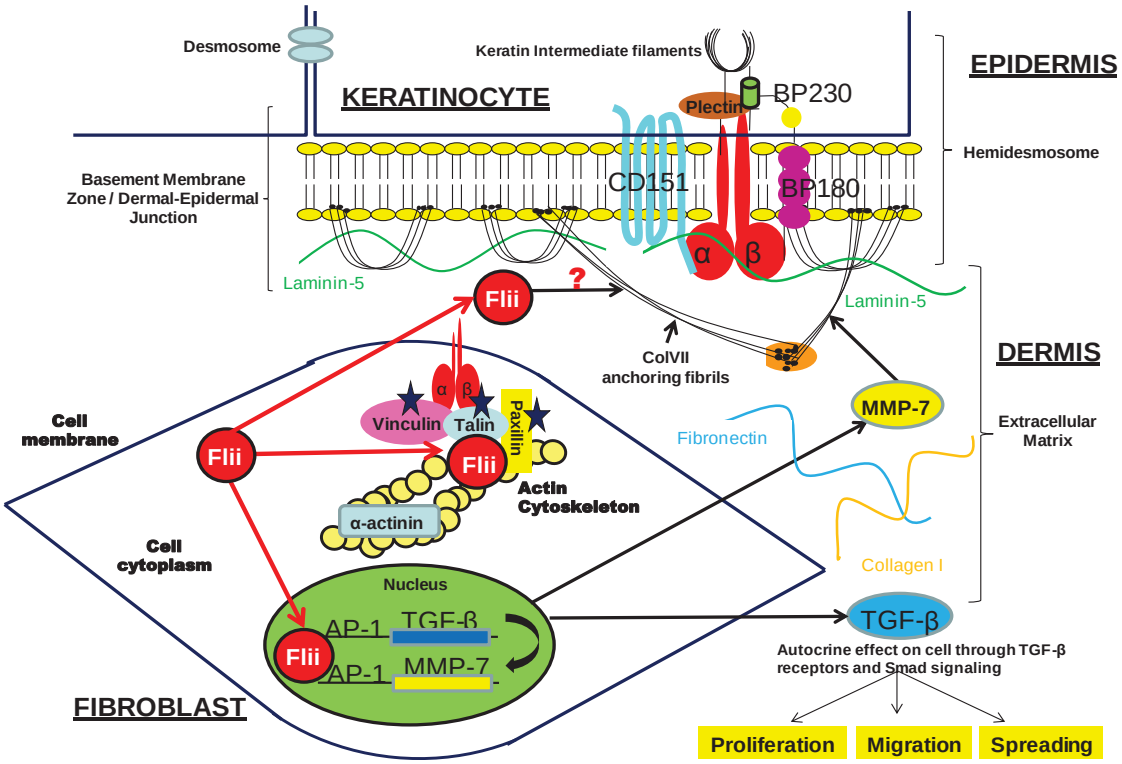

Figure 7.1: Schematic diagram of Flightless involvement with the cytoskeletal protein complex regulating cellular adhesion and migration response during wound healing. Figure indicates Flii cytoskeletal involvement, translocation to the nucleus and secretion to the extracellular matrix (red arrows) indicating a multifunctional response during wound healing. Subsequent proposed interactions are indicated with the black arrow. Flii association with structural and signaling cytoplasmic proteins (blue star) forming the cytoskeletal complex and regulating the integrin mediated cellular adhesion and migration. Flii effect on expression of hemidesmosome protein components including integrin receptors, laminin-5, CD151 and ColVII may explain altered fragile skin and hemidesmosome formation observed in Flii over-expressing mice skin.

reduced active Rac1 and Cdc42 and this may contribute to the reduced adhesion, migration and proliferation observed in $Flii^{Tg/Tg}$ mice and their impaired wound healing, a process dependent on effective cellular motility and adhesion.

While initial focal complex formation is not dependent on cell contraction, the presence of actin stress fibers can play a critical role in the structure of focal adhesions (Bach *et al*., 2010). We have observed enhanced fibrillar adhesions in response to increased Flii expression suggesting impaired turn-over of focal adhesion structures. Flii associates with paxillin and this may inhibit paxillin activation via the Src kinase and result in down regulation of downstream signaling proteins Src and p130Cas, as shown in Flii over-expressing fibroblasts *in vitro* and mice wounds *in vivo*. In contrast to Flii over-expressing cells, Flii deficient fibroblasts have active actin dynamics with lamellipodial crawling on the extracellular matrix and an actin meshwork at the leading edges of the cells, increased focal complex formation, regular turnover of focal adhesion structures and decreased contractibility. This suggests that Flii deficiency allows cells to adhere and migrate efficiently over the wound bed yet have a controlled response in contraction of collagen fibers resulting in a more controlled response during wound repair.

A recent study has described a direct relationship between focal complex formation and hemidesmosome protein complexes in live cells (Ozawa *et al*., 2010). Movement of cells across the wound bed is mediated by focal complex formation and hemidesmosome protein complexes which assemble new hemidesmosomes. In cells migrating to repopulate the wounds, focal complexes cluster in the direction of the wounds and their disassembly

marks the site for new hemidesmosome formation. Proteins or treatments which inhibit focal complex formation result in altered formation of hemidesmosome complexes, reduced hemidesmosome assembly and/or disassembly and decreased cell migration (Ozawa *et al*., 2010). Flii over-expressing fibroblasts exhibit decreased formation of focal contacts and wounds of Flii over-expressing mice exhibit decreased expression of hemidesmosome proteins CD151, laminin and laminin-binding integrin receptors. Moreover, unwounded skin of Flii over-expressing mice has a decreased number and altered structure of hemidesmosomes. Decreased expression of hemidesmosome proteins and impaired cellular formation of adhesion structures would result in decreased adhesion and impaired cellular migration. These findings could therefore explain the decreased adhesion and migration of Flii over-expressing cells which underpin the impaired wound healing observed in Flii over-expressing mice.

Considering the role of Flii in actin remodelling and its effect on regulation of adhesion signaling proteins and tension-mediated adhesion, we speculate that Flii may also coordinate the actin filaments with formation and turnover of adhesion structures, to regulate cellular adhesion and directional migration during wound healing. The exact mechanism of Flii activity in mediating cellular adhesion is not yet clear however our results suggest that this might be mediated both by Flii cytoskeletal activity and its nuclear receptor co-activator function as there appears to be a regulated balance of actin dynamics and altered levels of number of adhesion signaling proteins. In Figure 7.2 a model is outlined indicating the effect of Flii over-expression on paxillin phosphorylation and a subsequent downstream adhesion signaling pathway which may underpin the impaired wound healing observed in Flii over-expressing mice. As Flii is increased in response to

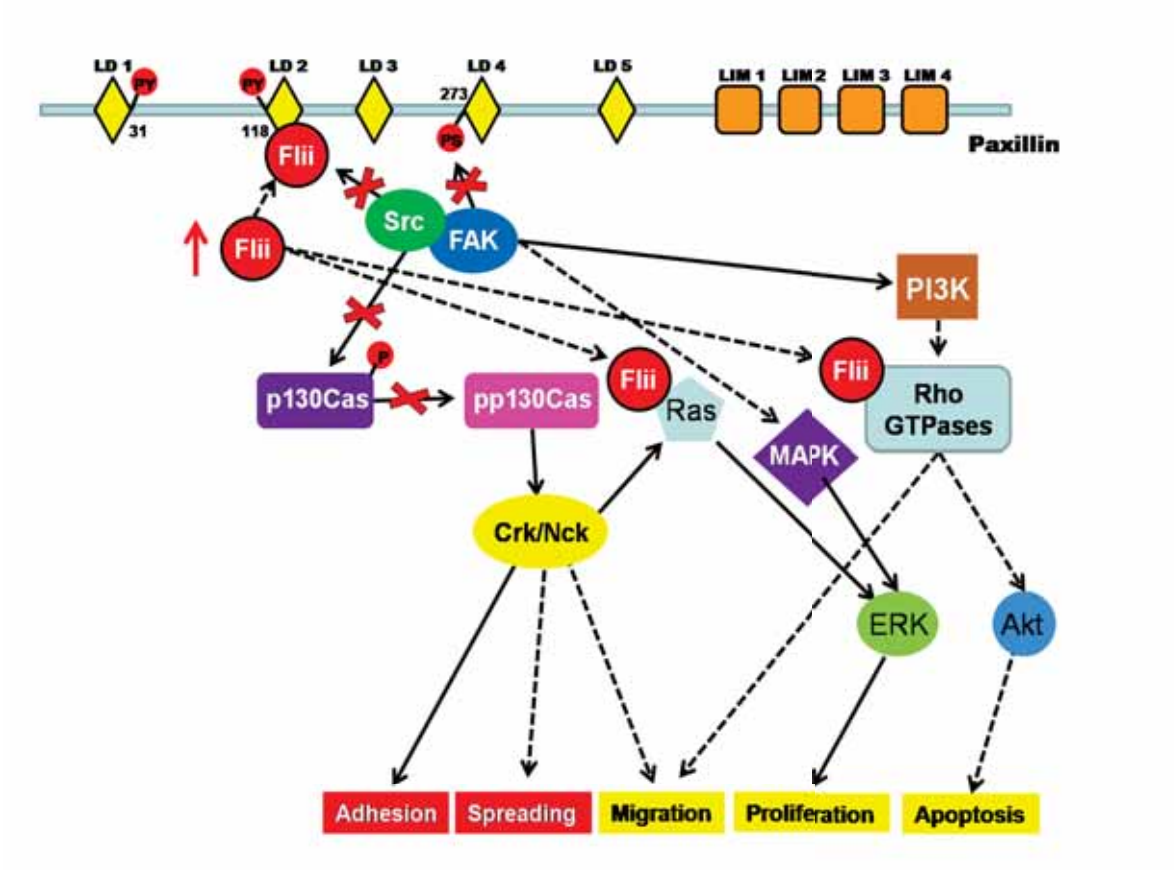

Figure 7.2: The effect of Flightless over-expression on paxillin phosphorylation and subsequent inhibition on downstream adhesion signaling pathway. Flii over-expression and binding to paxillin results in inhibition of paxillin phosphorylation on tyrosine residue 118 by inhibition of Src kinase binding (red cross). This subsequently may inhibits FAK binding to the adjacent LD motif and downstream phosphorylation of p130Cas by Src kinase (red cross) hence inhibiting adhesion signaling pathway with indirect effects on cellular pathways and modulation of Rho GTPases hence regulating cell migration, proliferation and apoptosis. Direct interactions = full line arrows, indirect interactions = dotted line arrows.

wounding and in non-healing blister wounds of EB patients this model may also account for the processes taking place during normal wound healing as well as impaired wound healing seen in EB patients.

7.4 Function of Flightless in Epidermolysis Bullosa skin blistering

This thesis presents results showing that Flii is an important regulator of cellular adhesion with the ability to modulate hemidesmosome structures between skin layers and focal adhesion structures at the cellular level. Consequently, it was hypothesized that Flii might be an important factor in the adhesion-based genetic skin blistering diseases EB. Persistent blistering and inflammation, frequent infections, poor nutrient uptake and decreased oxygen supply to the skin cause blister lesions to develop into chronic non-healing wounds (Bruckner-Tuderman, 2009). There is no cure or therapy for treatment of EB and patient care is dependent on wound management (Kopecki *et al*., 2009b, Mellerio *et al*., 2007).

Different approaches are being investigated to develop new treatments for EB patients, including systemic, gene or cell and protein based therapy. Many have been tested in mouse models, including embryonic bone marrow cell transplantation (Uitto, 2009), injection of recombinant human type VII collagen (Remington *et al*., 2009, Woodley *et al*., 2004a, Woodley *et al*., 2004b), and injection of gene corrected fibroblasts (Woodley *et al*., 2003, Woodley *et al*., 2007) or wild-type fibroblasts (Kern *et al*., 2009). While these

approaches showed promising results in ameliorating phenotypic features of murine EB, each has multiple technical and safety challenges and only a few have been trialled in a clinical setting. The systemic treatment with agents or transplant of healthy donor cord blood cells resulted in varied clinical outcomes and unpredictable side effects (Bruckner-Tuderman, 2002, Tolar *et al*., 2009) while *in vivo* gene therapy in JEB patients *(De Luca et al*., 2009) resulted in significant challenges in delivery of the therapeutic gene (Aumailley *et al*., 2006), efficient targeting of stem cells, integration of genetic material with the host genome and limiting patient's immune response (Ferrari *et al*., 2005). The continual self-renewal process of the epidermis and its compartmentalization also creates a problem in gene therapy, as the introduced gene must not interfere with the highly regulated developmental process of proliferation and differentiation in order to achieve a stable transmission and long-lasting phenotypic correction (Dunnwald *et al*., 2001, Featherstone and Uitto, 2007, Li *et al*., 2004, Tumbar *et al*., 2004). Protein or cell replacement therapy have also been reported for DEB and JEB patients (Aumailley *et al*., 2006, Kern *et al*., 2009, Uitto, 2008, Uitto, 2009) and clinical trials using allogenic fibroblasts for treatment of RDEB (Poocheron *et al*., 2008, Tamai *et al*., 2009, Wong *et al*., 2008) have shown promising results. However, a recent randomised clinical trial with a higher number of patients (Yan *et al*., 2009) showed no significant difference in wound healing in response to injection of the allogeneic fibroblasts compared to placebo. Moreover, current approaches in clinical trials have only been shown to be efficient in a small number of patients and further research is required to optimise current methods or develop novel approaches to improved wound healing in EB patients (Mavilio *et al*., 2006).

Defects in hemidesmosome formation, ColVII anchoring fibrils and integrin-mediated cellular adhesion underlie EB, however the molecular pathways which define the different clinical EB phenotypes are not yet fully understood. Unravelling these processes should identify potential new approaches and targets for improving wound repair and management of individuals suffering from EB. Because of Flii role in skin fragility, formation of hemidesmosomes and ColVII anchoring fibrils and effects on cellular adhesion structures and wound healing the role of Flii in skin blistering was examined. In this thesis, human samples and two different mouse models of skin blistering disease were used to investigate the role of Flii protein in EB. Flii expression was significantly up-regulated in 34 patients suffering from different subtypes of four main EB types. As Flii expression was observed in the different EB subtypes, the involvement of Flii is most likely in response to the wound repair of blister lesions rather than the pathology of the disease. Two mouse models, an autoimmune, inflammation-mediated ColVII murine model of EBA and the immunocompetent ColVII hypomorphic mouse model of RDEB, were used to investigate the role of Flii in blister formation and repair in EB.

In agreement with increased Flii expression in human blister wounds, Flii activity was also elevated in both murine models of EB. Based on this data it can be hypothesized that the Flii increase in response to blistering is mediated by mechanical trauma involving TGF-β mediated skin fibrosis and/or skin inflammation in response to wounding. Studies in our laboratory have shown that Flii over-expressing mouse wounds exhibit prolonged inflammation (Adams *et al.*, 2008) which may also contribute to the more severe skin blistering observed in the EBA mice model. Recent results in our laboratory have shown that Flii is expressed in skin inflammatory cells, including neutrophils and macrophages

further supporting the role for Flii in inflammation (Cowin AJ., unpublished data). Flii has also recently been implicated in Toll-like Receptor (TLR)-mediated inflammatory responses where its interaction with other signaling proteins negatively mediates a switch on – switch off mechanism of induction and repression of TLR signaling in a time-course, dependent cooperative manner (Dai *et al*., 2009). Moreover, a recent study has suggested that Leucine Rich Repeat Flightless Associated Protein 1 (LRRFlP1) mediates the production of pro-inflammatory cytokine interferon-β (Yang *et al*.), however the role of Flii in mediating inflammation during wound healing has not yet been investigated in detail. Further studies examining the role of Flii in inflammation especially in the context of wound healing may hold clues to the mechanisms of blister induction in inflammation-mediated conditions like EBA.

While elucidating the exact consequences of Flii action in blister lesions was beyond the scope of this study our results clearly identify a negative role for Flii in skin blistering. In agreement with a diffuse arrangement of ColVII anchoring fibrils and more fragile thinner skin, described in Chapter 3, Flii over-expressing mice had decreased expression of ColVII and more severe blister formation post-induction of EBA despite appearing phenotypically normal. However, anchoring fibrils have been shown to "tolerate" not only quantitative reduction but also qualitative abnormalities of ColVII to a great extent, and the DEB patient blistering phenotype is only evident when patients have between 0 - 30% of normal ColVII levels (Bruckner-Tuderman *et al*., 1999). This suggests that Flii over-expressing mice which appear phenotypically normal and survive to adulthood may be predisposed to severe blistering due to their altered anchoring fibril biology and weaker, more fragile skin. In contrast, Flii deficiency results in reduced skin

blistering and elevated ColVII and integrin expression, suggesting that lowering Flii expression could be beneficial for improved wound healing of blister wounds. The results presented in Chapter 6 also identify a cell-specific effect of Flii on fibroblast adhesion and demonstrate that impaired adhesion and proliferation in wild-type EBA induced fibroblasts can be rescued by using Flii neutralizing antibodies. These findings are supportive of studies which illustrated that fibroblasts might be a better target for novel DEB treatment development than keratinocytes. Fibroblasts were able to supply higher amounts of ColVII at the new dermal-epidermal junction and better restore the anchoring fibrils when compared to keratinocytes (Chen and Woodley, 2006, Goto *et al*., 2006).

Results in Chapter 6 further suggest that Flii does not affect the synthesis and secretion of pro-ColVII polypeptides, however it may associate with the NC-1 domain of ColVII. These findings suggest that extracellular secreted Flii may interfere with dimer assembly into functional anchoring fibrils and hemidesmosome assembly and its over-expression may result in more fragile skin, diffuse arrangement of anchoring fibrils and increased tendency for skin blistering as observed with Flii over-expressing mice. Previous studies have shown that Flii is a negative regulator of wound healing and results described in this thesis now also show that Flii is a negative contributing factor to blister formation in EB. Importantly results in this thesis show that reduction of Flii either genetically or using neutralizing antibodies reduces the incidence and severity of blisters and improves cellular responses required for wound repair of blister lesions.

Results presented in this thesis suggest that Flii effects on fibroblast differentiation and collagen contraction may be mediated through TGF-β. We have also shown that addition of TGF-β reduces the Flii association with ColVII suggesting that TGF-β may be involved in Flii interaction with extracellular matrix proteins. Studies have shown that TGF-β signaling is involved in activation of the ColVII promoter (Naso *et al*., 2003). TGF-β binds to the ColVII promoter (Vindevoghel *et al*., 1998) and modulators of TGF-β1 may be valuable in prevention of excessive fibrosis in EB patients. TGF-β also modulates ColVII production and regulates the Kindler syndrome protein in integrin-mediated adhesion (Kloeker *et al.*, 2004, Konig and Bruckner-Tuderman, 1994). Studies in our laboratory have shown that Flii modulates TGF-β1 (Adams *et al*., 2009, Adams *et al*., 2008). Reducing *Flii* gene expression either in wounds of $Flii^{+/-}$mice or *in vitro* fibroblasts by siRNA reduced TGF-β1 levels while Flii over-expression results in marked upregulation of TGF-β1. In agreement with these findings, in this thesis we describe significantly decreased TGF-β expression in blister wounds of $Flii^{+/-}$ EBA-induced mice. Consequently, Flii affect on wound healing of blister lesions could also be partly mediated through TGF-β/Smad signaling and may explain possible mechanisms involved in a cell's ability to recognise the changes in the actin cytoskeleton and respond with changes in gene transcription during wound healing. A schematic model describing one possible interpretation of Flii in wound healing of blister lesions is illustrated in Figure 7.3. Overall, our results describe the negative role for Flii in skin blistering and identify Flii as a possible target for development of novel therapy aimed at improved wound healing in EB patients.

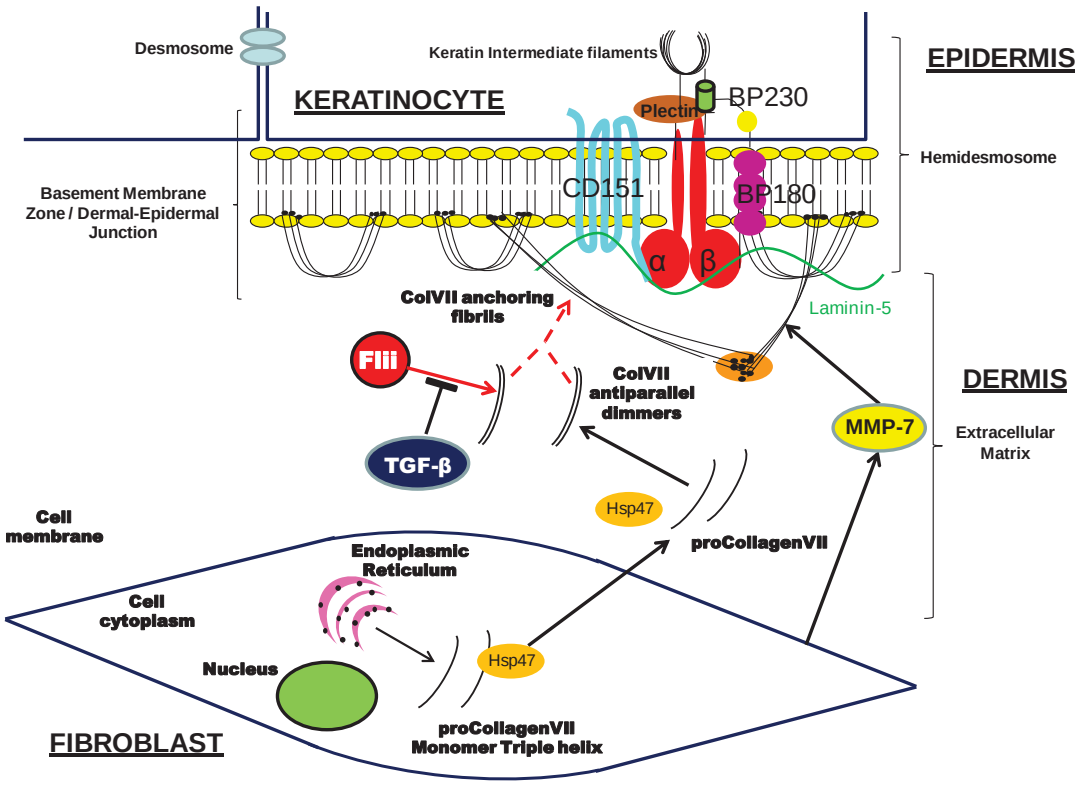

Figure 7.3: Schematic diagram of a possible mechanism behind Flightless effect on wound healing of blister lesions and more severe skin blistering observed in Flightless over-expressing mice. Flii over-expressing mice exhibited more severe blistering post EBA-induction presumably due to decreased network and altered arrangement of ColVII anchoring fibrils. Diagram illustrates Flii association with ColVII (full red line arrow) altering dimmer assembly into functional anchoring fibrils (dotted red line arrow) which may explain increased blistering and altered hemidesmosome formation observed in Flii over-expressing mice skin. Flii association with ColVII is weakened by TGF-β1.

Current studies have shown promising results with the Flii antibody based therapy aimed at improving wound healing in small animal models *(Adams et al., 2009, Adams et al.,* 2008, Cowin *et al*., 2007, Kopecki *et al*., 2009a). While Flii antibody therapy is still to be tested in EBA-induced mice and EB patients in clinical trials, this approach of mechanistic-based treatment for improved wound healing may improve the quality of life for EB patients. Flii is secreted *in vitro* from fibroblasts (Cowin *et al*., 2007) and recent studies have shown that it is also present in human acute wound fluid and blood plasma (unpublished data, Cowin A.J.) which would suggest that Flii may also be present in the blister wound fluid and sera of EB patients. Attenuation of Flii in human wounds could therefore be beneficial to wound repair. In support of this, intradermal injection of specific FnAb (polyclonal affinity purified IgG antibodies raised against a specific peptide of the LRR domain of Flii) to both full thickness murine incisional wounds and partial thickness burn injury significantly improved wound healing, with decreased wound areas and improved wound reepithelisation (Cowin *et al*., 2007, Adams *et al*., 2009). Application of FnAb to *in vitro* cell cultures resulted in up to 5 fold increase in proliferation of keratinocytes and fibroblasts and up to 28% decreased area of incisional wounds after 7 days (unpublished data, Cowin A.J.).

Current research is underway to investigate the efficacy of FnAb in a larger pig model of wound healing. Preliminary result from this model indicate improved wound healing of both incisional and excisional pig wounds treated with FnAb with smaller wound area, increased granulation tissue and reduced scarring. If similar results in response to FnAb therapy could be repeated in the human wound injuries and EB patients leading to improved cellular proliferation, decreased wound area and improved healing

time of wounds (≥ 25% on histology as compared to the normal healing process) this would be clinically significant. These findings may lead to development of novel therapeutic interventions for improved wound healing of chronic wounds in EB patients, significantly improving their quality of life and reducing physiological effects associated with EB.

7.5 Future directions

This study is the first to identify a role for Flii in cellular adhesion and demonstrate Flii association with structural and signaling proteins present at the dermal epidermal junction. Flii may be an important nexus for the coordination of numerous signaling pathways mediating cellular adhesion, migration and proliferation during wound healing. Flii may modulate epithelial interactions through cytoskeletal complexes which suppress integrin-dependent signalling pathways. Further investigations aimed at determining the precise interaction or binding sites between Flii and proteins identified in this study; and understanding the mechanisms by which the cell orchestrates these interactions in a spatiotemporal and context-specific manner are of vital importance. Flii is a secreted protein and future studies should examine the mechanisms behind Flii secretion as well uptake or internalisation of secreted or extracellular Flii to fully understand the role of Flii in wound healing. Results presented in this thesis have described the Flii dependent changes on focal adhesion turnover and cellular spreading and migration. Future studies should aim to investigate whether α-actinin knockdown and/or constitutively active Rac1 or Cdc42 are sufficient enough to restore the spreading and focal turnover in Flii over-expressing cells. The results presented in this thesis describe Flii as a multifunctional

protein interacting with numerous proteins. Experiments using Flii immunoprecipitation and mass-spectrometry studies could be used screen and determine different proteins that Flii is complexed with. Understanding the spatiotemporal modulation of protein-protein interactions by Flii, and therefore its regulation of downstream signaling pathways would allow us to modulate specific interactions while allowing other interactions to occur and hence manipulate specific signaling pathways. These findings would be vital for further therapy development aimed at improved wound healing.

Further investigations aimed at determining the mechanism behind the role of Flii in wound healing and, specifically, healing of blister lesions, are required before these findings can lead to applications in a clinical setting. Future studies need to more closely investigate the relationship between Flii expression and ColVII synthesis, secretion and assembly into the functional anchoring fibrils by examining anchoring fibril stability in vivo, and effect of Flii on ColVII secretion chaperone Hsp47 or intracellular ColVII proteolytic enzyme matrilysin (MMP7) using immunohistochemistry and zymography studies. Moreover, production of the full length recombinant Flii protein (rFlii) would allow researchers to determine whether the Flii effects on cytoskeletal stability and gene expression intracellularly are more important than extracellular functions of Flii during wound healing and blister formation. Intracellular functions of Flii can be investigated by transfecting primary Flii deficient cells with GFP-tagged constructs containing truncated forms of Flii gene including specific regions of the LRR domain or gelsolin domain in combination with biochemical immunoprecipitation, microscopy-based direct binding analyses and immunofluorescence. The effect of truncated Flii proteins on Flii localization and secretion can be determined using the GFP tag. Meanwhile, extracellular function of

Flii can be investigated by treating keratinocytes and fibroblasts with the different concentrations of rFlii and examining the cellular adhesion, migration and proliferation. Moreover, blocking TGF-β gene expression by siRNA, in conjunction with exogenous addition of rFlii in EBA fibroblasts would further confirm function of extracellular Flii and show whether rFlii can induce myofibroblast formation in the absence of TGF-β, highlighting the extent to which Flii contributes to the excessive fibrosis and pseudosyndactyly observed in EB patients.

The effect of Flii on tissue inflammation during wound healing has not yet been investigated. However, Flii effect on inflammation may be important in wound healing of blister wounds, which often resemble chronic wounds and exhibit excessive inflammation. A role for Flii in wound healing of blister wounds and subsequent effect on inflammation could be further investigated by comparing Flii expression in mechanical trauma induced suction blisters in healthy individuals which have been shown to have delayed and mild inflammation up to 24hrs post blister induction (Koivukangas and Oikarinen, 2003) versus Flii expression in highly inflamed skin conditions like psoriasis and inflammatory skin blistering conditions like EBA. Based on current studies indicating improved wound healing in response to Flii neutralising antibody therapy (Adams *et al*., 2009, Cowin *et al*., 2007) future studies need to examine the effect of FnAb on the healing of blister lesions in both EBA and ColVII hypomorphic mice models and investigate the levels of Flii in blister fluid and sera of patients with different subtypes of EB. Lastly, using the small and large animal models of wound healing and EB, examination of FnAb dosage, topical delivery or number of intradermal injections and antibody clearance rate will need to be fully

investigated in order to establish a treatment regime that would lead to improved wound healing and decreased fibrosis before such therapy could proceed to human clinical trials.

7.6 Conclusion

The aim of this study was to investigate the effect of differential Flii expression on skin architecture, cellular responses, adhesion mediated cell signaling, and blister formation. Despite analysis of *Flii* function in a variety of organisms little was known about the molecular mechanisms underlying function of Flii during wound healing and in skin homeostasis in general. The multidimensional approach of both *in vitro* and *in vivo* methodologies presented in this thesis has revealed several novel findings and contributed to a better understanding of the involvement of Flii in maintaining skin homeostasis and regulating the processes of wound healing. In particular, the studies presented here have revealed a role for Flii in cellular adhesion and have identified Flii as a possible target for development of novel therapies for improved wound healing in EB patients. The aims and hypothesis of this study were supported and the significance of the new knowledge gained may open doors to significant changes in clinical practice and contribute to better therapeutic design by which healing of blisters in patients with EB might be improved.

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APPENDIX

Appendix contains the copies of the publications that have stemmed from the results presented in this thesis.

Kopecki, Z. and Cowin, A.J. (2008) Flightless I: An actin-remodelling protein and an important negative regulator of wound repair.
International Journal of Biochemistry & Cell Biology, v.40 (8), pp. 1415-1419, 2008

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International Journal of Biochemistry & Cell Biology, v.40 (8), pp. 1555-1569, 2008

ACCETED FOR PUBLICATION IN JOURNAL OF INVESTIGATIVE DERMATOLOGY ON 6TH FEB 2011

Regulation of focal adhesions by Flightless I involves inhibition of paxillin phosphorylation via a Rac1-dependent pathway

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Short title: Flii regulation of focal adhesion

Abstract

Flightless I (Flii) is an actin remodeling protein which influences diverse processes including cell migration and gene transcription and links signal transduction with cytoskeletal regulation. Here we show that Flii modulation of focal adhesions and filamentous actin stress fibres is Rac1-dependent. Using primary skin fibroblasts from Flii over-expressing (*Flii*$^{Tg/Tg}$), wild-type and Flii deficient (*Flii*$^{+/-}$) mice we show that elevated expression of Flii increases stress fibre formation via impaired focal adhesion turn-over and enhanced formation of fibrillar adhesions. Conversely Flii knockdown increases the percentage of focal complex positive cells. We further show that a functional effect of Flii at both the cellular level and in mice wounds in vivo is through inhibiting paxillin tyrosine phosphorylation and suppression of signaling proteins Src and p130Cas both of which regulate adhesion signaling pathways. Flii is up-regulated in response to wounding and over-expression of Flii inhibits paxillin activity and reduces adhesion signaling by modulating the activity of the Rho family GTPases. Over-expression of constitutively active Rac1 GTPase restores the spreading ability of *Flii*$^{Tg/Tg}$ fibroblasts and may explain the reduced adhesion, migration and proliferation observed in *Flii*$^{Tg/Tg}$ mice and their impaired wound healing, a process dependent on effective cellular motility and adhesion.

Introduction

Flightless I (Flii) is an actin-remodeling protein and a nuclear receptor co-activator, linking signal transduction with cytoskeletal regulation (Kopecki and Cowin, 2008). Flii is a member of the gelsolin family of actin-remodeling proteins that regulate actin by severing pre-existing filaments and/or capping filament ends to enable filament reassembly into new cytoskeletal structures (Campbell et al, 1997). Consistent with this Flii co-localises with molecules involved in regulating cytoskeletal reorganization, including members of the Rho family of GTPases: Ras and Cdc42 (Davy et al, 2001). Flii has also been identified as an important negative regulator of wound healing (Adams et al, 2008; Cowin et al, 2007; Kopecki et al, 2008; Kopecki et al, 2009). In mice heterozygous for *Flii* gene knockout, healing is enhanced whilst *Flii* over-expression leads to significantly impaired healing, with reduced cellular proliferation and delayed epithelial migration (Cowin et al, 2007). Flii deficient fibroblasts and keratinocytes have improved cellular adhesion and spreading resulting in accelerated scratch wound closure (Cowin et al, 2007; Kopecki et al, 2009). Flii is upregulated in response to wounding yet its over-expression impairs healing and this may appear to be contradictory. However in an attempt to regain tissue integrity and prevent possible wound infection, upregulation of proteins and cytokines has been observed including transforming growth factor β1 which leads to more rapid wound closure but adverse scar formation (Shah et al., 1995).

Focal adhesion dynamics and downstream signaling are important processes involved in cellular adhesion and migration and fundamental to the wound healing process. Initial integrin mediated cell-matrix adhesions termed focal complexes develop underneath lamellipodia and are driven by actin polymerization. They are highly dynamic structures that exist for a limited amount of time.

A proportion of stable focal complexes develop into elongated focal adhesions which are associated with contractile stress fibres (Berrier and Yamada, 2007). Focal adhesions function as both adhesion sites and signal transduction mechanosensors allowing cells to adapt to the changing extracellular matrix protein composition and the cellular environment encountered during wound healing (Bershadsky, 2006). Focal adhesions have the ability to transform into fibrillar adhesions that can modify the structure and rigidity of the extracellular matrix hence contributing to tissue remodeling during wound repair (Arnaout et al, 2007). The vital function of focal adhesions is to anchor the polymerized actin filament stress fibres into bundles which provide contractile force required for effective translocation of the cell body during cellular migration however impaired turn-over of these adhesion structures results in impaired cellular migration (Bach et al, 2009). Rho family of GTPasees act as GTP-dependant molecular switches that bind and modulate the activities of effector molecules regulating actin cytoskeleton including formation of focal adhesions and actin stress fibers (Tominaga et al., 2000).

Closely regulated turn-over and phosphorylation of focal adhesion proteins allow cells to respond appropriately to their environment. Focal adhesion proteins like paxillin provide a point of signal convergence, a platform for protein kinases such as Src to interact with other signaling proteins hence allowing downstream adhesion signaling pathways to proceed (Goetz 2009). Here we examine the effect of differential *Flii* gene expression on integrin mediated focal adhesion formation and turn-over; its association with adaptor, structural and signaling proteins involved in focal adhesion dynamics and its effects on downstream adhesion signaling.

Results

Flii over-expressing fibroblasts have increased actin stabilization and focal adhesions

Flii null mutations are embryonic lethal due to defective gastrulation associated with disorganised actin cytoskeleton (Campbell et al, 2002). Mice heterozygous for *Flii* have 50% gene expression with a corresponding reduction in Flii protein (Cowin et al, 2007). Using fibroblasts from Flii deficient ($Flii^{+/-}$), wild-type and Flii over-expressing ($Flii^{Tg/Tg}$) mice we examined the effect of differential *Flii* gene expression on filamentous actin (F-actin) distribution and focal adhesion formation by coimmunostaining of phalloidin and paxillin respectively. Both $Flii^{+/-}$ and wild-type fibroblasts developed F-actin networks with lamellipodial crawling on the extracellular matrix and an actin meshwork at the leading edges of the cells (Fig 1a). In contrast, increased F-actin was observed in $Flii^{Tg/Tg}$ fibroblasts with formation of prominent ventral stress fibres linking focal adhesion sites across the cell periphery (Fig 1a). A significant increase in F-actin content was observed in $Flii^{Tg/Tg}$ vs. wild-type fibroblasts at 15 minutes post-seeding and between $Flii^{Tg/Tg}$, wild-type and *Flii* deficient cells at 60 minutes and 24 hours post-seeding, indicating a maintained response to cellular adhesion (Fig 1b).

Paxillin staining of focal adhesions revealed formation of focal adhesions at leading edges of $Flii^{+/-}$ and wild-type fibroblasts and increased peri-nuclear expression of paxillin indicative of active focal adhesion dynamics while $Flii^{Tg/Tg}$ fibroblasts had individual focal adhesion staining around the entire cell periphery (Fig 1a). Assessment of $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ fibroblasts revealed a significant increase in the number and size of focal adhesions in $Flii^{Tg/Tg}$ fibroblasts (Fig 1c-d). Example of focal adhesion and complex structures in wild-type cells is shown in Fig 1e. Examining the focal complex formation in $Flii^{+/-}$, wild-type and $Flii^{Tg/Tg}$ fibroblasts showed a

significant decrease in the percentage of *Flii*$^{Tg/Tg}$ fibroblasts containing focal complexes compared to both *Flii*$^{+/-}$ and wild-type cells suggesting that *Flii* over-expression alters focal adhesion dynamics (Fig 1f-g). Time-course experiments monitoring the effect of Flii over-expression on initial focal complex formation at 10, 20, 30, 40, and 50 minutes post-seeding revealed that similar to WT cells, *Flii*$^{Tg/Tg}$ fibroblasts form initial focal complexes by 20 minutes post-seeding (Fig S1). These focal complexes progress to form focal adhesions by 40 minutes however unlike WT cells they fail to turn-over these focal adhesions into new focal complexes by 50 minutes (Fig S1). Even at 90 minutes no focal complexes are observed in *Flii*$^{Tg/Tg}$ fibroblasts (Fig 1f-g).

Reduced paxillin phosphorylation and increased α-actinin expression in Flii over-expressing fibroblasts impairs focal adhesion turn-over

To explore the role of Flii in focal adhesion dynamics we examined the phosphorylation of paxillin on tyrosine residue 118, a major site for downstream adhesion mediated signaling. Paxillin and p-paxillin immunostaining of *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts plated on a mixture of fibronectin and laminin extracellular matrix substrates showed decreased expression of p-paxillin in *Flii*$^{Tg/Tg}$ fibroblasts both at the cell periphery and under the cell body (Fig 2a). Similarly, significantly decreased p-paxillin was observed in *Flii*$^{Tg/Tg}$ fibroblasts as compared to both *Flii*$^{+/-}$ and wild-type cells, (Fig 2c-d). The ratio of p-paxillin to total paxillin levels at individual focal adhesions showed significantly decreased levels of p-paxillin in *Flii*$^{Tg/Tg}$ fibroblasts (Fig 2b) which was also confirmed by assessment of paxillin and p-paxillin fluorescence intensity at individual focal adhesions using line scan analysis (Fig S2).

To determine if *Flii* affected structural and signaling proteins involved in integrin mediated cellular adhesion *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts were stained for talin, vinculin and α-actinin. No significant difference in the expression of talin in *Flii*$^{Tg/Tg}$ fibroblasts vs wild-type vs *Flii*$^{+/-}$ was observed. We saw increased but not significantly elevated expression of vinculin in *Flii*$^{Tg/Tg}$ fibroblasts but significantly increased expression of α-actinin in Flii$^{Tg/Tg}$ fibroblasts compared to both *Flii*$^{+/-}$ and wild-type counterparts (Fig 2e). Perinuclear α-actinin was mainly observed however it was also seen at sites of focal adhesions in *Flii*$^{+/-}$ and wild-type fibroblasts. Increased expression of α-actinin was observed in *Flii*$^{Tg/Tg}$ fibroblasts both in the perinuclear region of the cell and at the cell periphery at sites of focal adhesions (Fig 2f). Over expression of α-actinin is a marker of focal adhesions which exhibit decreased dynamics and impaired turn-over (Webb et al, 2004).

Flii over-expressing fibroblasts have enhanced fibrillar adhesion formation

To investigate the effect of Flii in fibrillar adhesion formation, tensin and fibronectin expression were analysed in *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts plated on collagen and laminin extracellular matrix substrates. Fibrillar adhesions are distinguished both by parallel staining of fibronectin to the actin stress fibres and colocalization between paxillin and tensin and are indicative of contractile, non-motile phenotype. *Flii*$^{Tg/Tg}$ fibroblasts exhibited enhanced fibrillar adhesion (Fig 3) with parallel fibronectin and actin stress fibres and colocalization between paxillin and tensin being observed (Fig 3a-b). In contrast, *Flii*$^{+/-}$ and wild-type fibroblasts minimal fibrillar adhesions but had increased perinuclear fibronectin expression and punctate tensin staining indicative of cells exhibiting a highly dynamic turn-over of adhesion structures.

Over-expression of Flii results in impaired cellular spreading and down regulation of adhesion signaling pathways

Spreading of *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts on different extracellular matrix substrates revealed impaired cellular spreading in Flii over-expressing fibroblasts compared to both Flii$^{+/-}$ and wild-type counterparts (Fig 4a). Dynamic regulation of Rho family of GTPases activity plays a major role in cell motility as both inhibition or constituent activation of these GTPases can alter cellular spreading and migration (Arthur et al., 2000). No significant difference was observed between levels of activated RhoA in *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts however *Flii* over-expression resulted in reduced activation of both Rac1 and Cdc42 (Fig 4b). To analyze the effect of Rac1 GTPase on cell spreading and focal adhesion turnover, *Flii*$^{Tg/Tg}$ fibroblasts were transfected with either GFP or GFP-Rac1 fusion constructs (Bargon *et al.*, 2005). Over-expression of constitutively active Rac1 resulted in significantly improved cellular spreading evidenced by a significant increase in cell size (Fig 4d) and the formation of lamellipodia in *Flii*$^{Tg/Tg}$ fibroblasts (Fig 4c).

Over-expression of *Flii* also had a profound effect on the focal adhesion signaling components Src tyrosine kinase and p130Cas. Significantly increased levels of total p130Cas levels (p130Cas + pp130Cas) in *Flii*$^{Tg/Tg}$ vs. *Flii*$^{+/-}$ fibroblasts but significantly decreased ratio of activated pp130Cas were observed compared to both wild-type and *Flii* deficient cells (Fig 5a-c). Furthermore we saw decreased expression of Src tyrosine kinase in *Flii*$^{Tg/Tg}$ fibroblasts vs. *Flii*$^{+/-}$ and wild-type fibroblasts (Fig 5a and d) suggesting that Flii over-expression down-regulates adhesion signaling pathways required for efficient cellular adhesion and migration.

Effect of Flii on paxillin activation and signalling in wounds in vivo

An incisional wound model was used to evaluate if these in vitro responses were observed in vivo. Over-expression of *Flii* resulted in significantly larger, more gaping wounds at 3 days post-injury while Flii deficiency (*Flii*$^{+/-}$) lead to significantly smaller wound area with improved wound appearance (Fig 6a-f and k). Increased p-paxillin was observed in keratinocytes (data not shown) and fibroblasts adjacent to the wound in *Flii*$^{+/-}$ mice (Fig 6d-f). In contrast, p-paxillin was significantly reduced in the wounds of *Flii*$^{Tg/Tg}$ mice (Fig 6g-j). Similarly, decreased expression of adhesion signaling proteins pp130Cas and Src (Fig 6l) were also observed in protein extracted from 3 day wounds in *Flii*$^{Tg/Tg}$ mice vs. wild-type and *Flii*$^{+/-}$. This study supports our in vitro findings and suggests that Flii function on cell adhesion and migration may be mediated by its negative effect on pp130Cas and Src.

To determine if *Flii* effects on actin stress fibre, paxillin activity and adhesion signaling affected collagen contraction, a fibroblast populated floating collagen gel contraction assay was used (Fig 6m). Despite observing increased stress fibres in *Flii*$^{Tg/Tg}$ fibroblasts in vitro (Fig 1a) no differences were observed in the rate of collagen gel contraction in *Flii*$^{Tg/Tg}$ vs. wild-type controls (Fig 6m). In contrast, decreased contraction of the collagen gels was observed in *Flii*$^{+/-}$ fibroblasts (Fig 6m-n) suggesting that the improved healing response observed in *Flii*$^{+/-}$ mice (Fig 6a-c) may be attributed more to improved turn-over of adhesion structures resulting in improved cellular migration and proliferation rather than a direct effect on wound contraction.

Discussion

Flii is an actin remodelling protein that mediates rapid remodelling of actin filaments and associates with actin arcs and membrane ruffles illustrating its role in actin dynamics, cellular adhesion and motility (Davy et al, 2000). Here we have demonstrated a novel role for Flii in the regulation of focal adhesions, affecting paxillin phosphorylation, downstream adhesion signaling and formation and stabilization of actin stress fibres. While both *Flii*$^{+/-}$ and wild-type fibroblasts showed active actin dynamics with actin meshwork involved in cellular spreading and migration, *Flii*$^{Tg/Tg}$ fibroblasts displayed impaired spreading and increased expression of ventral stress fibres linking the focal adhesions at the cell periphery. *Flii*$^{Tg/Tg}$ cells also exhibited significantly increased expression of filamentous F-actin over a time course of 24 hours illustrating a maintained response to cellular adhesion. *Flii* over-expression leads to increased numbers and larger focal adhesions around the periphery of the fibroblasts compared to *Flii*$^{+/-}$ and wild-type fibroblasts which only expressed active focal adhesions at leading edges of the cells. *Flii* over-expressing fibroblasts form initial focal complexes which proceed to focal adhesions however the turnover of these focal adhesions into new focal complexes appeared to be impaired. *Flii* over-expression also resulted in a decrease in the percentage of focal complex positive cells suggesting that *Flii* may impair focal adhesion turn-over and hence decrease the formation of new focal complexes.

Paxillin is an adaptor protein which facilitates the optimal signal integration through its ability to recruit multiple signaling proteins to the specific regions of the cell (Turner, 1998; Zaidel-Bar et al, 2007). We examined the phosphorylation of paxillin at tyrosine residue 118 in *Flii*$^{+/-}$, wild-

type and *Flii*$^{Tg/Tg}$ mouse wounds *in vivo* and primary fibroblasts *in vitro*. This site is phosphorylated by Src or FAK and is specifically activated at focal adhesions facilitating paxillin function in adhesion turn-over (Bach et al, 2009). *Flii*$^{Tg/Tg}$ fibroblasts had decreased levels of total paxillin and significantly reduced ratio of p-paxillin levels compared to both *Flii*$^{+/-}$ and wild-type fibroblasts. This was surprising considering the increased number of focal adhesions observed in *Flii*$^{Tg/Tg}$ fibroblasts, however compared to *Flii*$^{+/-}$ and wild-type fibroblasts decreased cytoplasmic paxillin staining was observed around the nucleus suggesting that the paxillin present in *Flii*$^{Tg/Tg}$ fibroblasts was predominantly associated with focal adhesions. Decreased paxillin tyrosine phosphorylation was also observed in *Flii*$^{Tg/Tg}$ mice wounds suggesting that over-expression of *Flii* and its association with paxillin may inhibit the interaction of paxillin with other signaling molecules resulting in decreased paxillin activation and impaired focal adhesion turn-over required for directional cellular movement. Indeed studies have shown that focal adhesions that remain stable in position and have impaired turn-over also contain low levels of phosphorylated paxillin (Zaidel-Bar et al, 2007). We observed that *Flii*$^{+/-}$ and wild-type fibroblasts had increased phosphorylation of paxillin and focal complex formation compared to *Flii*$^{Tg/Tg}$ fibroblasts supporting previous studies showing that paxillin phosphorylation enhances lamellipodial formation and stimulates formation of focal complexes (Zaidel-Bar et al, 2007).

Cells with increased ventral stress fibres and low levels of phosphorylated paxillin often show enhanced formation of fibrillar adhesions (Bach et al, 2009). *Flii*$^{Tg/Tg}$ fibroblasts had increased stress fibre formation and decreased paxillin phosphorylation with parallel staining of fibronectin and F-actin fibres and colocalization between paxillin and tensin which is indicative of enhanced fibrillar adhesions (Bach et al, 2009). *Flii*$^{+/-}$ and wild-type fibroblasts had significantly increased

tyrosine phosphorylation of paxillin and no evidence of fibrillar adhesions suggesting a high turn-over of adhesion structures. Moreover, *Flii*$^{+/-}$ fibroblasts showed decreased ability to contract collagen gels which could be attributed to the decreased TGF-β1 gene expression in these cells (Adams et al, 2008).

Incorporation of paxillin into focal complexes with talin enhances matrix-actin cytoskeleton bond and increasing resistance to mechanical stress (Alon et al, 2005, Moser et al, 2005). It has been hypothesized that Flii over-expression may result in talin sequestration leading to decreased integrin activation and impaired wound healing (Kligys and Jones, 2009). However our results indicate that although we see increased number of focal adhesions in *Flii*$^{Tg/Tg}$ fibroblasts there is no significant difference in talin or vinculin expression between *Flii*$^{+/-}$, wild-type or *Flii*$^{Tg/Tg}$ fibroblasts suggesting that the role of Flii in focal adhesions is more likely to be through paxillin phosphorylation than integrin mediated cellular adhesion and migration.

Transformation of focal complexes into focal adhesions requires the activation of RhoA which activates Daam1 and participates in the Src-regulated tyrosine phosphorylation of multiple substrates including paxillin (Tominaga et al 2000). A recent study suggested that *Flii* promotes the GTP-bound active RhoA mediated relief of the autoinhibition of Daam1 hence modulating the actin assembly activity (Higashi et al., 2010). Examining the effect of *Flii* on activation of RhoA we found no significant difference in activation of RhoA in *Flii*$^{+/-}$, wild-type or *Flii*$^{Tg/Tg}$ fibroblasts. This was not surprising as *Flii*$^{Tg/Tg}$ fibroblasts show effective focal adhesion formation but decreased formation of novel focal complexes, RhoA activates Rho-kinase and

dominant active Rho-kinase induces the stress fiber formation in Swiss 3T3 fibroblasts while dominant negative Rho-kinase inhibits the wound-induced cell migration (Kaibuchi et al., 1999). Consequently, a tight control of Rho family of GTPases is instrumental in cellular adhesion, spreading and motility. *Flii*$^{Tg/Tg}$ fibroblasts have reduced active Rac1 and Cdc42 both of which are required for formation of focal complexes, cell spreading and formation of lamellipodia and filopodia respectively (Geiger and Bershadsky, 2001; Lunn and Rozengurt, 2004). Increased expression of active Rac1 in *Flii*$^{Tg/Tg}$ fibroblasts restored the ability of these cells to spread and form lamellipodia suggesting that Flii effects may be Rac1 dependant.

Signaling of small Rho GTPases has previously been linked to Src kinase activity where Src activation of p-130Cas leads to activation of Ras-MAP kinase signalling pathway (Ben-Ze'ev, 1997). Reduced Src kinase expression in *Flii*$^{Tg/Tg}$ fibroblasts was observed which may contribute to the decreased paxillin phosphorylation and reduced suppression of RhoA resulting in impaired focal adhesion turn-over and increased formation of stress fibres observed in these cells. Interestingly, compared to *Flii*$^{+/-}$ fibroblasts, *Flii*$^{Tg/Tg}$ fibroblasts had increased total levels of p130Cas, a major Src substrate, however the ratio of activated pp130Cas to total p130Cas was significantly decreased in these cells compared to both *Flii*$^{+/-}$ and wild-type fibroblasts. Phosphorylation of p130Cas initiates Rac1 activation, increases membrane ruffling and lamellipodial extensions (Sharma and Mayer, 2008). Consequently, decreased activated p130Cas in *Flii*$^{Tg/Tg}$ fibroblasts may reduce Rac1 activity and therefore help to explain Flii negative effect on focal adhesion turnover and cell spreading.

Materials and methods

Reagents and antibodies

Laminin (L-6274), Collagen type I (C-8919), Fibronectin (F-1141) and Collagen Solution (C-4243) (Sigma-Aldrich, Australia). Antibodies: tensin (H-300), paxillin-pY^{118} (Santa Cruz Biotechnology, CA, USA); talin (05-385), vinculin (05-386), α-actinin (05-384), Src (clone GD11) (Upstate Biotechnology, NY, USA); paxillin, p130Cas (BD Biosciences, NJ USA); phalloidin-fluorescein isocyanate (FITC), β-tubulin antibody and DAPI nucleic acid stain (Sigma-Aldrich, Australia); Alexa-Fluor488 donkey anti-goat IgG and Streptavidin-Cy3 Alexa-Fluor555 conjugate (Invitrogen, Australia); species specific horseradish peroxidase-conjugated antibodies (DAKO Corporation, Botany, Australia) and species specific biotinylated IgG antibodies (Vector Laboratories, CA, USA).

Mice

All experiments were approved by the Animal Ethics Committee's of the Adelaide Child Youth & Women's Health Service and the Australian National University following the Australian Code of Practice for the Care and the Use of Animals for Scientific Purposes. All studies were performed in mice with the BALB/c background. *Flii* heterozygous null mice (*Flii*$^{+/-}$) and mice carrying the complete human *Flii* gene on a cosmid transgene were as previously described (Campbell et al, 2002). Heterozygous transgenic mice (*Flii*^{Tg}) were made by crossing *Flii*$^{+/+}$ with cosmid transgene *Flii*$^{+/-}$. These transgenic mice were intercrossed to obtain animals homozygous for the transgene (*Flii*$^{Tg/Tg}$). Heterozygous and transgenic mice were genotyped as previously described (Campbell, 2002).

Primary Fibroblast Isolation and Culture

Primary fibroblasts were isolated from 12 week old BALB/c Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ mice skin and collected in ice-cold Ham's Nutrient Mixture F12 (SAFC Biosciences, KS, USA). Fibroblasts were grown from skin explants as previously described in (Kopecki et al, 2009) and cultured in 10% FCS/DMEM. Low passage cells (P<5) were used in all experiments.

Immunofluorescence Analysis

Briefly primary fibroblasts, grown on coverslips pre-treated with poly-L-lysine (50μg/ml) and coated with a mixture of extracellular matrix proteins, namely: fibronectin (20μg/ml) and laminin (10μg/ml) or collagen (10μg/ml), were washed in 1xPBS and fixed in 4% paraformaldehyde for 10min at room temperature. They were permeabilized using a 0.2% Triton-X-100 and 0.5% BSA in 1xPBS for 5min at room temperature prior to application of primary antibodies (1:200 or 1:500 as previously optimised) for 1hr. Further incubations with fluorescent Alexa-Fluor488 donkey anti-goat IgG (2mg/ml) or species specific, biotinylated antibodies with Streptavidin Alexa-Fluor555 conjugate (2mg/ml) were performed. Sections were washed in 1xPBS and mounted in Fluorescent mounting medium (DAKO Corporation, Australia). Negative controls included replacing primary antibodies with species specific IgG. For verification of staining, non-specific binding was determined by omitting primary or secondary antibodies. All control sections had negligible immunofluorescence.

Integrated fluorescence intensity was determined using AnalySIS software package (Soft-Imaging System GmbH, Germany) and images captured using Leica Spectral Confocal

Microscope as previously described (Kopecki et al, 2009). Focal adhesion and ratio images were prepared following background subtraction. Phosphorylation of paxillin at individual focal adhesions was determined by drawing polygons around individual focal adhesions and obtaining pixel intensities which were used to obtain ratio distribution graph of p-paxillin/(p-paxillin plus paxillin) (Bach et al, 2009). Line scans of individual focal adhesions were performed on 5-pixel width lines. Only paxillin positive focal adhesions at the periphery of cells were included in the analysis; adhesions at cell centre were excluded.

Strict criteria were used to distinguish between focal complexes and focal adhesions based on size, localization and composition. Focal complexes are smaller thin punctuate dot-like structures localized mainly at cell periphery while focal adhesions are much larger thicker arrow head-like structures localized both at leading cell edge of spreading cells and cell periphery of round non-spreading cells. Example of wild-type cells with focal complexes or focal adhesions clearly labelled is shown in Figure 1e. These criteria were used to quantify the percentage of fibroblasts containing focal complexes by two independed blinded observers. A time-course experiment examining the effect of Flii over-expression on initial focal complex formation and progression to focal adhesions was performed on wild-type and *Flii*$^{Tg/Tg}$ fibroblasts using the paxillin staining of adhesion structures over a period of 50 minutes post seeding. F-actin concentration in primary *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts was as previously described (Clark et al, 1998).

Cell spreading

Spreading of *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts on coverslips coated with different extracellular matrix substrates was performed as previously described in Kopecki et al., 2009.

Cells were acetone fixed at 30, 60 and 120 minutes post seeding. Following blocking in 3% normal horse serum, phalloidin-FITC (Sigma-Aldrich, Australia) was added at 1:200 for 1 hour staining in the dark. Representative images of cell spreading on different extracellular matrix substrates were taken using AnalySIS software package (Soft Imaging System GmbH, Germany), illustrating filamentous actin fibers. All control sections had negligible immunofluorescence.

Rho GTPase Pull Down Activation Assay

Primary *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts were grown to 80% confluence, lysed and cell lysates analysed for levels of active small GTP binding proteins RhoA, Rac and Cdc42 using the RhoA/Rac1/Cdc42 Activation (Cell Biolabs, Inc., Australia). Active forms of RhoA, Rac1 and Cdc42 were detected using the Rhotekin RBD and p21-activated protein kinase PBD Agarose beads which selectively isolate and pull down the active forms of RhoA, Rac1 and Cdc42 from cell lysates. The precipitated active GTP-RhoA or Rac1 or Cdc42 and total levels of RhoA, Rac1 and Cdc42 were detected by Western blot analysis using monoclonal anti-RhoA or Rac1 or Cdc42 antibody according to the manufacturer's directions.

Transient transfection

EGFP was expressed from pEGFPC1 (Clontech, USA). Constitutively active Rac1 (pEGFP.RacL61) construct was obtained from Beric Henderson (Westmead Millenium Institute) with the kind permission of Mark Phillips (New York, University School of Medicine) and its construction has been described previously (Bargon *et al.*, 2005). Flii over-expressing fibroblasts were electroporated with a Nucleofactor^{TM} (Amaxa GmbH, Germany) according to the

manufacturer's recommendations. To determine the effects of constitutively active Rac1 GTPase on cellular spreading of *Flii* over-expressing fibroblasts images of 70 GFP positive transfected cells were taken at 48hrs post transfection and 120min post seeding and analysed for cell spreading by measuring cell area using AnalySIS program software package (Soft Imaging System GmbH Germany). Constructs were verified by standard Western blot analysis of transfected cell lysates.

Protein extraction and Western blotting

Protein was extracted from *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ fibroblasts using standard protein extraction protocols (Cowin et al, 2006). Sample proteins (10µg) were run on a 10% SDS-PAGE gels at 100V for 1hr and transferred to nitrocellulose by wet transfer. Membranes were blocked in 15% skimmed milk solution for 10min and primary antibodies added in PBS containing 5% skimmed milk, 0.3% Tween. Appropriate species-specific secondary horse radish peroxidise-conjugated antibodies were added for a further 1hr at room temperature. Stringent washes were performed before detection of horse radish peroxidise and exposure using GeneSnap analysis program (SynGene, MD, USA). Membranes were stripped and re-probed for β-tubulin (Sigma-Aldrich, Australia) for normalization of protein levels.

Murine surgical techniques

Flii$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ female 12-week-old mice (n=6 animals per group), were anaesthetized using gaseous isofluorane, 5% induction, 2% maintenance and wounded using standard protocols (Cowin et al, 2006; Kopecki et al, 2009). Briefly, two equidistant, 1cm full thickness incisions were made through the skin and panniculus carnosus on the flanks of the

mice extending 3.5–4.5cm from the base of the skull, 1cm either side of the spinal column. At 3 days post-wounding, mice were euthanized prior to harvesting of the wounds. Both left and right wounds were collected from each mouse and bisected. Half was fixed in 10% buffered formalin, the other half snap frozen for protein extraction. Unwounded skin was collected from each mouse group. Wound area was quantified by two independent blinded observers using AnalySIS software package (Soft-Imaging System GmbH, Germany) as previously described (Cowin et al., 2007).

Floating collagen gel contraction (FCGC) assay

3-D collagen gels were prepared by mixing 8 parts of chilled collagen solution with 1 part 10xDMEM containing 10% FCS and pH adjusted to 7.2-7.6 using 0.1M NaOH as previously described (Geary et al, 2008). Primary fibroblasts from *Flii*$^{+/-}$, wild-type and *Flii*$^{Tg/Tg}$ skin ($1x10^5$ cells/ml) were added to the collagen gel mixture which was added to 48 well flat bottomed plate (500µl/well) and allowed to set for 120min at 37ºC 5% CO_2. The gel was dislodged by addition of 1x DMEM media (1ml/well). The degree of contraction at 24, 48 and 72hr was determined from floating gel images using Image Pro-Plus software (MediaCybernetics Inc, MD, USA).

Statistical analysis

Statistical differences were determined using the Student's t-test or an ANOVA. For data not following a normal distribution, the Mann-Whitney U test was performed. A P value of less than 0.05 was considered significant.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by grants from the NHMRC and Channel 7 Children's Research Foundation. RA is supported by The Sylvia and Charles Viertel Charitable Foundation. AJC is supported by an NHMRC Senior Research Fellowship.

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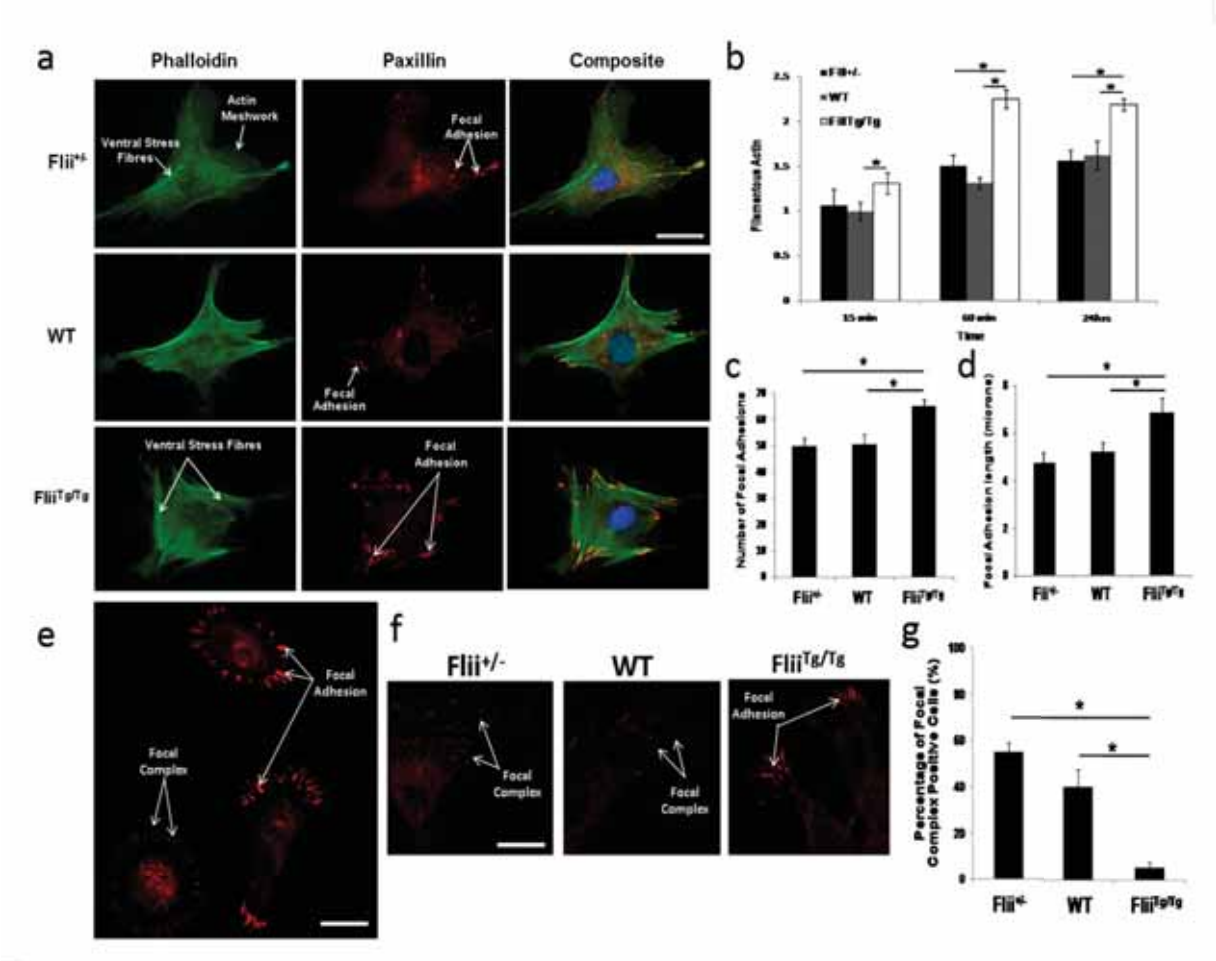


Figure 1. Flightless I over-expression stabilizes actin filaments and increases focal adhesion formation. (**a**) *Flii*$^{+/-}$, WT and *Flii*$^{Tg/Tg}$ fibroblasts coimmunostained with phalloidin (green) and paxillin (red) at 180 minutes post-seeding. Bar=25μm. (**b**) Quantification of F-actin in *Flii*$^{Tg/Tg}$ fibroblasts compared to WT fibroblasts at 15 minutes and at 60 minutes and 24 hours. (**c-d**) *Flii*$^{Tg/Tg}$ fibroblasts have significantly increased number of longer focal adhesions compared to both *Flii*$^{+/-}$ and WT fibroblasts. (**e**) Representative image of WT cells showing focal adhesion and focal complex structures. (**f**) *Flii*$^{+/-}$ and WT fibroblasts stained with paxillin show focal complex formation at 90 minutes post-seeding. (**g**) *Flii*$^{Tg/Tg}$ fibroblasts have significantly reduced percentage of focal complex positive cells compared to *Flii*$^{+/-}$ and WT fibroblasts at 90 minutes post-seeding. n=100 cells. Bar=10μm in **a** and 1μm in **e** and **f**. Mean +/- SEM. *P<0.05.

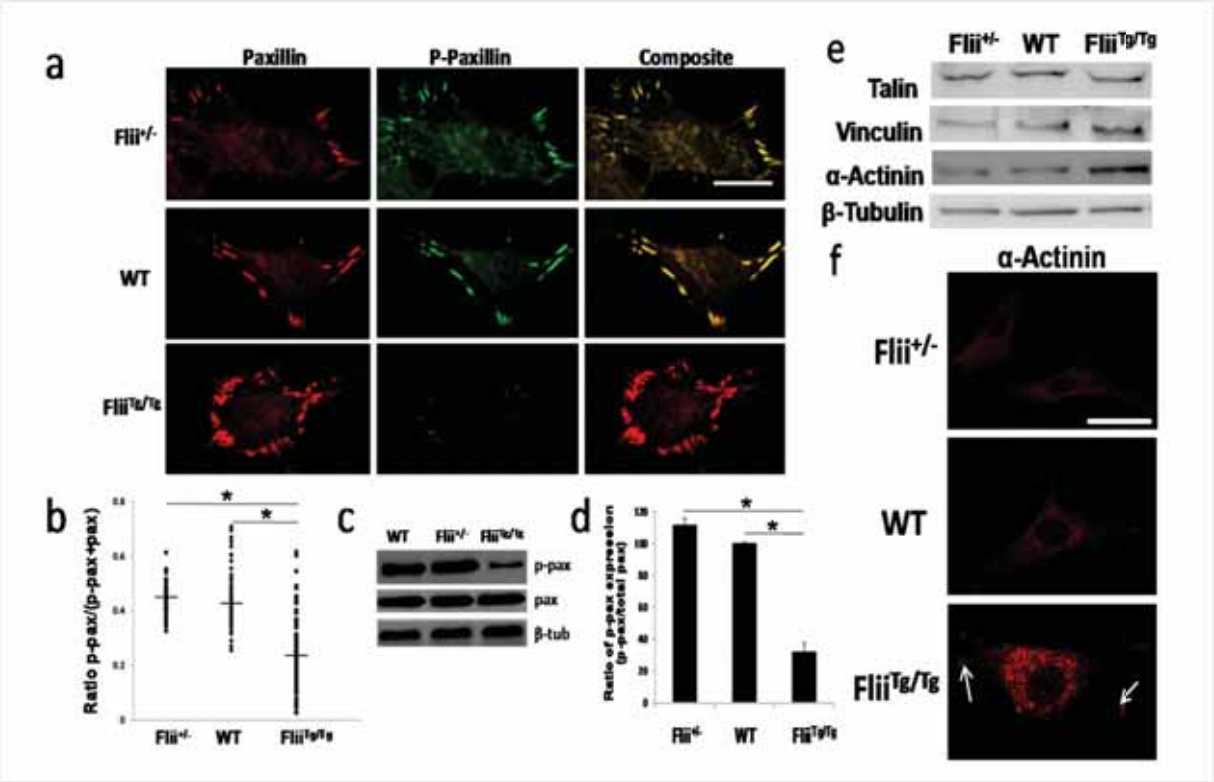


Figure 2. Flightless I over-expressing fibroblasts have reduced paxillin phosphorylation and increased α-actinin expression. (**a**) Paxillin (red) and p-paxillin (green) immunostained *Flii*$^{+/-}$, WT and *Flii*$^{Tg/Tg}$ fibroblasts plated at 180 minutes post-seeding. (**b**) Distribution of the ratios of p-paxillin at individual focal adhesions (n=100 from >5 different cells) p-pax=phosphopaxillin; pax=total paxillin. (**c**) Western blot analysis of p-paxillin, total paxillin and β-tubulin levels. (**d**) Graph showing the ratio of p-paxillin/total paxillin. Mean +/- SEM. *P<0.05. (**e**) Western blot analysis of talin, vinculin, α-actinin and β-tubulin levels. (**f**) α-actinin staining of *Flii*$^{+/-}$, WT and *Flii*$^{Tg/Tg}$ fibroblasts plated on fibronectin and laminin substrates illustrates increased expression of α-actinin in Flii$^{Tg/Tg}$ fibroblasts. Bar=10μm.

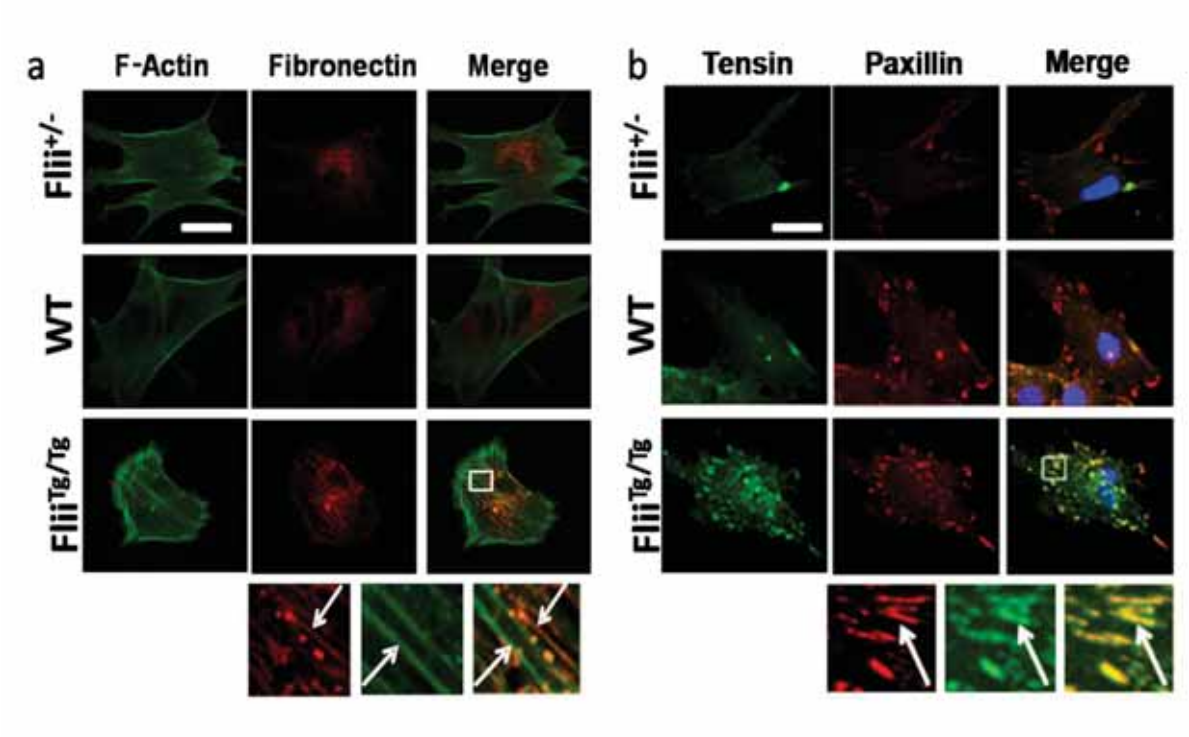


Figure 3. Enhanced fibrillar adhesion formation in Flightless I over-expressing fibroblasts. Cells plated onto collagen and laminin coated coverslips were fixed after 180 minutes and coimmunostained for F-actin (green) and fibronectin (red) (**a**) and Tensin (green) and paxillin (red) (**b**). Bar=10μm. The boxed region in the *Flii*$^{Tg/Tg}$ merged cell images is magnified at higher magnification (x100) below. Arrows indicate F-actin (green) and fibronectin (red) (in **a**) and paxillin (red) and tensin (green) (in **b**) in positive fibrillar adhesions.

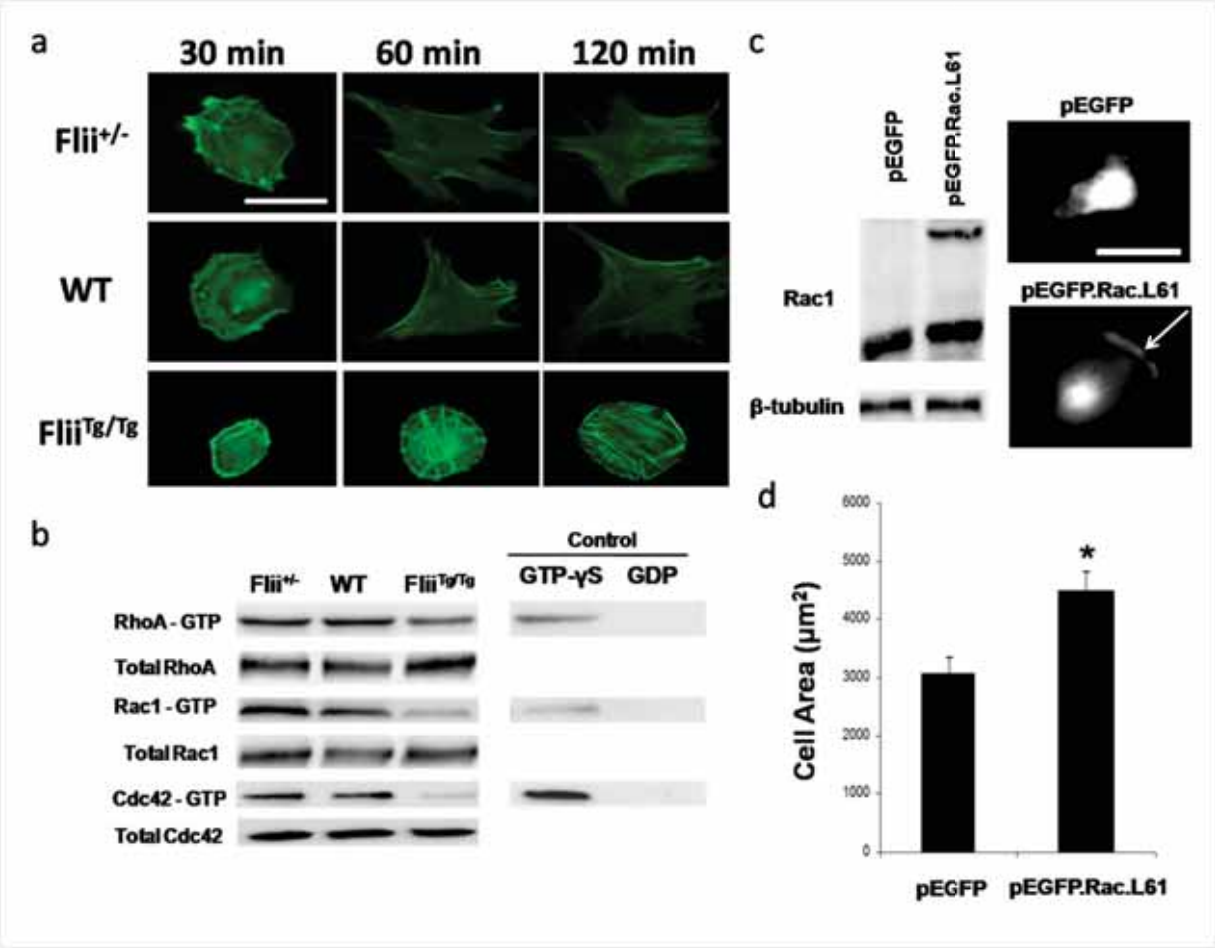


Figure 4. Reduced activation of Rho GTPases affects the spreading of Flightless I over-expressing fibroblasts. (**a**) Spreading of Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ fibroblasts stained with phalloidin reveals impaired spreading of Flii$^{Tg/Tg}$ fibroblasts. (**b**) Total and active levels of RhoA, Rac1 and Cdc42 in cell lysates of sub-confluent Flii$^{+/-}$, wild-type and Flii$^{Tg/Tg}$ fibroblasts as determined by pull down activation assay and Western blotting. Flii$^{Tg/Tg}$ fibroblasts have decreased activation of Rac1 and Cdc42 GTPases required for focal complex formation and turnover of adhesion structures. (**c-d**) Over-expression of constitutively active Rac1 GTPase in Flii$^{Tg/Tg}$ fibroblasts induces lamellipodia formation (arrow) and significantly increases cell spreading. Bar=10μm.

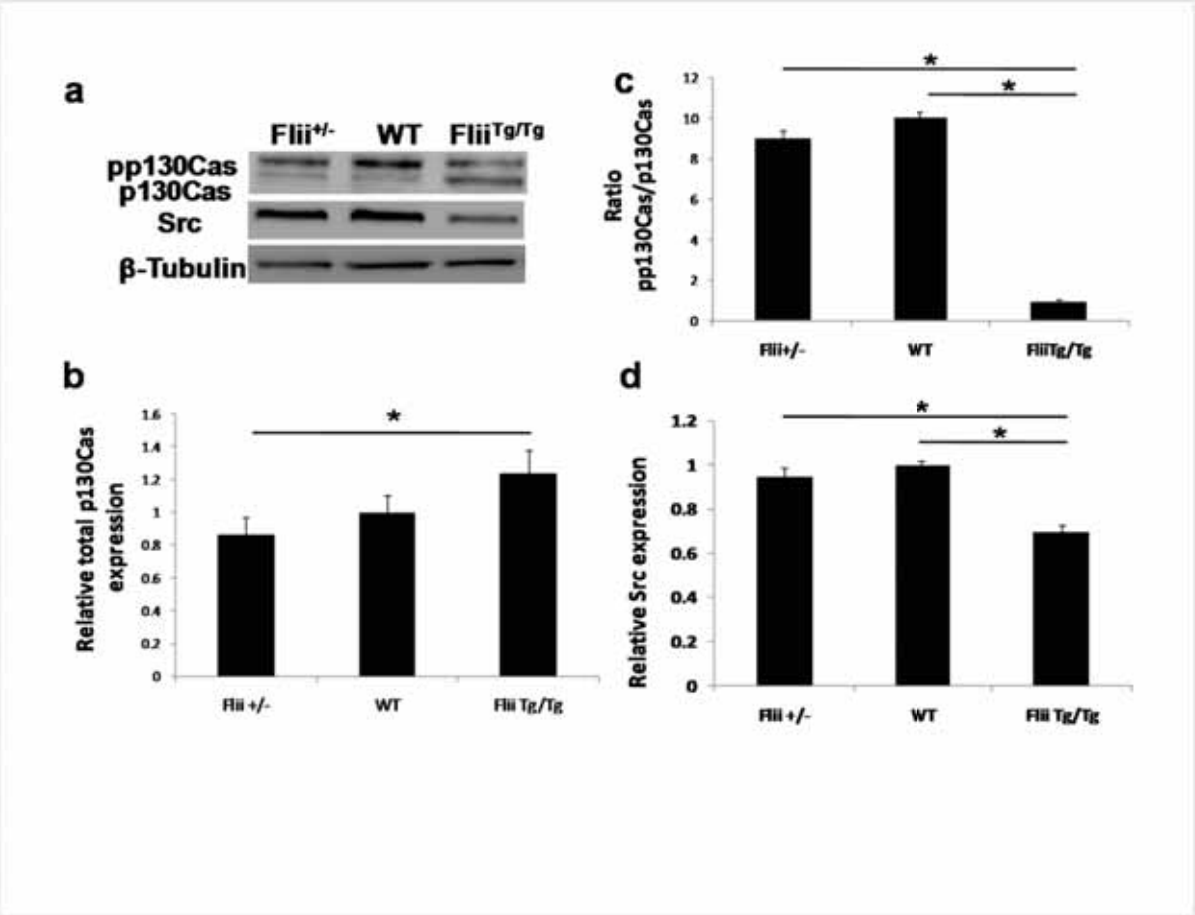


Figure 5. Over-expression of Flightless I results in down regulation of adhesion signalling pathways. (**a**) Western Blot analysis of p130Cas, pp130Cas, Src and β-tubulin protein levels. (**b**) Band densitometry illustrating total p130Cas (p130Cas + pp130Cas) expression and (**c**), the ratio of pp130Cas and p130Cas protein levels. *Flii*$^{Tg/Tg}$ fibroblasts show significantly increased total p130Cas expression compared to Flii$^{+/-}$ fibroblasts but decreased levels of active pp130Cas compared to both Flii$^{+/-}$ and WT fibroblasts. (**d**) Graphical representation of Src protein expression determined by densitometry. *Flii*$^{Tg/Tg}$ fibroblasts show decreased levels of Src proteins compared to both *Flii*$^{+/-}$ and WT fibroblasts. The data show the mean of three independent repeats. Mean +/- SEM . *P<0.05.

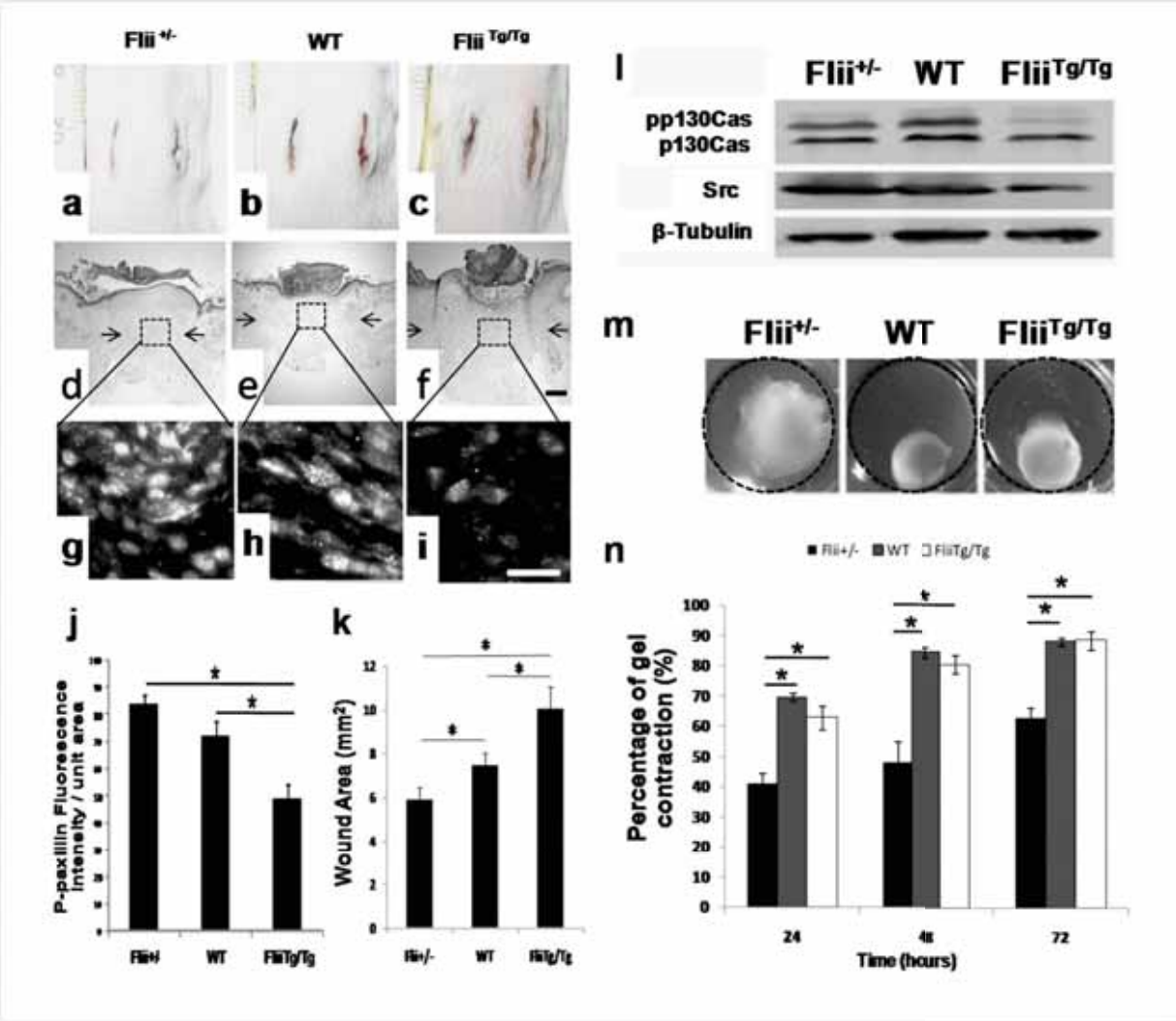
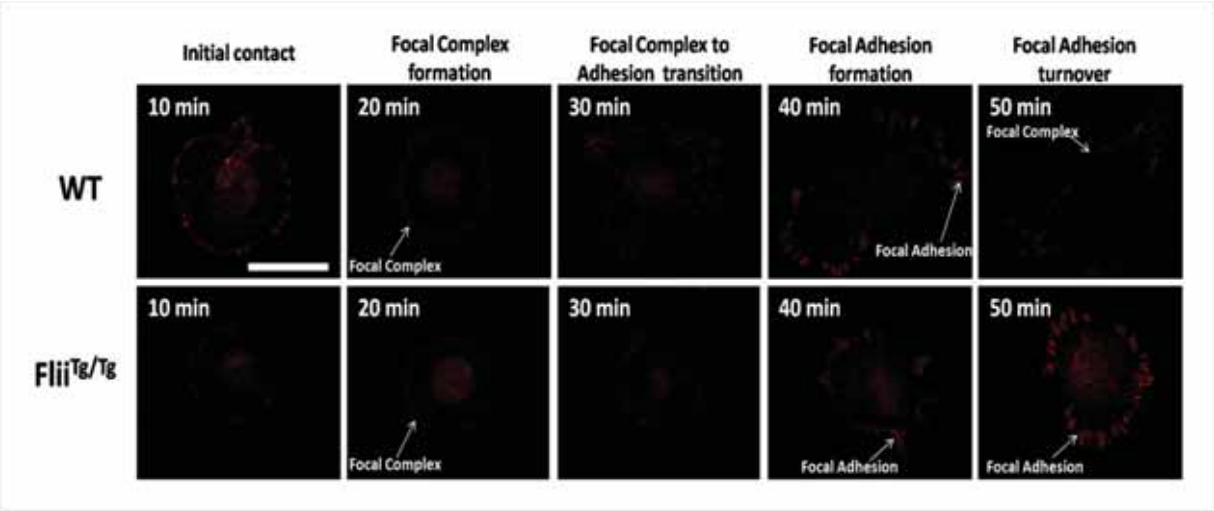
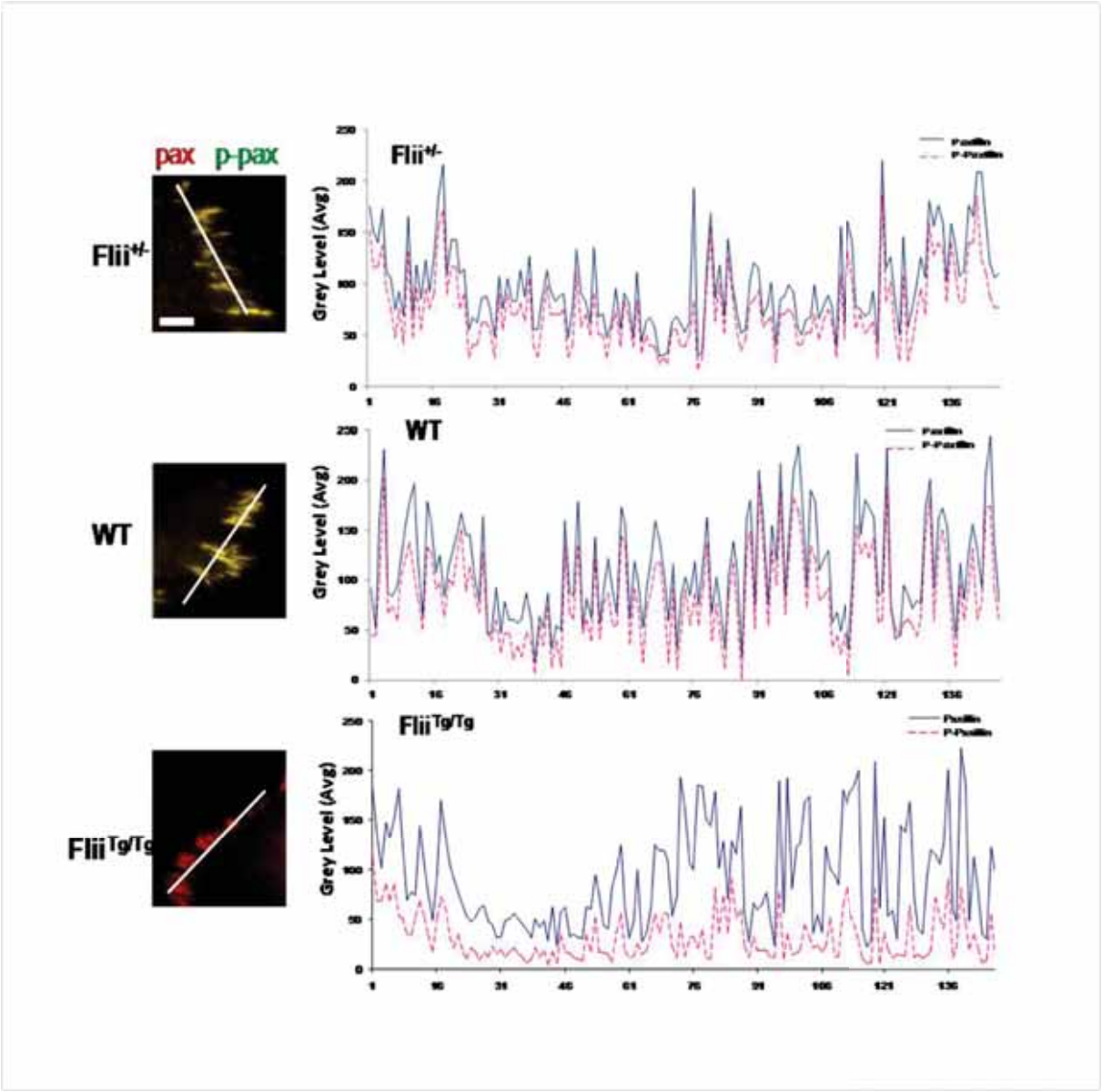


Figure 6. Effect of Flightless I on paxillin activation and signalling in wounds in vivo. (**a-f and k**) Representative 3 day images of full thickness incisional wounds, (**g-i**) immunofluorescent staining of p-paxillin and (**j**) p-paxillin. *Flii*$^{Tg/Tg}$ mice wounds show reduced paxillin staining compared to *Flii*$^{+/-}$ and WT counterparts, n=6. Bar=500μm (d-f) and 30μm (g-i). (**l**) Western Blot showing decreased expression of Src and p130Cas in wounds of *Flii*$^{Tg/Tg}$ mice. (**m**) 3-D floating collagen gel contraction assay at 24hrs. Magnification x6.5. dotted line = well size and original gel size (**n**) Flii$^{+/-}$ fibroblasts have significantly reduced collagen gel contraction compared to both WT and *Flii*$^{Tg/Tg}$ fibroblasts at 24, 48 and 72 hours. n=5. Mean +/- SEM. *P<0.05.



Supplementary Figure 1. Effect of Flightless I on initial focal complex formation. Representative images of paxillin stained wild-type and *Flii*$^{Tg/Tg}$ fibroblasts adhesion and spreading over a 50 minutes time-course highlighting differences in formation and turnover of adhesion structures. *Flii* over-expressing fibroblasts form initial focal complexes which proceed to focal adhesions but may have inhibited focal adhesion turnover to form new focal complexes. Bar=10µm.



Supplementary Figure 2. Effect of Flightless I on paxillin phosphorylation. Line scans showing fluorescence intensity of individual focal adhesions. Representative composite images of *Flii*$^{+/-}$, WT and *Flii*$^{Tg/Tg}$ fibroblast focal adhesions stained for paxillin (red) and p-paxillin (green), composite = yellow. Fluorescence intensity expressed as grey level average, solid blue line (paxillin), and dotted red line (p-paxillin). n=150 individual focal adhesions. Bar=1μm.

Kopecki, Z., Murrell, D.F. and Cowin, A.J. (2009) Raising the roof on epidermolysis bullosa (EB): a focus on new therapies.
Wound Practice and Research, v.17 (2), pp. 72-80, May 2009