

**Functional RNAi analysis of novel
regulators of adhesion with further
characterisation of the role of
LZTFL1 in keratinocytes and heart
disease**

Susann Bruche

Imperial College London

National Heart and Lung Institute

Submitted for the degree of Doctor of Philosophy of Imperial College
London

Declaration of Originality

I herewith declare that all data presented in this thesis is my own work and any contributions from others are clearly acknowledged.

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Abstract

Adherens junctions mediate intercellular adhesion in most tissues. Cell-cell contacts need to be regulated in a variety of biological processes including developmental morphogenesis, wound healing and transendothelial migration. Actin remodelling is crucial for adherens junction control and members of the family of Rho small GTPases are key regulators of adherens junction formation, maintenance and turnover. Although only a few Rho GTPases have been shown to date to control adherens junctions, they govern a large number of cellular processes downstream of cadherin interaction by activating different effector proteins. Considering the complexity of adherens junction biology, a complex network of actin-binding proteins and Rho GTPase effectors are likely to mediate adherens junction assembly and preservation. Therefore, we performed RNAi screens and identified several actin-binding proteins and Rho GTPase effectors as novel adherens junction regulators. Here, I validate the role of several of these proteins during adherens junction assembly and further investigate the mechanisms involved for a few of them.

Skin and heart are under constant mechanical stress and therefore require specialised cell-cell contacts to ensure tissue integrity. In addition to adherens junctions, epithelial cells and cardiomyocytes possess desmosomes and both complexes warrant junction stability. In this study, I identify LZTFL1 as a protein involved in the stabilisation of cell-cell contacts. LZTFL1 is a tumour suppressor and is involved in trafficking towards cilia. Here I show that LZTFL1 depletion negatively affects adherens junction-associated E-cadherin and actin as well as desmosome-coupled desmoplakin and keratin 14. I further demonstrate that LZTFL1 can bind to α -catenin and keratin 14 and thus might be able to mediate crosstalk between different cell-cell contact complexes.

In cardiovascular disease, contraction and mechanical coupling of neighbouring cardiomyocytes need to adapt to changes in pressure and volume load. In accordance with its cell-cell contact-stabilising function in epithelial cells, I detect changes in LZTFL1 expression levels in different cardiac pathologies. These changes are specific to certain pathologies and disease stages and likely represent adaptations to altered mechanical requirements of the heart.

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Abbreviations

Abbreviation	Full name
ADF	Actin-depolymerizing factor
ADP	Adenosine diphosphate
AJ	Adherens junctions
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
APC	Adenomatous polyposis coli
aPKC	Atypical protein kinase C
ARVC	Arrhythmogenic right ventricular cardiomyopathy
ATP	Adenosine triphosphate
AU	Arbitrary unit
BAR	Bin/amphiphysin/Rvs
BBS	Bardet-Biedl syndrome protein
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CIP4	Cdc42-interacting protein 4
CK1	Casein kinase 1
DAPI	4',6-diamidino-2-phenylindole
DCM	Dilated cardiomyopathy
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPEC	Diethylpyrocarbonate

Dsc	Desmocollin
Dsg	Desmoglein
DTT	Dithiothreitol
EB	Epidermolysis bullosa
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EF1 α	Elongation factor 1 alpha
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eGFP	Enhanced green fluorescence protein
EMT	Epithelial to mesenchymal transition
EPLIN	Epithelial protein lost in neoplasm
EST	Expressed sequence tag
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FER	Feline sarcoma-related
FGFR2	Fibroblast growth factor receptor 2
FISH	Fluorescence <i>in situ</i> hybridization
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
G-actin	Globular actin
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
Gem	Gemini of Cajal bodies
GFAP	Glial fibrillary acidic protein

GLUT4	Glucose transporter type 4
GSK-3 β	Glycogen synthase kinase 3 beta
GST	Glutathione S-transferases
GTP	Guanosine triphosphate
HCM	Hypertrophic cardiomyopathy
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HPRT	Hypoxanthine phosphoribosyltransferase
HSD	Honest significant difference
IF	Intermediate filaments
IGF1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IP	Immunoprecipitation
IPTG	Isopropyl β -D-1-thiogalactopyranoside
JAM-A	Junctional adhesion molecule-A
LB	Lysogeny broth
Lef	Lymphoid enhancer-binding factor
LIC	Ligation independent cloning
LIM	Lin-1, Isl-1, and Mec-3
LPP	Leupeptin, pefabloc, pepstatin
LZTFL1	Leucine zipper transcription factor like 1
MDCK	Madin-Darby canine kidney
MI	Myocardial infarct
MHC	Myosin heavy chain
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLP	Muscle LIM protein
mRFP	Monomeric red fluorescence protein

mRNA	Messenger ribonucleic acid
MT	Microtubules
mTFP	Monomeric teal fluorescence protein
MTSS1	Metastasis suppressor 1
MyBP-C	Myosin binding protein C
NCAM	Neural cell adhesion molecule
NLS	Nuclear localisation signal
OPT	Oct1/PTF/transcription
ORF	Open reading frame
Par6	Partitioning defective 6
PBS	Phosphate-buffered saline
PcG	Polycomb
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PDZ	PSD-85, Dlg, ZO-1
PF	Pemphigus foliaceus
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIPES	Piperazine-N,N'-bis
PKC/D	Protein kinase C/D
PKP	Plakophilin
PMA	Phorbol 12-myristate 13-acetate
PML	Promyelocytic leukemia
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein phosphatase 2A
PTCH1	Patched 1
PV	Pemphigus vulgaris
PVDF	Polyvinylidifluoride

qPCR	Quantitative real-time PCR
Rab	Ras-like in the brain
RCM	Restrictive cardiomyopathy
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
ROCK	Rho-associated protein kinase
Rho	Ras homologous
RTK	Receptor tyrosine kinases
RPLP0	Ribosomal protein, large, P0
SCAR	Suppressor of cAMP receptor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH3	Src homology 3
SHH	Sonic Hedgehog
SHRSP	Spontaneously hypertensive stroke-prone rat
siRNA	Small interfering ribonucleic acid
SMO	Smoothened
SNX-1	Sorting nexin-1
TAC	Transverse aortic constriction
TBS	Tris buffered saline
TCF	T-cell factor
TEV	Tobacco Etch Virus
TJ	Tight junctions
TNF α	Tumour necrosis factor alpha
TNM	Tumour-Node-Metastasis
tRNA	Transfer ribonucleic acid
Ub	Ubiquitylation

VASP	Vasodilator-stimulated phosphoprotein-like
VEGFR	Vascular endothelial growth factor receptor
VH	Vinculin homology
WASP	Wiskott–Aldrich syndrome protein
WAVE	WASP family Verprolin - homologous protein
WH2	WASP-homology 2
Wnt	Wingless- integrated
YAP1	Yes-associated protein 1
ZASP	Z-band alternatively spliced protein

Chapter 1 Introduction

1.1 Multicellularity and specialisation

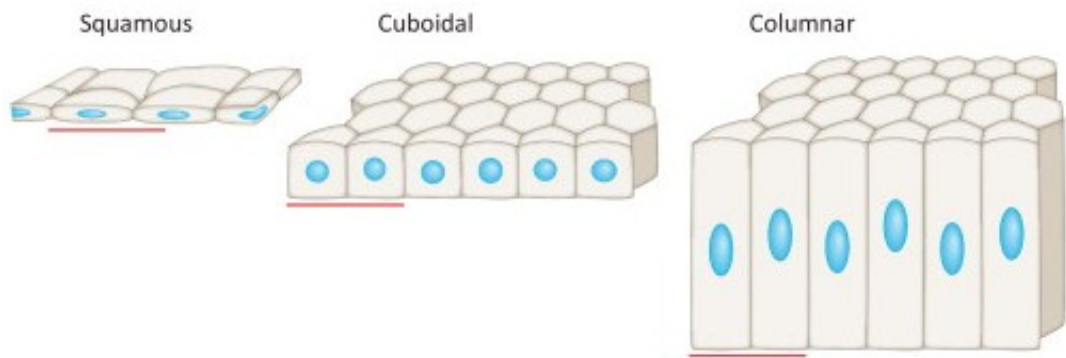
The evolution of multicellularity allowed the specialisation of cells within the multicellular organism. This eventually led to the development of tissues comprising differentiated and organised cells, which fulfil a specific, common function. Different tissues are then grouped and layered to form organs. Multicellularity required the development of cell-cell adhesion proteins to hold cells together and of molecules that permit intercellular communication.

1.2 Epithelial cells

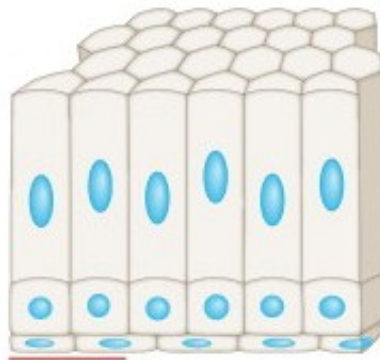
Epithelial cells form the epithelium, a tissue that functions to separate inside from outside, plays a role in absorption and secretion and forms a mechanical barrier to protect underlying tissues from water loss or insults by pathogens, physical and chemical stress (Powell, 1981). Epithelial cells are found throughout the body, lining organs and the outer surface of the body. Epithelia come in different shapes, which is an adaptation to the distinct function the tissue performs in the different organs. They are categorised by morphology as squamous, cuboidal and columnar and can be single layered (simple epithelia) or multi-layered (stratified epithelia) (Figure 1.1). Squamous epithelium is flat and allows passage by diffusion and filtration in organs such as air sacs of the lungs. Simple cuboidal and columnar epithelia function in absorption and secretion in organs such as kidney tubules and digestive tract. Stratified epithelia largely have protective functions and are for example found in the skin (Presland and Jurevic, 2002). Furthermore, some epithelia consist of a single layer of cells, but the nuclei are not aligned at one level therefore giving a multi-layered appearance (pseudostratified epithelia). Pseudostratified epithelia are found in highly proliferative tissues such as the digestive system and have a role in secretion (Lee and Norden, 2013).

Key features of epithelial cells are apical/basal polarity, which is required to define inside and outside of the epithelium, and the presence of specialised cell-cell contacts, which are required for barrier function but are also involved in establishing cellular polarization (Braga, 2000; McCaffrey and Macara, 2011).

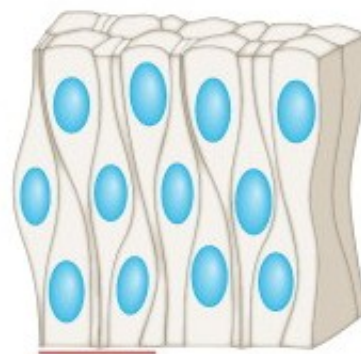
(a) Simple epithelia



(b) Stratified epithelia (e.g., columnar stratified)



(c) Pseudostratified epithelia



TRENDS in Cell Biology

Figure 1.1: Types of epithelial cells Epithelia are classified by their morphology as squamous, cuboidal and columnar. Based on the number of cell layers they are distinguished into (a) simple epithelia with one layer or (b) stratified epithelia with multiple layers. (c) Pseudostratified epithelia have one cell layer, but nuclei are not aligned and epithelia therefore appear multilayered. Figure from (Lee and Norden, 2013).

1.3 Muscle cells

The function of muscle cells is contraction in a rapid and repetitive manner. Mammals possess three different types of muscle cells that differ in structure and function (Figure 1.2). Skeletal muscle cells are required for voluntary movement (Alberts et al., 2002). They are very long and contain several nuclei because they are formed by fusion of muscle precursor cells, myoblasts. Cardiac muscle cells are small with only one or two nuclei. They are connected on their transverse ends with neighbouring cells and their specialised shape allows them to connect to more than two neighbour cells which might aid the spreading of signals throughout the heart. Cardiac muscle is the most persistent muscle and beats continuously throughout life without tiring. Both skeletal and cardiac muscles have a striated appearance because their cytoskeleton is highly organised. Smooth muscle cells surround inner organs such as the digestive system and blood vessels and contract and relax slowly and involuntarily. They are spindle shaped with a single nucleus and do not possess the same ordered structure in their cytoskeleton that causes striation in the other muscle types.

1.4 Rho GTPases

The cytoskeleton is responsible for shaping and structuring cells, but also to generate forces that allow cell movement and shape changes and therefore needs to be dynamic. The three main components of the cytoskeleton are actin filaments, microtubules (MT) and intermediate filaments (IF).

The Rho small GTPases are a family of signalling molecules with 20 members that have diverse effects on the cytoskeleton (Aspenström et al., 2004; Boulant et al., 2011). Rho GTPases have a guanine nucleotide binding site and most family members exist in two states, active and inactive, dependent on their binding to guanine nucleotide triphosphate (GTP) or guanine nucleotide diphosphate (GDP), respectively. Rho GTPases mediate their signalling function through the recruitment and activation of downstream effector molecules. GTP binding promotes conformational changes, which allow effector protein binding (Bishop and Hall, 2000). Conformational changes upon GTP hydrolysis to GDP disrupt the interaction and the effector dissociates from the GTPase.

Rho GTPase activity is regulated by guanine nucleotide exchange factors (GEF), by GTPase-activating proteins (GAP) and by guanine-nucleotide dissociation inhibitors (GDIs). GEF positively regulate GTPases by stabilizing the nucleotide-free form of the

GTPase. This stimulates the release of bound GDP, which is then preferentially replaced by GTP due to its higher cellular concentration as compared to GDP (Spiering and Hodgson, 2011; Vetter and Wittinghofer, 2001). The intrinsic GTP hydrolysis rate of GTPases is slow, but is promoted by GAP, which thus serve as negative regulators of GTPases by 'switching them off' (Spiering and Hodgson, 2011). Finally, GTPases are negatively regulated by GDI, which prevent dissociation of GDP from the GTPase as well as sequester GTPases in the cytoplasm and thereby prevent them from signalling (Spiering and Hodgson, 2011).

Although Rho GTPases have actin-independent functions, such as effects on cell cycle progression, gene transcription and vesicular trafficking, the majority of their roles are exerted through the regulation of the actin cytoskeleton (Etienne-Manneville and Hall, 2002; Narumiya and Yasuda, 2006; Villalonga et al., 2006). Early studies showed that the best-characterized GTPases RhoA, Rac1 and Cdc42 each promote different actin-based structures. Active RhoA causes focal adhesion and stress fibre formation in fibroblasts (Ridley and Hall, 1992). Rac1 activation drives the formation of lamellipodia (Ridley et al., 1992), whilst Cdc42 activity leads to filopodia-like protrusions (Kozma et al., 1995; Nobes and Hall, 1995). Formation of distinct actin-rich structures by the three GTPases is dependent on the downstream activation of distinct actin-regulatory proteins. To date, more than 100 effector proteins have been identified for RhoA, Rac1 and Cdc42 including actin-polymerizing formins, protein and lipid kinases, scaffold proteins and membrane deforming BAR proteins, highlighting the complexity of Rho GTPase signalling (Hall, 2012; Sit and Manser, 2011).

RhoA-mediated actin regulation is a key component of cytokinesis (Piekny et al., 2005). In several cell types, RhoA has been shown to associate with the actomyosin ring at the cleavage furrow and to promote its contractility through myosin light chain phosphorylation via the RhoA effectors citron kinase and ROCK (Di Cunto et al., 2000; Kosako et al., 2000; Madaule et al., 1998; Yamashiro et al., 2003). The RhoA effectors ROCK and myotonic-dystrophy-kinase-related Cdc42-binding kinase (MRCK) are further required to anchor F-actin to cell membranes through activation of ezrin-radixin-moesin (ERM) family proteins (Niggli and Rossy, 2008). Active ERM proteins are the able to bridge F-actin and a variety of transmembrane proteins.

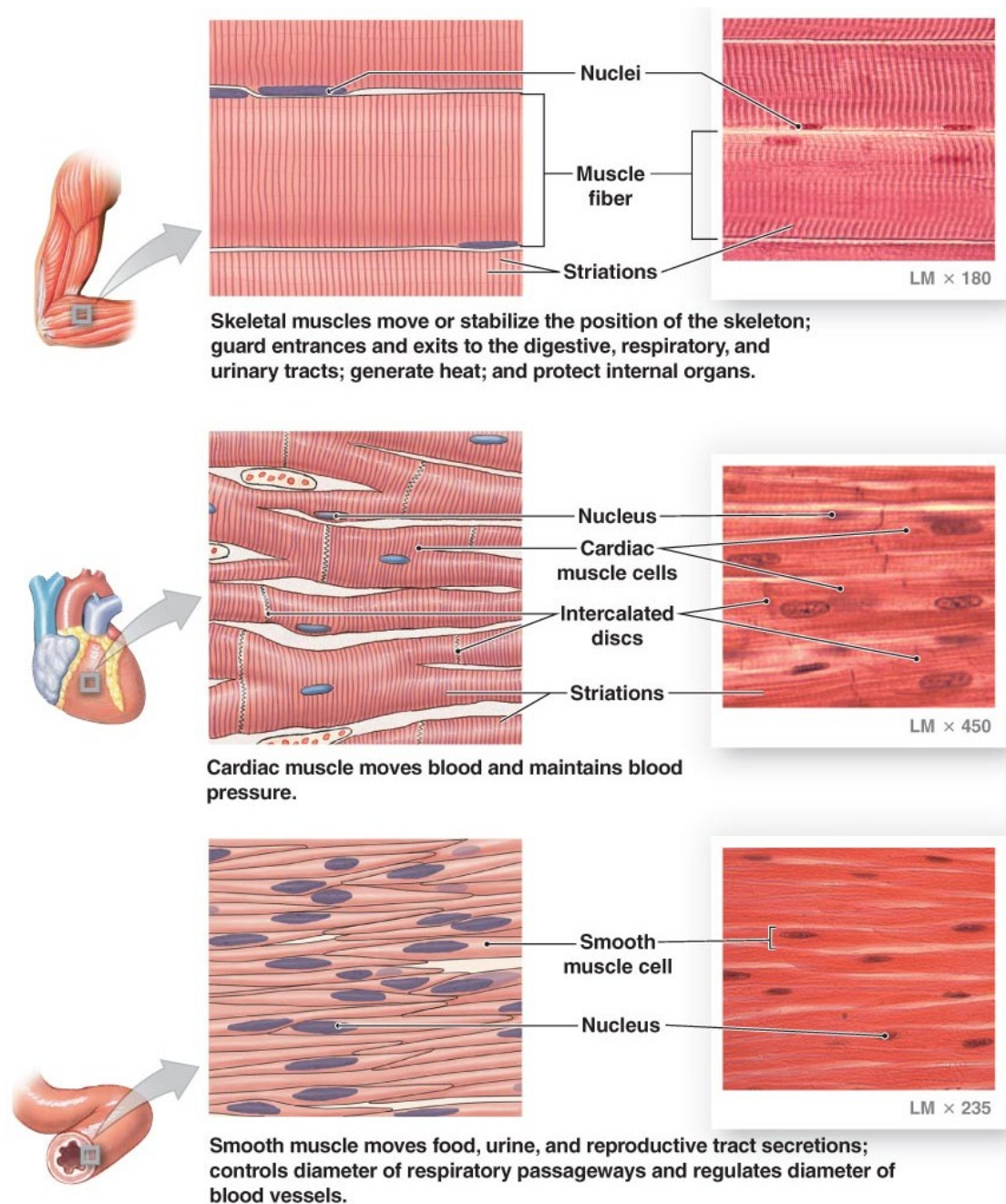
Rho GTPase activity is also involved in directed cell migration in 2D-cultures, which requires actin polymerization and membrane elongation at the front of the cell and actomyosin contraction at centre and rear of the cell (Raftopoulou and Hall, 2004). Active Rac1 is recruited to the leading edge of the cell in response to chemotactic

signals (Gardiner et al., 2002; Itoh et al., 2002b; Kraynov et al., 2000) and drives the assembly of branched-actin networks that push the cell forwards (Raftopoulou and Hall, 2004). Cdc42 is required for the directionality of migration possibly by spatial organisation of Rac signalling (Allen et al., 1998; Etienne-Manneville and Hall, 2002). At the rear of the cell, RhoA activation generates contractile forces through myosin II phosphorylation by ROCK (Raftopoulou and Hall, 2004; Riento and Ridley, 2003).

1.5 Cytoskeleton

1.5.1 Cytoskeletal components

MT are the stiffest filaments (Gittes et al., 1993) and are required as tracks for organelle and vesicle transport and as component of mitotic spindles for chromosome segregation (Howard and Hyman, 2003). MT can self-assemble into hollow tubes from heterodimers of α - and β -tubulin. Tubulin is a GTPase and dimers assemble to filaments in their high affinity status when bound to GTP. For depolymerisation, GTP is hydrolysed to GDP, which destabilises the filament (Howard and Hyman, 2003). MT are polar filaments, but dimers can be added or removed from both filament ends. However, one end grows faster than the other and is referred to as plus end while the other end is the minus end. Minus ends are usually anchored in the centre of cells at centrosomes and grow from there outwards towards the cell periphery (Howard and Hyman, 2003).



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Figure 1.2: Types and function of muscle cells. Schematic drawings and light microscopic images of the three muscle types skeletal muscle, heart muscle and smooth muscle. Functions of the different muscle types are described below the schematics. Image modified from <http://www.highlands.edu/academics/divisions/scipe/biology/faculty/harnden/2121/notes/tissues.htm>

Actin filaments are less rigid but can be bundled or cross-linked by accessory proteins to form stiffer, higher order structures such as branched networks (e.g. at the leading edge) and linear fibre bundles (e.g. in stress fibres) (Fletcher and Mullins, 2010). Actin dynamics are involved in migration, in the formation of cell protrusions, in the stabilisation of cell-cell contacts and in the generation of contractile forces. Similarly to MT, actin filaments are also used as tracks for the transport of cellular components.

IF are a large family of highly flexible filaments that are expressed in a tissue-specific manner (Herrmann et al., 2007). To name a few, vimentin is found in mesenchymal cells, muscle expresses desmin, epithelial cells keratin and neurons contain neurofilaments. IF are found in the cytoplasm or in the nucleus attached to the nuclear membrane. In the cytoplasm, IF regulate cell shape and organelle localisation. Nuclear IF are called lamins and are involved in the organisation of heterochromatin and nuclear protein complexes.

1.5.2 Actin formation and regulation

Actin monomers called G-actin form polar, helical filaments called filamentous actin (F-actin) by self-assembly, but require nucleation factors to start polymerisation (Pollard et al., 2000). Filaments can grow and shrink from either end, but elongation and depolymerisation are faster at the barbed compared to the pointed end. The nucleation complex Arp2/3 acts as template for actin polymerization and can bind to the sides of pre-existing actin filaments, thereby promoting the formation of branched actin networks (Goley and Welch, 2006). Arp2/3 needs to be activated by nucleation promoting factors such as members of the WASP/SCAR family, which themselves are activated by Rho small GTPases. Nucleation factors of the formin family drive linear actin filament polymerization and are directly stimulated by Rho small GTPases (Higgs, 2005).

G-actin exists in the cell in concentrations that are about 1000-fold higher than the critical concentration required for polymerisation (Pollard et al., 2000). Therefore, to stop actin filament growth, filament ends are hindered from extending by binding of capping proteins and sequestering of G-actin by monomer-binding proteins. To continue elongating filaments, capping proteins can be removed and this is sufficient to overcome the sequestration of monomers. Severing of existing filaments into shorter fragments with free ends can also induce filament elongation (Pollard et al., 2000).

Monomeric actin is bound to ATP when polymerizing into filaments and ATP is subsequently hydrolysed into ADP-P_i (Pollard et al., 2000). Dissociation of the gamma-phosphate is slow and ADP-actin allows the binding of actin depolymerizing proteins ADF/cofilin. Depolymerisation of actin by ADF/cofilin is regulated by phosphorylation events downstream of Rho GTPase signalling (Pollard et al., 2000). Monomer-binding proteins then bind ADP-actin monomers and ADP is exchanged for ATP, leaving ATP-actin ready for reuse in another round of polymerization (Blanchoin and Pollard, 1998).

1.5.3 Regulation of intermediate filaments

Structurally, all IF contain a roughly 45 nm-long central α -helical domain and non- α -helical N- and C-terminal domains (Figure 1.3 A) (Herrmann et al., 2007). Two IF monomers align in parallel and form a coiled-coil dimer. Keratins form heterodimers containing an acidic and a basic keratin type, e.g. keratin 5 and 14 in basal keratinocytes, while vimentin and desmin IF can form both homo- and heterodimers. The composition of the dimers can greatly influence IF mechanical and protein-binding properties. Four of these dimers then associate to form an anti-parallel, half-staggered tetramer (Figure 1.3 B). Eight of these tetramers align laterally to form a circular unit-length filament. These short filament units are then aligned longitudinally and compacted radially to form long, mature filaments. Because of the anti-parallel alignment of the polar coiled-coil dimers in the tetramer, IF filaments have no polarity and accordingly unit-length filaments are added to both ends of the IF during elongation.

IF disassembly can be achieved either by targeting IF monomers for degradation or by dismounting them into unit-length filaments for reuse. While the first mechanism is mainly employed during stress and pathology, the second mechanism seems to be prevalent under normal conditions (Windoffer et al., 2011). Subunits are then temporarily prevented from incorporation into IF through modifications or binding to IF-associated proteins.

Most IF filaments undergo constant turnover, but filaments anchored to cell-cell adhesive complexes called desmosomes, cell-substratum contacts named hemidesmosomes and the nucleus are less dynamic (Strnad et al., 2002; Windoffer et al., 2011). Upon attachment, those fibres are thought to exit the turnover cycle and become stabilised, a process termed maturation (Windoffer et al., 2011). In this model, dynamic IF extension and disassembly in the cell periphery might be required to probe

the membrane for new contacts and connecting to adhesive complexes eventually results in IF maturation.

IF are characterised by high flexibility, extensibility, breakage-resistance and strain-hardening and cooperate with other skeletal filaments to define the mechanical properties of the cell (Herrmann et al., 2007). Increased mechanical and biochemical stability of IF is achieved by IF bundling through IF-binding proteins or by spontaneous self-organisation (Kim et al., 2010; Lee and Coulombe, 2009; Windoffer et al., 2011). IF undergo a variety of different posttranslational modifications under distinct cellular circumstances including modifications such as phosphorylation, sumoylation and glycosylation (Figure 1.3 C) (Coulombe and Omary, 2002; Hyder et al., 2008; Snider and Omary, 2014). Modifications influence spontaneous IF bundling but also bundling through IF-associated proteins (Coulombe and Omary, 2002; Windoffer et al., 2011). In addition, modifications affect IF stability (Hyder et al., 2008; Snider and Omary, 2014; Windoffer et al., 2011), for example phosphorylation of the intermediate filament glial fibrillary acidic protein (GFAP) in astrocytes or Keratin 8 in glandular epithelia attenuates their turnover (Ku and Omary, 2000; Takemura et al., 2002).

However, IF not only provide structural support to cells but are also involved in cell signalling since they are scaffolds for a large number of different proteins including components of the translational machinery as well as metabolic and signalling proteins (Hyder et al., 2008; Kim and Coulombe, 2007; Snider and Omary, 2014; Windoffer et al., 2011). Modifications can influence IF signalling function by creating new binding sites for IF-binding proteins.

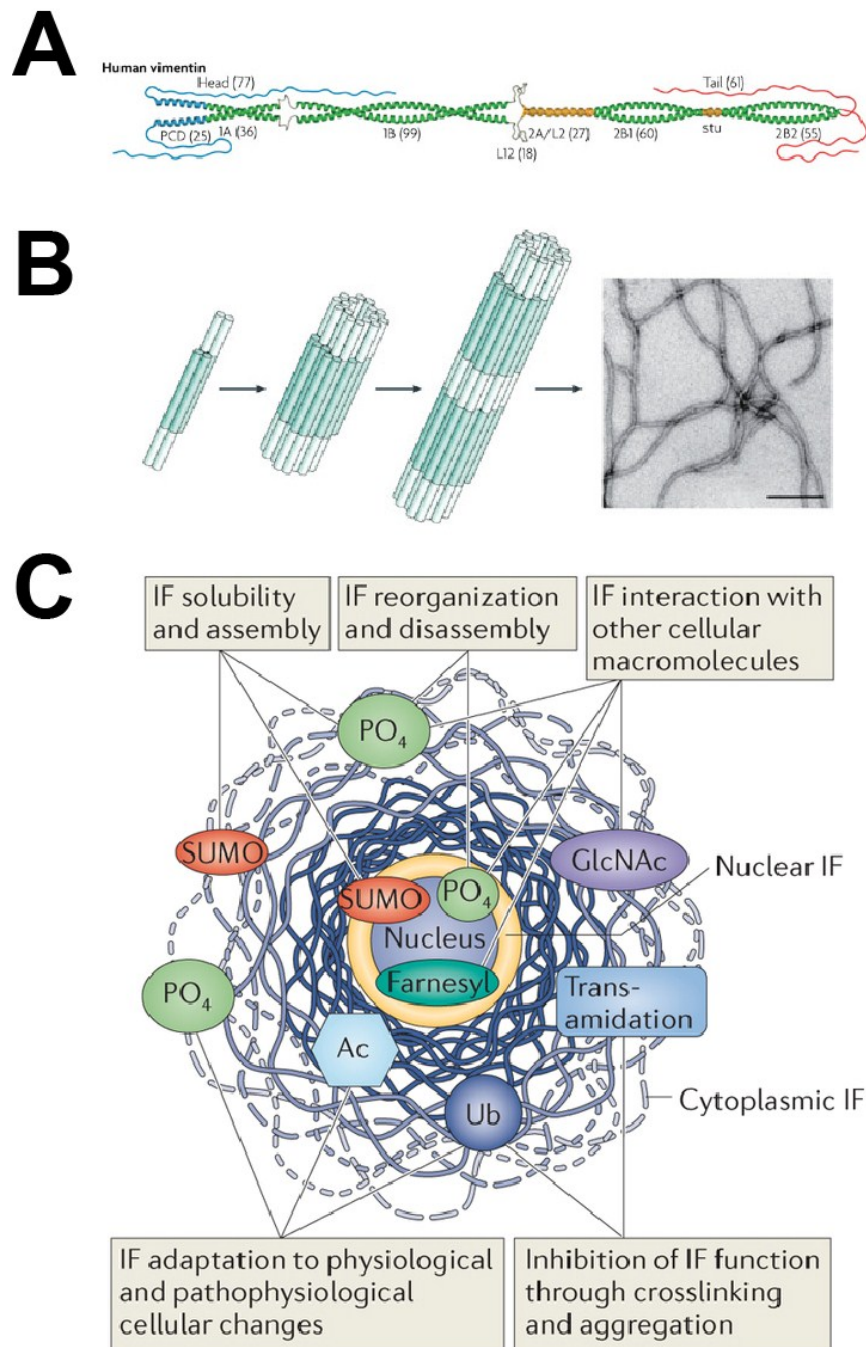


Figure 1.3: Intermediate filament structure, assembly and modification. A) Model of a human vimentin homodimer based on structural data and structure prediction. Vimentin and other intermediate filaments (IF) contain a central α -helical rod domain divided into the segments 1A, 1B, 2A, 2B1 and 2B2. Vimentin also contains an α -helical pre-coil domain (PCD). Linker regions connecting the α -helical segments are labelled L12 and L2. Left-handed coiled-coil segments are shown in green. Regions that are predicted to form nearly parallel α -helical bundles as well as the conserved so-called stutter (stu) region that interrupt the coiled-coil arrangement are represented in yellow. Non- α -helical linkers are shown in grey. The unstructured N- (head) and C-terminal (tail) domains are coloured blue and red, respectively. Numbers in brackets refer to number of amino acids in each respective domain. B) Eight IF tetramers associate laterally to form a unit-length filament. These short filaments anneal longitudinally to form elongated filaments which then radially compact to yield mature IF. An electron microscopy image of negatively stained preparations show *in vitro* assembled mouse recombinant desmin. Scale bar = 100 nm. C) Summary of IF post-translational modifications and their function. Phosphorylation (PO₄), sumoylation (SUMO), O-linked β -D-N-acetylglucosamine-mediated glycosylation (GlcNAc), lysine acetylation (Ac), ubiquitylation (Ub), farnesylation (Farnesyl), transamidation. Images and legends adapted from (Herrmann et al., 2007; Snider and Omary, 2014).

1.5.4 Cytoskeleton in epithelial cells

Epithelial cells are polarised cells with apical and basal domains that are structurally and functionally distinct. Short, randomly oriented MT form a mat between apical membrane and Golgi apparatus, which is required to organise the Golgi apparatus (Mays et al., 1994). In addition, MT are arranged longitudinally along the apical-basolateral axis with the minus ends towards the apical domain. These filaments are involved in directional transport between apical and basolateral domains (Mays et al., 1994).

Although actin filaments are widely distributed throughout epithelial cells, the most prominent actin populations are found at the apical-basolateral border. One population of actin is found close to the membrane underlying cell-cell contacts and is called junctional actin (Zhang et al., 2005). Junctional actin is required to stabilise cell adhesive complexes in the membrane. In addition, in the same domain a circumferential ring of actin bundles is encircling the cell and is referred to as thin bundles (Zhang et al., 2005). These thin bundles not only reinforce adhesive complexes but are also required to gain lateral cell height. Actin is also found at sites of cell-substratum contact (focal adhesions) and as bundles in apical microvilli, if present in the cell (Mays et al., 1994).

Keratin is the main IF filament found in epithelial cells that spans the cytoplasm and inserts at cell-cell contacts into desmosomes. Keratin IF are formed of heterodimers containing an acidic type I (K9-K10, K12-K20, K23-K24) and a basic type II (K1-K8, K76-K80) keratin monomer (Moll et al., 2008). Different types and differentiation stages of epithelial cells express distinct keratin pairs. Many simple epithelia are characterised by the expression of K8/K18, while others possess K7/K19. The pair K5/K14 is expressed in basal keratinocytes of stratified squamous epithelium, although the pair K5/K15 is also found. Suprabasal layers of stratified epithelium contain K6/K16, K1/K10 or K4/K13, while the upper spinous and granular layers contains K2/K9 (Moll et al., 2008). The type of keratin expressed is determining the mechanical properties of the cell, but can also influence cell signalling and transport (Bragulla and Homberger, 2009).

1.5.5 Cytoskeleton in cardiomyocytes

The actin cytoskeleton in cardiomyocytes is highly organised into sarcomeres. Sarcomeres are repetitive cytoskeletal units that are connected end to end to form fibrils (Figure 1.4). Sarcomeres are made up of overlapping actin and myosin filaments, which slide past each other in an ATP-dependent manner to shorten sarcomeres and cause myocyte contraction. Many other proteins also form part of the sarcomere and regulate contraction. Tropomyosin stabilises actin filaments and binds the troponin complex of troponin-T, -I and -C, which regulates the interaction of myosin with actin filaments in a Ca^{2+} -dependent manner. Actin filaments are anchored at the ends of the sarcomere in the Z-disc on a scaffold of α -actinin where filaments from neighbouring sarcomeres are connected and oriented. A large protein called titin is spanning half the sarcomere from the Z-band to the centre of the sarcomere (M-band). Titin binds to actin and α -actinin in the Z-band and to myosin in the region of overlap between myosin and actin (A-band). Titin functions as a spring to aid the return of the sarcomere to its initial length after contraction. Other functions include a role in sarcomerogenesis as molecular ruler, centring of myosin during contraction and organisation of the link between α -actinin and actin in the Z-disc (Sarantis et al., 2012).

Many additional proteins have structural and regulatory functions in the sarcomere and especially in the Z-disc as reviewed by (Frank and Frey, 2011; Frank et al., 2006; Lange et al., 2006). For example, it has been noted that the Z-disc is a signalling hub that permits binding of transcription factors, signalling proteins and enzymes such as the transcriptional regulator muscle LIM protein (MLP) or protein kinases C and D (PKC, PKD) which bind to the Z-disc scaffold protein Z-band alternatively spliced protein (ZASP)/Cypher.

Neighbouring cardiomyocytes are connected on their transverse ends via specialised cell-cell contacts called intercalated discs (Forbes and Sperelakis, 1985) (see below). They not only link cardiomyocytes but also form the insertion site for sarcomeres, where they become aligned precisely with the fibrils of neighbouring cells to optimise contraction and allow cells to act as a functional syncytium.

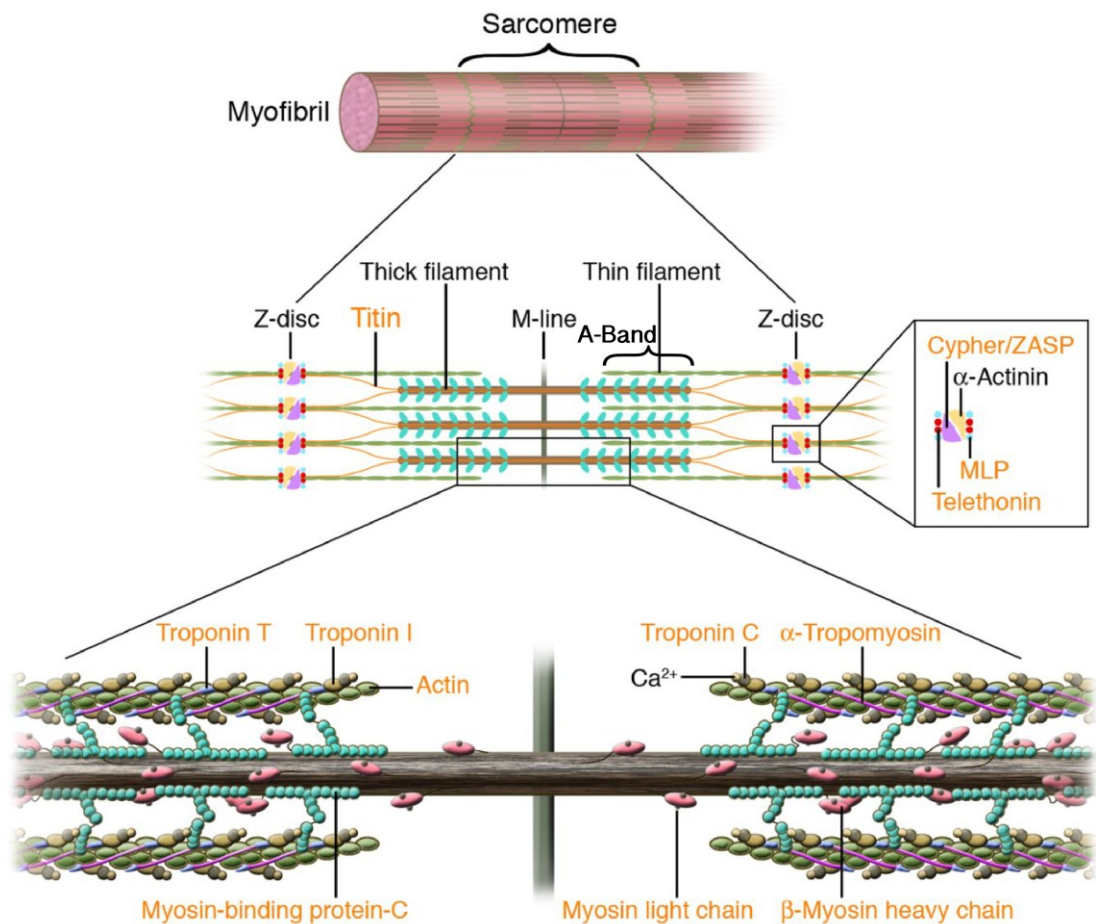


Figure 1.4: Sarcomere structure. Schematic image of a sarcomere within a myofibril. Actin thin filament and myosin thick filament form the core of the sarcomere. Troponin C, I and T and tropomyosin bind on actin filaments and regulate the interaction with myosin. Titin spans the sarcomere from the Z-disc to the M-line and is stabilised in the Z-disc by telethonin. Myosin-binding protein-C is located on myosin in the A-band. The Z-disc contains a scaffold of α -actinin, but also contains many other proteins such as the transcriptional regulator muscle LIM protein (MLP) or the scaffold PDZ/LIM protein Z-band alternatively spliced protein (ZASP)/Cypher. The figure is modified from (Morita et al., 2005).

Aside the sarcomere, cytoskeleton exists similar to other cells in the form of intermediate and actin filaments and microtubules (Sarantitis et al., 2012). Desmin is the intermediate filament found in cardiomyocytes and is required for nucleus positioning. It surrounds Z-discs and connects Z-discs of neighbouring muscle strands to one another and to cell-cell contacts, the nuclear envelope and costameres. Costameres are lateral membrane bound protein complexes that convey signals between extracellular matrix and cardiomyocytes. It is thought that these connections are required to transduce contraction and extracellular signals between different compartments and influence nuclear function to adapt to changed conditions and requirements. Actin filaments similarly connect neighbouring Z-discs and intercalated discs and costameres, but the exact function of extra-sarcomeric actin is unknown. Microtubules are involved in anchoring cellular organelles, transport and signal transmission. They can also regulate cell flexibility through rapid polymerization and depolymerisation, thereby enabling a quick change of cytoskeletal organisation of cells.

1.6 Cell-cell contact complexes and function

Cell-cell adhesive complexes are required to hold cells together in tissues and are particularly important in tissues such as epithelia and cardiac muscle. They allow epithelia to act as barrier and provide integrity to muscle to resist the mechanical stress during contraction. They act as anchors for the cytoskeleton and also perform as signalling hubs. Other complexes in the membranes of neighbouring cells are specialised in intercellular communication.

1.6.1 Tight junctions

Tight junctions (TJ) are found in many epithelial cell types in vertebrates and are the most apical of the cell-cell contact complexes. In the four cellular layers of stratified skin epithelium (basal, spinous, granular, cornified layer), they are only found in the granular layer (Pummi et al., 2001). TJ composition is highly complex (Anderson and Van Itallie, 2009; González-Mariscal et al., 2003). Claudins are the main transmembrane protein component and are complemented by tetraspan membrane proteins with a Marvel domain (e.g. occludin, tricellulin) and IgG superfamily proteins such as JAM-A. These transmembrane proteins interact with a large number of cytoplasmic plaque proteins comprising adapter proteins and different classes of signalling proteins. Via some of these plaque proteins TJ are bound to F-actin and microtubules.

TJ are required to form a semipermeable diffusion barrier between neighbouring cells. Loss of some TJ components is detrimental as claudin-1-knockout mice die shortly after birth from dehydration (Furuse et al., 2002) and JAM-A knock-down in epithelia cell cultures causes monolayers to become leaky (Liu et al., 2000). In their function as signalling hub, they influence gene transcription to control proliferation and they are also thought to be involved in epithelial polarization since several polarity complexes are found at TJ (Matter and Balda, 2003; Shin et al., 2006).

1.6.2 Adherens junctions

In the apical-basal plane, adherens junctions (AJ) are found below TJ and above a third adhesive complex called desmosomes. Of the three junction types AJ were the first to develop during evolution (Oda, 2012). Likely as a result, AJ are the first adhesive complex that forms during epithelia biogenesis. TJ and desmosomes follow subsequently and their formation is abolished when AJ assembly is impaired (Gumbiner et al., 1988; Lewis et al., 1994).

Classical cadherins form the transmembrane part of AJ. Their ectodomain consists of five extracellular cadherin domains, which are seven stranded β -barrel domains (Brasch et al., 2012). Linker regions, each of which can bind three Ca^{2+} ions, connect these domains. Calcium binding is required to stabilise the ectodomain in a conformation that allows homophilic *trans* interaction with cadherin molecules on opposing membranes (Nagar et al., 1996). A process called strand swapping, where the N-terminal part of the β -A strand is swapped between the respective first extracellular domains of two opposing cadherins, mediates homophilic interaction (Boggon et al., 2002; Parisini et al., 2007; Patel et al., 2006). In addition to *trans* interactions, neighbouring cadherins on the same cell interact by *cis* interactions, which are mediated by a lateral interface between the extracellular domain 1 of one cadherin molecule and the extracellular domain 2 of the adjacent cadherin (Boggon et al., 2002; Harrison et al., 2011).

Different types of classical cadherins mediate AJ in distinct cell types (Geiger and Ayalon, 1992; Halbleib and Nelson, 2006). E-cadherin is the main component in epithelial cells, while N-cadherin is found in muscle and neural tissue. Skeletal muscle also expresses M-cadherin (Donalies et al., 1991), while some epithelial cells additionally possess P-cadherin. VE-cadherin is mediating AJ in endothelial cells and R-cadherin is found in forebrain and bone. Since cadherins form homophilic

interactions, this tissue specific expression of cadherin molecules facilitates cell sorting during development (Halbleib and Nelson, 2006).

Cadherins have a conserved cytoplasmic tail (Geiger and Ayalon, 1992) that contains binding sites for members of the catenin family, which facilitate tethering of AJ to the actin cytoskeleton and regulate junction stability and turnover (Figure 1.5 A). The juxtamembrane portion of the cadherin cytoplasmic tail is the binding site for p120 catenin (p120^{CTN}) and the C-terminus is for β -catenin (Oda, 2012).

P120^{CTN} is a member of the Armadillo family of proteins. Armadillo proteins are characterised by repeats of Armadillo domains in the centre of the proteins, nine in the case of p120^{CTN}, which are flanked by distinct N- and C-termini (McCrea and Gu, 2010). These Armadillo repeats are arranged in α -helices and together form a superhelical structure, which acts as protein-binding scaffold. P120^{CTN} is believed to aid cadherin clustering and to regulate cellular cadherin levels by stabilising cadherin at AJ (Pieters et al., 2012; Xiao et al., 2007; Yap et al., 1998). It does this by masking internalisation motifs at the cadherin tail and loss of p120^{CTN} leads to cadherin internalisation and lysosomal degradation (Miyashita and Ozawa, 2007; Nanes et al., 2012). In addition, p120^{CTN} stabilises cadherin by promoting actin remodelling through Rho GTPase regulation (Anastasiadis, 2007).

Beta-catenin is also an Armadillo family protein and contains 12 Armadillo repeats and a conserved helix (Helix-C) between the last repeat and the C-terminal domain. Beta-catenin is a core component of AJ but also regulates transcription in response to Wingless-integrated (Wnt) signalling (Valenta et al., 2012). Wnt signalling is critical during embryonic development, but is also required in adult life to retain tissue homeostasis and regulate cell renewal (Angers and Moon, 2009). Due to its importance for development, β -catenin knock-out is embryonic lethal (Haegel et al., 1995).

In response to Wnt signalling, β -catenin translocates from the cytoplasmic pool to the nucleus, where it binds to the transcription factors T-cell factor (TCF)/Lymphoid enhancer-binding factor (Lef) and promotes transcription. The β -catenin helix-C binds transcriptional co-activators, while the Armadillo repeats superhelix is the interaction site with TCF/Lef. Canonical Wnt signalling is switched off by degradation of β -catenin when bound to a destruction complex containing the scaffold proteins axin and adenomatous polyposis coli (APC) and the kinases glycogen synthase kinase 3 β

(GSK-3 β), casein kinase 1 (CK1) and protein phosphatase 2A (PP2A) (Valenta et al., 2012). The superhelix is not only the interaction site with TCF/Lef but also with APC and with cadherin. The binding of these three proteins is mutually exclusive and competition for β -catenin binding is important for canonical Wnt signalling regulation (Valenta et al., 2012).

Association between β -catenin and cadherin occurs immediately after synthesis and the complex is transported together to AJ (Hinck et al., 1994). The interaction prevents degradation of either protein. E-cadherin blocks the β -catenin superhelix and thereby prevents β -catenin binding to the destruction complex, while β -catenin masks an ubiquitin ligase recognition motif on the E-cadherin tail (Huber and Weis, 2001). At AJ, β -catenin binds α -catenin with a part of its N-terminus and first Armadillo repeat (Pokutta and Weis, 2000).

Alternatively to β -catenin, plakoglobin, another member of the Armadillo family, can be bound at the C-terminal portion of the cadherin tail (Ozawa et al., 1989; Ozawa et al., 1990). Binding to β -catenin or plakoglobin is mutually exclusive but both types of complexes exist in the same cell (Butz and Kemler, 1994; Näthke et al., 1994). In conditional knock-out models plakoglobin can compensate for β -catenin at AJ but not in Wnt signalling (Huelsen et al., 2000). As β -catenin, plakoglobin interacts with α -catenin at AJ (Aberle et al., 1994; Hülsken et al., 1994).

Alpha-catenin is crucial for AJ stability as it links AJ to the actin cytoskeleton (Rimm et al., 1995) and ablation of α -catenin leads to loss of AJ and epithelial polarity (Vasioukhin et al., 2001a). Alpha-catenin is homologous to the actin-binding protein vinculin (Herrenknecht et al., 1991; Nagafuchi et al., 1991) and contains three vinculin homology (VH) domains (Figure 1.6). The VH1 domain mediates dimerization and β -catenin/plakoglobin binding, while the VH2 domain contains a binding site for the actin nucleating protein formin-1. The M-domain comprises parts of the VH2 domain and is required to mediate intercellular adhesion. It is thought that this domain might aid cadherin clustering. Alpha-catenin binds actin through the VH3 domain (Kobiela and Fuchs, 2004).

Originally α -catenin was believed to be a direct linker between β -catenin and F-actin, but it has now been shown that binding to β -catenin and F-actin appears to be mutually exclusive (Yamada et al., 2005). Instead, it is likely that α -catenin mediates the link by recruiting an actin-binding protein(s). Many candidates for such α -catenin-actin bridging

proteins have been suggested upon others formin-1 and EPLIN (reviewed in (Yonemura, 2011)). Formin-1 nucleates unbranched actin filaments. Chimera containing the β -catenin binding site of α -catenin and the actin polymerization domains of formin-1 were able to rescue AJ formation in α -catenin null keratinocytes (Kobielak et al., 2004). EPLIN, on the other hand, is an inhibitor of actin depolymerization and similar to formin-1, chimera of full-length EPLIN and E-cadherin mutants deleted of the β -catenin binding region could induce actin-bound AJ in cadherin-deficient L cells (Abe and Takeichi, 2008). Another example is Ajuba, an actin-binding and bundling protein, which localises to AJ through α -catenin binding. Similar to β -catenin, Ajuba is not only found at AJ but also in the nucleus where it regulates differentiation and proliferation (Kanungo et al., 2000; Marie et al., 2003; Nola et al., 2011). In addition, α -catenin has a masked vinculin-binding site that becomes unmasked as actin pulls on α -catenin during cellular tension development. Vinculin is an actin binding protein and in turns recruits more actin filaments to strengthen the AJ (Thomas et al., 2013; Yonemura et al., 2010). Precise mechanisms of actin binding to AJ need yet to be uncovered, but it has become clear in recent years that the process is more complex than previously imagined.

In addition to AJ bound α -catenin, there also exists a large cytoplasmic pool of α -catenin and actin regulatory functions independently of AJ have been assigned to α -catenin (Benjamin et al., 2010; Gottardi and Gumbiner, 2004; Schneider et al., 1993). While α -catenin exists as a heterodimer with β -catenin at AJ, in the cytosol α -catenin can exist as monomer or homodimer. A recent study exploited homodimer formation to specifically deplete the cytoplasmic pool by overexpressing an α -catenin construct with a mitochondrial targeting sequence. Cells deprived of cytosolic α -catenin exhibited increased actin dynamics as evident by increased cell migration, membrane dynamics and broader distribution of Arp2/3 at the leading edge (Benjamin et al., 2010).

Alpha-catenin also functions in transcriptional regulation. It is a negative regulator of Wnt signalling by stabilising β -catenin binding to the destruction complex and thus promoting β -catenin degradation (Choi et al., 2013). However, α -catenin can also enter the nucleus where it binds to β -catenin/TCF transcriptional complexes and attenuates their transcriptional activity (Choi et al., 2013; Daugherty et al., 2014; Giannini et al., 2000). In addition, α -catenin mediates contact inhibition of growth by regulating the proliferation-controlling Hippo pathway. The transcriptional co-activator of the Hippo signalling pathway Yes-associated protein 1 (YAP1) is found in the nucleus in non-confluent cells but shifts to the cytoplasm and AJ upon cell-cell contact formation and

this change in localisation is dependent on α -catenin (Schlegelmilch et al., 2011; Silvis et al., 2011). Alpha-catenin can bind phosphorylated YAP1 via the scaffolding protein 14-3-3 (Schlegelmilch et al., 2011) and recruits YAP1 to AJ in confluent cells. In the absence of contacts, YAP1 is free and can be dephosphorylated by PP2A, which allows entry to the nucleus (Schlegelmilch et al., 2011). To date it is unclear how α -catenin transduces the signal from AJ to the nucleus to cause YAP1 translocation.

1.6.3 Desmosomes

Desmosomes are the third and most basal cell-cell adhesive complex that is only found in tissues that require strong mechanical strength such as skin epithelia and cardiac muscle. Desmosomes have a similar composition as AJ and contain members of the cadherin and Armadillo family, but IF are the cytoskeletal component underlying desmosomes (Figure 1.5 A).

There are two desmosomal cadherins, desmocollin (Dsc) and desmoglein (Dsg). Desmocollin has three subtypes (Dsc1-3) and desmoglein has four (Dsg1-4), all of which are expressed in a tissue-specific manner (Garrod and Chidgey, 2008). Dsc2 and Dsg2 are found in all tissues that possess desmosomes, while the other desmosomal cadherins are expressed in a differentiation-specific manner in stratified epithelial such as the epidermis. Different Dsc and Dsg subtypes are expressed within the same cell. The exact arrangement of Dsc and Dsg in desmosomes has not been finally concluded, but it appears that Dsc and Dsg can form both homo- and heterophilic dimers *in vitro* and in cells overexpressing desmosomal cadherins (Chitaev and Troyanovsky, 1997; Syed et al., 2002; Troyanovsky et al., 1999). However, recent evidence by crosslinking studies in epithelial cells suggests that *trans* interactions occur homophilic and subtype-specific *in vivo* (Nie et al., 2011). The mode of *trans* interaction is likely to involve β -strand swapping similarly to classical cadherins (Garrod and Chidgey, 2008).

The ectodomain of desmosomal cadherins consists of four extracellular cadherin domains and an extracellular anchor domain, while the cytoplasmic domain contains an intracellular anchor domain and an intracellular cadherin-like sequence domain. Desmogleins also have additional cytoplasmic domains, but their function is unknown (Garrod and Chidgey, 2008).

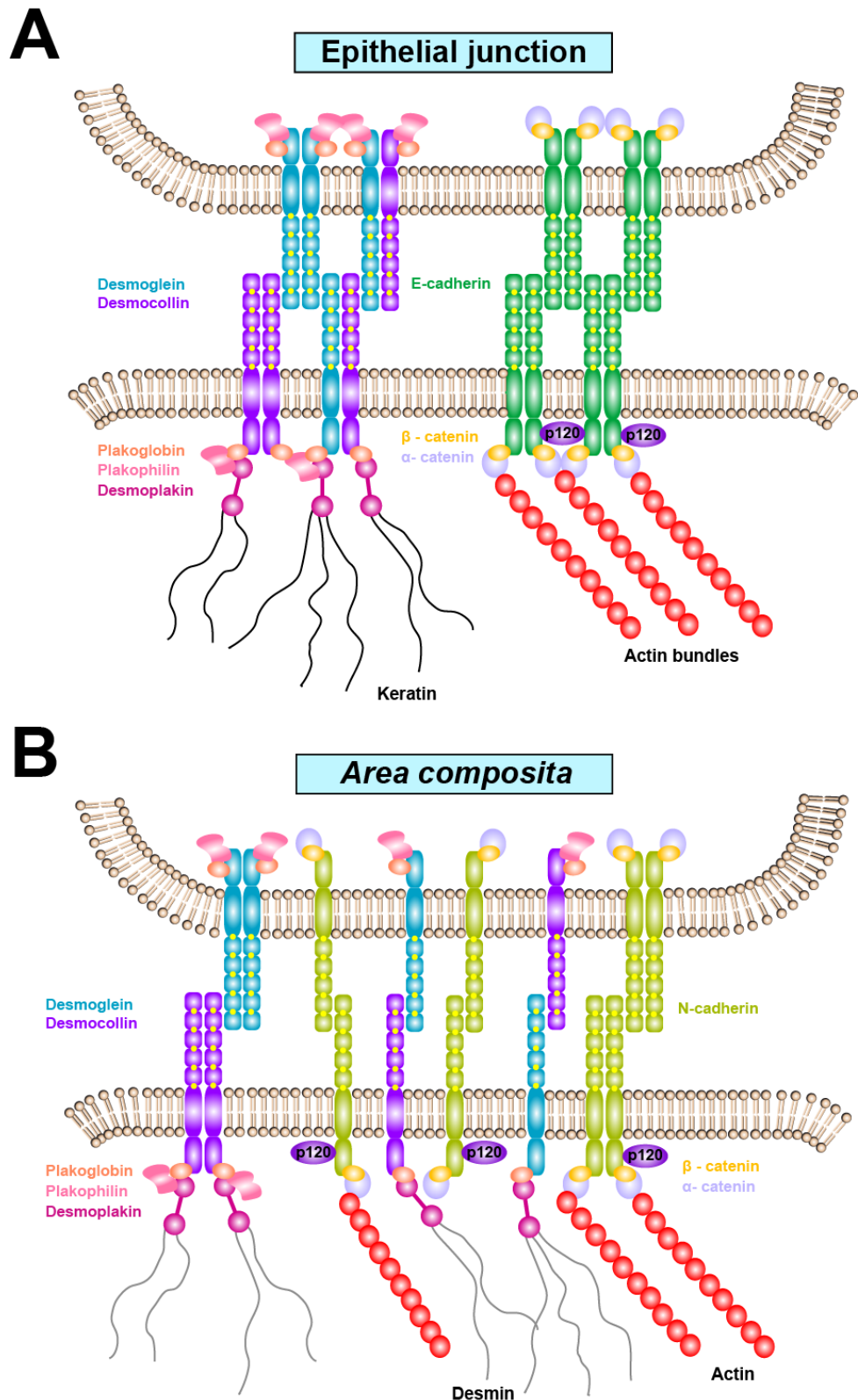


Figure 1.5: Composition of cell-cell contacts in epithelial cells and cardiomyocytes. Schematic representation of A) cell-cell contacts in basal epithelial cells or B) the *area composita* in cardiomyocytes. E-cadherin, N-cadherin, desmoglein and desmocollin span the membrane and mediate adhesion. At the E- or N-cadherin cytoplasmic tail are bound p120^{CTN} and β-catenin. Alpha-catenin binds to β-catenin and links AJ to F-actin. Desmosomal cadherins are bound to plakoglobin and plakophilin, which connect to desmoplakin. Desmoplakin links desmosomes to the A) keratin or B) desmin cytoskeleton. Adherens junctions (AJ) and desmosomes are A) spatially separated or B) exist side-by-side in the same area.

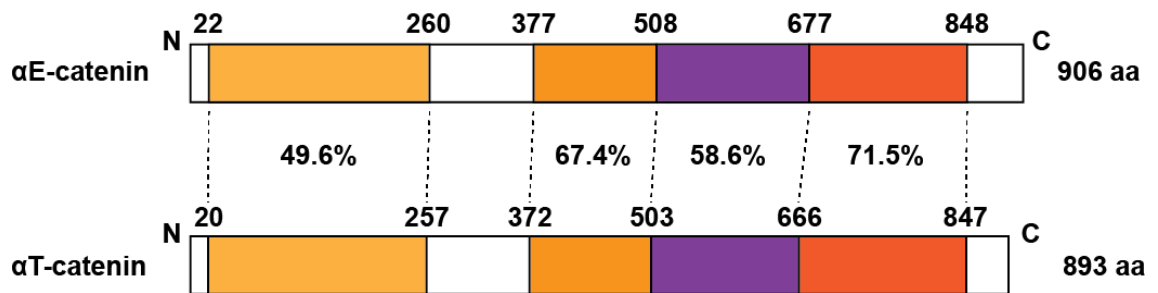


Figure 1.6: Sequence comparison between αE-catenin and αT-catenin. Schematic protein alignment between αE- and αT-catenin. The light orange box represents the vinculin homology (VH) domain 1 and is the domain that mediates β-catenin/plakoglobin binding and dimerization. The darker orange box labels the largest part of the VH2 domain which comprises binding sites for formin-1 and a part of the binding site for vinculin. The purple box corresponds to the M-domain required for mediating intercellular adhesion. The red box corresponds to the VH3 domain which mediates F-actin binding and contains the second part of the vinculin binding site. Numbers above the boxes represent residues flanking the domains. Total protein length in amino acids (aa) is given on the right. Numbers in the centre of the alignment show amino acid identity (%) between the corresponding domains. The figure is based on (Janssens et al., 2001).

Plakoglobin and plakophilin (PKP), members of the Armadillo family that contain twelve or nine Armadillo repeats, respectively, bind to the intracellular cadherin-like sequence domain (McCrea and Gu, 2010). Plakoglobin also binds to classical cadherins at AJ, but its binding affinity is greater for desmosomal cadherins and thus a larger proportion is found at desmosomes (Chitaev et al., 1996). The binding of plakoglobin to AJ is required to initiate desmosome formation and plakoglobin also seems to regulate desmosome size (Lewis et al., 1997; Palka and Green, 1997). Plakoglobin provides a binding site to recruit desmoplakin to desmosomes, but it does not recruit α -catenin to desmosomes because its desmosomal cadherin binding site largely overlaps with its α -catenin binding site (Chitaev et al., 1998; Kowalczyk et al., 1997; Witcher et al., 1996). There are three types of plakophilin (PKP1-3) that are expressed in a tissue- and differentiation-specific manner (Hatzfeld, 2007). Plakophilins also bind desmoplakin and additionally plakoglobin and IF such as cytokeratins and vimentin and are required for proper clustering of the desmosomal plaque (Bornslaeger et al., 2001; Hatzfeld, 2007).

Desmoplakin is essential for desmosome formation and IF tethering. Its N-terminal domain is required to bind to plakoglobin and plakophilin, the central domain allows oligomerization and the C-terminus links to IF (Garrod and Chidgey, 2008). Linking desmosomal complexes to IF is crucial for desmosomal integrity (Huen et al., 2002). The extent of IF binding by desmoplakin is likely regulated by desmoplakin phosphorylation and thereby allows to control adhesive strength of desmosomes (Hobbs and Green, 2012).

Desmosomes possess two adhesive states, a weaker state and a strongly adhesive state referred to as hyperadhesive. The hyperadhesive state is the normal status of desmosomes in adult tissue and in this state they are independent of calcium, i.e. they do not disassemble when calcium is removed (Garrod et al., 2005; Matthey and Garrod, 1986; Watt et al., 1984). It is thought that the hyperadhesive state is achieved by highly regular arrangement of desmosomal cadherins by *cis* interactions and possibly conformational changes of the extracellular domain that traps already bound calcium ions thereby making them resistant to ion chelation (Garrod and Chidgey, 2008; Garrod et al., 2005). Desmosomal plaque proteins mediate the spatial organisation of desmosomal cadherins. They engage with neighbouring plaque proteins to form highly structured spatial arrangements and thus align the desmosomal cadherins to which they are bound (Garrod and Chidgey, 2008). Desmosomes change to the weaker adhesive state under conditions that require tissue remodelling and cell movement

such as wound repair (Wallis et al., 2000). Phosphorylation events are required for this status change and likely modify plaque proteins to interfere with their ordered arrangement and their interaction with IF (Garrod and Chidgey, 2008; Hobbs and Green, 2012).

1.6.4 Composite junctions and area composita

In different cell types, composite junctions have been observed where AJ complexes contain desmosomal plaque proteins and are thus able to bind intermediate filaments. In endothelial cells these junctions have been termed *complexus adhaerens* and have been observed in human vascular and lymphatic endothelial cells (Hämmerling et al., 2006; Kowalczyk et al., 1998; Valiron et al., 1996). Experiments testing the assembly of these junctions in VE-cadherin-overexpressing L-cell fibroblasts and COS cells show that VE-cadherin binds plakoglobin which recruits desmoplakin and links VE-cadherin to vimentin (Kowalczyk et al., 1998). *Complexus adhaerens* are crucial for microvasculature integrity and it is therefore thought that the interaction with IF is necessary to provide further junctional stability by complementing the cadherin-F-actin link (Gallicano et al., 2001). Similar composite junctions are found in chicken lens, where N-cadherin is bound to vimentin (Leonard et al., 2008). In addition, a recent study in *Xenopus* mesendoderm shows C-cadherin connectivity to keratin and demonstrates the importance of this complex for mechanosensing and collective cell migration in *Xenopus* development (Weber et al., 2012).

Transverse cell-cell contacts in cardiomyocytes are called intercalated discs and contain AJ complexes and desmosomal protein complexes side by side, as compared to the clear spatial separation of both complexes in epithelial cells. These mixed adhesive plaques are therefore referred to as *area composita* (Figure 1.5 B) (Borrmann et al., 2006; Franke et al., 2006). In addition, intercalated discs contain patches of gap junctions and areas without plaques. Cardiomyocyte desmosomes contain desmoglein 2 and desmocollin 2 as transmembrane proteins, which are linked to the intermediate filament desmin via plakoglobin, desmoplakin and plakophilin 2 (Saffitz, 2011). AJ complexes in cardiomyocytes consist of N-cadherin, which is bound either to β -catenin or plakoglobin (Hertig et al., 1996a). In cultured adult rat cardiomyocytes, the complex containing β -catenin is predominant in freshly isolated cells while plakoglobin-containing AJ prevail after several days in culture (Hertig et al., 1996a). Bound to β -catenin or plakoglobin is α E-catenin, which links AJ to actin and in cardiomyocytes mediates the attachment of terminal sarcomeres to the intercalated disc (Luo and Radice, 2003).

Interestingly, an α E-catenin homologue, α T-catenin, exists in cardiomyocytes side-by-side with α E-catenin (Janssens et al., 2001). This protein has an overall identity with α E-catenin of 58% (Figure 1.6) and is mainly expressed in heart and testis and at low levels in brain, kidney, liver and skeletal muscle (Janssens et al., 2001). In fact, its expression level in heart is higher than that of α E-catenin. AlphaT-catenin localises to intercalated discs and can bind both the AJ component β -catenin and the desmosome component plakophilin-2 (Goossens et al., 2007; Janssens et al., 2001). It is thought that α T-catenin can link AJ via plakophilin 2 to desmin and that this interaction reinforces and strengthens junctions (Goossens et al., 2007). It might also be required to form the *area composita* by drawing AJ and desmosome components together and bridging them.

1.6.5 Gap junctions

Gap junctions are found in practically all cells in solid tissue and mediate electrical, chemical and metabolic coupling of cells by allowing diffusion of ions and small molecules (Goodenough and Paul, 2009). They are formed by tetraspan integral membrane proteins of the connexin family. Six connexins form a half channel in the membrane of one cell, which is combined head-to-head with a half-channel of the adjacent cell to assemble a full gap junction. These channels do not stay single, but cluster with other gap junctions into plaques. The same cell can express different connexins and a half channel therefore can be homomeric or heteromeric. Based on their composition, the physiological properties of gap junctions vary including the permeability for ions and small molecules. Gap junctions can switch between open and closed state to regulate intercellular diffusion.

1.7 Formation of AJ in epithelial cells

1.7.1 Initiation of AJ formation

The calcium-dependence of classical cadherins has enabled researchers to study assembly and disassembly of AJ. Cells grown in low calcium medium do not exhibit AJ, but AJ formation can be induced by addition of calcium to the medium (O'Keefe et al., 1987). Conversely, established AJ can be broken down by chelating calcium with EDTA or replacing standard medium with low calcium medium (Kartenbeck et al., 1991; Kartenbeck et al., 1982).

In subconfluent epithelial cells, E-cadherin is mostly located in cytoplasmic vesicles and the Golgi apparatus, while some is found on the cell surface (Le et al., 1999). Cadherin at the cell surface is involved in the earliest steps of AJ formation. Epithelial cells constantly form cell protrusions to probe for adjacent cells (Adams et al., 1998; McNeill et al., 1993; Vaezi et al., 2002; Vasioukhin et al., 2000). If contact is made, cadherin molecules, that reach the contact site by diffusion, cluster through *cis*-interactions and engage with cadherins at adjacent cells to form discrete puncta (Adams et al., 1998; Adams et al., 1996; Hong et al., 2010; Vaezi et al., 2002; Vasioukhin et al., 2000). These early contacts then activate a cascade of events, which are regulated in part by Rho small GTPases.

The Rho small GTPase Rac1 is recruited to E-cadherin and activated after initial contact formation (Nakagawa et al., 2001; Noren et al., 2001). Different signalling pathways have been indicated in Rac1 activation at cadherin-catenin complexes. In MDCKII cells, treatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin attenuates Rac1 activation downstream of E-cadherin engagement, but had no effect on Rac1 recruitment to contact sites (Nakagawa et al., 2001). However, in primary keratinocytes epidermal growth factor receptor (EGFR) signalling but not PI3K is involved in Rac1 activation (Betson et al., 2002).

Active Rac1 mediates actin remodelling. It recruits the actin regulator WASP family verprolin-homologous protein 2 (WAVE2) to initial contacts, which is required to activate the actin polymerization protein Arp2/3 (Figure 1.7) (Yamazaki et al., 2007). In addition, the Arp2/3-stabilising protein cortactin is recruited to cell-cell contact sites where it acts as a scaffold for actin polymerization proteins (Han et al., 2014; Helwani et al., 2004). Actin polymerization by Arp2/3 is required to assemble actin underneath cadherin puncta to stabilise E-cadherin in the membrane (Verma et al., 2004;

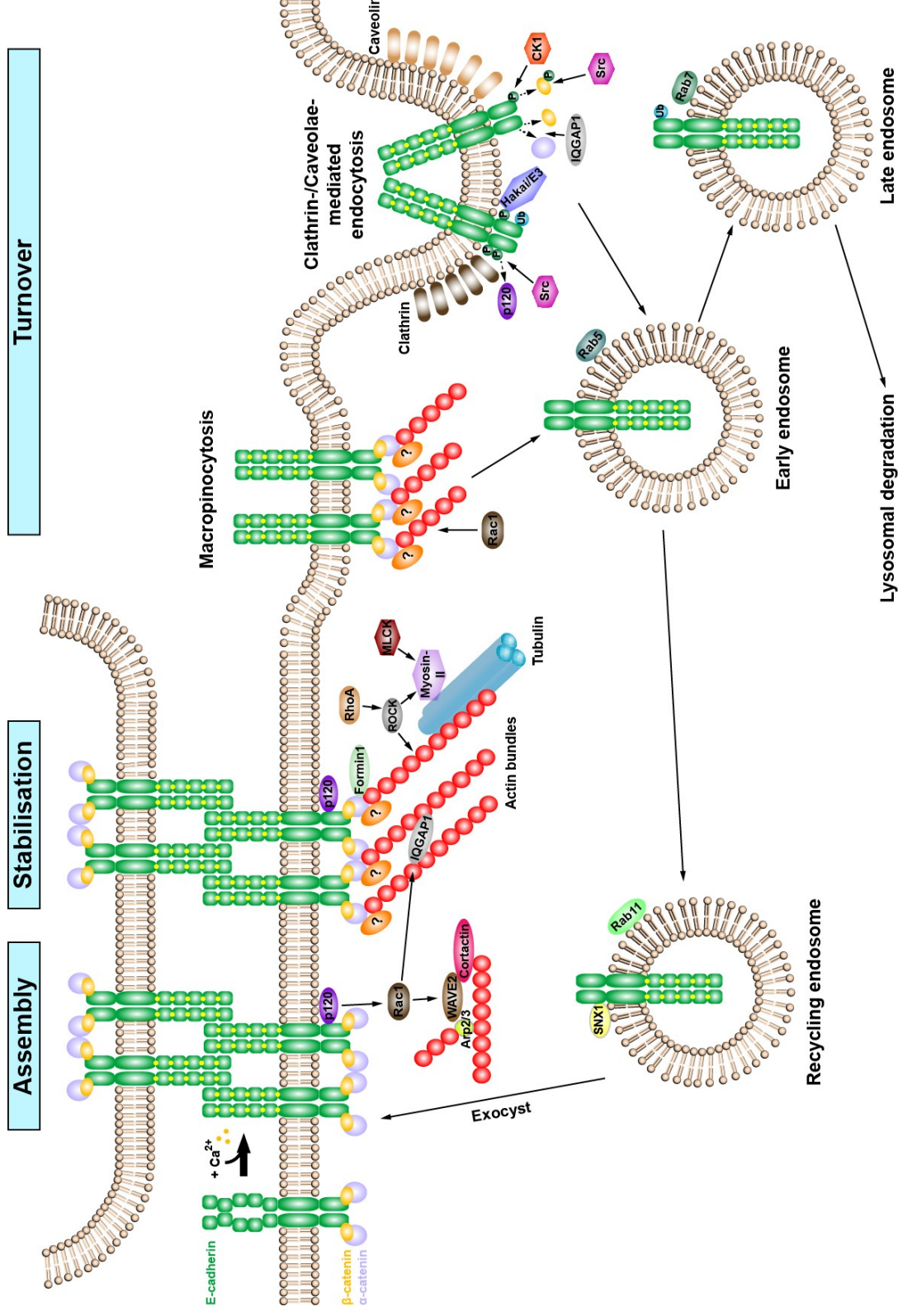
Yamazaki et al., 2007). The actin population underlying cadherin is called junctional actin (Zhang et al., 2005). In addition, actin remodelling enables cell-cell contact expansion outwards from the initial site of contact (Verma et al., 2004; Yamada and Nelson, 2007; Yamazaki et al., 2007).

Contact expansion also requires E-cadherin transport. It is thought that E-cadherin is delivered to the *zonula adherens* both by lateral movement as well as vesicle-based transport (Cavey et al., 2008; de Beco et al., 2012). Vesicular transport requires endocytosis of E-cadherin at distant membrane domains and recycling of the receptor to the site of cell-cell contact formation (de Beco et al., 2012). Microtubules (MT) are essential for AJ formation and might be required for the vesicular transport of AJ components to developing junctions (Ligon and Holzbaur, 2007). However, another study demonstrated that E-cadherin accumulation at the cell surface in response to calcium is not perturbed by MT disruption (Stehbens et al., 2006). Instead, MT disruption causes the loss of myosin II from mature junctions. Lateral diffusion is slowed down soon after cell-cell contact initiation to retain E-cadherin clusters at the contact site. This process is dependent on the actin network forming beneath AJ and requires myosin-II-dependent contractility (Cavey et al., 2008; Hong et al., 2010; Kusumi et al., 1993; Sako et al., 1998). Thus, myosin II is recruited to nascent AJ and is needed to concentrate E-cadherin at junctions by limiting diffusion. It is likely that MT are required for myosin II localisation to AJ, which in turn promotes E-cadherin concentration at junctions (Cavey et al., 2008; Shewan et al., 2005; Stehbens et al., 2006).

1.7.2 Maturation

Junction maturation is characterized by the formation of actin bundles parallel to AJ and requires the recruitment and activation of actin regulators that promote the polymerization of linear filaments such as formin-1 (Kobielak et al., 2004; Vasioukhin et al., 2000; Zhang et al., 2005). Cortical actin bundles then compact towards the membrane to stabilise adhesion complexes and increase lateral cell height (Zhang et al., 2005). Actin bundle compaction is dependent on active myosin II (Zhang et al., 2005) and myosin II-dependent actin contractility is also required to fully expand cell-cell contacts (Yamada and Nelson, 2007). Recruitment and activation of myosin II is dependent on myosin light chain kinase (MLCK) and Rho kinase (ROCK) (Yamada and Nelson, 2007). ROCK is an effector of the GTPase RhoA, which becomes activated during AJ maturation at the edges of the expanding cell-cell contacts (Matsui et al., 1996; Yamada and Nelson, 2007).

Figure 1.7: Signalling pathways involved in AJ formation, stabilisation and turnover. Initiation of AJ formation activates Rac1, which recruits WAVE2 to initiate Arp2/3-dependent actin polymerization. The actin-binding protein cortactin is also recruited to nascent AJ and aids actin polymerization. During junction maturation formin 1 binds to α -catenin and promotes polymerisation of unbranched actin, which can be bundled by Rac1-recruited IQGAP1. RhoA is activated and mediates myosin-II recruitment through ROCK. MLCK also aids myosin-II recruitment and myosin-II transport to AJ is dependent on microtubules. Myosin-II then mediates actin contraction to stabilise E-cadherin at contact sites. E-cadherin can be removed from contacts by Rac-1-initiated macropinocytosis or by clathrin- or caveolin-mediated endocytosis. Casein kinase 1 (CK1) or Src kinase destabilise E-cadherin- β -catenin interaction by phosphorylation of the E-cadherin tail or β -catenin, respectively. IQGAP causes dissociation of α -catenin from β -catenin and thereby destabilises E-cadherin in the membrane. Src kinase phosphorylates the E-cadherin juxamembrane domain and p120^{CTN} to displace p120^{CTN} which allows binding of the ligase Hakai and E-cadherin ubiquitination and degradation. E-cadherin not targeted for degradation is recycled back to the membrane in a SNX1- and exocyst-dependent manner.



1.7.3 Remodelling

Mature AJ are not static structures, but are constantly turned over (Le et al., 1999). In addition, AJ disassembly is necessary in many physiological processes such as tissue movement during development, in response to different growth factors and in endothelial cells to allow transendothelial migration of leukocytes (Bryant et al., 2007; Bryant et al., 2005; Gavard and Gutkind, 2006; Kamei et al., 1999; Lu et al., 2003; Ogata et al., 2007). Depending on the stimulus, different pathways of cadherin internalisation have been described including clathrin-dependent and caveolin-dependent endocytosis as well as macropinocytosis (Akhtar and Hotchin, 2001; Le et al., 1999; Paterson et al., 2003).

The interaction of cadherin with catenins stabilises AJ and prevents cadherin endocytosis (Davis et al., 2003; Dupre-Crochet et al., 2007; Kuroda et al., 1998; Xiao et al., 2005). Removal of catenins from AJ has been identified as an initial step of endocytosis in many studies. Phosphorylation of serine 846 in the cytoplasmic tail of E-cadherin by CK1 leads to destabilisation of the E-cadherin- β -catenin interaction and triggers E-cadherin endocytosis (Dupre-Crochet et al., 2007). Similarly, phosphorylation of N-cadherin by Src kinase causes displacement of β -catenin (Qi et al., 2006). At the same time, phosphorylation of β -catenin by Src or Abl kinase also destabilises the cadherin- β -catenin interaction (Rhee et al., 2002; Roura et al., 1999). Loss of p120^{CTN} is a common feature of many tumours (Thoreson and Reynolds, 2002) and causes the internalisation of cadherins by unmasking endocytotic motifs in the juxtamembrane domain of cadherin (Davis et al., 2003; Miyashita and Ozawa, 2007; Nanes et al., 2012). Disruption of the interaction between α - and β -catenin also destabilises AJ (Kuroda et al., 1998). Dissociation of the two catenins may require IQGAP, an effector of Rac1 and Cdc42 that competes with α -catenin for the binding site at β -catenin (Kuroda et al., 1996; Kuroda et al., 1998; McCallum et al., 1996). A proposed mechanism for junction stabilisation via active Rac1 and Cdc42 is sequestering of IQGAP, which prevents E-cadherin endocytosis and instead mediates actin bundling through the F-actin-crosslinking domain of IQGAP (Fukata et al., 1999; Izumi et al., 2004).

Growth factor-mediated receptor tyrosine kinase (RTK) activation can cause a variety of cellular responses including cell migration (Lemmon and Schlessinger, 2010). Migration is essential during tissue morphogenesis and wound healing but also tumour cell invasion. Epithelial cell migration requires remodelling or breakdown of cell-cell contacts (Normanno et al., 2006). Epidermal growth factor (EGF) stimulation in A431

carcinoma cells induces caveolin-dependent internalisation of E-cadherin (Lu et al., 2003). Instead, EGF treatment of SCC12f keratinocytes caused activation of Rac1 and the formation of large intracellular vesicles resembling macropinosomes where E-cadherin co-localises with EGFR, β -catenin and p120^{CTN} (Bryant et al., 2007). E-cadherin is internalised from a pool of cadherin not actively involved in AJ and is transported into late endosomes from where it is recycled back to the cell surface in a sorting nexin-1 (SNX1)-dependent manner (Bryant et al., 2007).

Internalisation and recycling of cadherin is useful for short-term remodelling of cell-cell contacts where total cellular cadherin levels remain constant (Kowalczyk and Nanes, 2012). However, for long-term remodelling e.g. during epithelial to mesenchymal transition (EMT), cadherin needs to be permanently removed from the membrane by targeting internalised E-cadherin for degradation (Janda et al., 2006; Palacios et al., 2005). Expression of the oncogenic kinase v-Src causes EMT by phosphorylation of cadherin at the cytoplasmic tail, which disrupts binding to p120^{CTN} and recruits the E-cadherin-specific E3 ubiquitin-ligase Hakai. Ubiquitination by Hakai induces internalisation of E-cadherin and without de-ubiquitination, E-cadherin is degraded in the lysosome (Behrens et al., 1993; Fujita et al., 2002; Palacios et al., 2005).

1.8 Desmosome formation

Our understanding of desmosome formation to date is very limited, since desmosomes are widely insoluble which makes studying them difficult. Early studies demonstrated that desmosomal components are soluble after synthesis, but then quickly become insoluble (Pasdar and Nelson, 1988a; Pasdar and Nelson, 1989). After synthesis in the endoplasmic reticulum, desmoglein 1 becomes core-glycosylated and is transported to the Golgi apparatus where it is complex glycosylated and subsequently turns insoluble (Pasdar and Nelson, 1989). From the Golgi complex, desmosomal components are transported to the membrane, but in the absence of cell-cell contacts they are not stabilised and soon degraded. However, if junctions are formed desmosomes are incorporated at junctions and their half-life is strongly extended (Pasdar and Nelson, 1988a; Pasdar and Nelson, 1988b; Pasdar and Nelson, 1989).

Dsg2 and Dsc2 are transported to the membrane in vesicles that travel along MT. Interestingly, Dsg2 and Dsc2 are not co-transported but exist in distinct vesicles whose interaction with MT is mediated by kinesin-1 or kinesin-2, respectively. Transport of Dsg2 also requires plakophilin 2 (Nekrasova et al., 2011). An exocyst complex

containing Sec3 is likely involved in docking of the vesicles to the membrane at sites of developing desmosomes (Andersen and Yeaman, 2010).

Minutes after cell-cell contact initiation, first plakophilin 2 and then desmoplakin starts appearing in puncta at the contact site (Godsel et al., 2010; Godsel et al., 2005). Subsequently, desmoplakin and plakophilin 2-containing particles form in the cytoplasm (Godsel et al., 2005). Plakophilin 2 recruits protein kinase C alpha (PKC α) to the cytoplasmic complexes, which phosphorylates desmoplakin to regulate its interaction with keratins (Bass-Zubek et al., 2008). In addition, the membrane-bound pool of plakophilin 2 is required to locate RhoA to forming junctions, which is essential for cortical actin remodelling (Godsel et al., 2010). The desmoplakin/plakophilin 2 complexes then move from the cytoplasm to nascent cell-cell contacts and their transport is dependent on RhoA-remodelled F-actin and PKC α -regulated desmoplakin-keratin interaction but does not require MT (Bass-Zubek et al., 2008; Godsel et al., 2005).

As junctions mature, desmosomes become very stable and immobile and gain a hyperadhesive state that is believed to depend on a highly regular spatial organisation of cadherins and plaque proteins (Garrod and Chidgey, 2008; Windoffer et al., 2002). Despite that, mature desmosomes remain somewhat dynamic as has been demonstrated by time-lapse fluorescence microscopy and fluorescence recovery after photobleaching (FRAP). It is thought that in addition to junctional desmosomal cadherins a second pool exists in the membrane that is not assembled in desmosomes and that both pools are in constant exchange (Windoffer et al., 2002).

Advances have also been made in the identification of signalling events that regulate desmosome assembly, adhesiveness and turnover. Several phosphorylation events have been shown to govern desmosome status (reviewed in (Nekrasova and Green, 2013)). In addition, different reports show that EGFR signalling negatively regulates desmosome adhesion. EGFR activation induces Dsg2 internalisation, protease-dependent cleavage of Dsg2 extracellular domain and phosphorylation of plakoglobin to destabilise its association with desmoplakin (Gaudry et al., 2001; Klessner et al., 2009; Miravet et al., 2003; Yin et al., 2005).

1.9 Interdependence of AJ and desmosomes

AJ and desmosome formation are interdependent. As mentioned above, AJ formation is required for the initiation of desmosome assembly and requires plakoglobin binding to nascent AJ (Gumbiner et al., 1988; Lewis et al., 1994; Lewis et al., 1997). In epidermis, P-cadherin can compensate effects of E-cadherin depletion on cell-cell contact formation and tissue integrity. It was therefore suggested that desmosome formation is sensitive to cadherin levels but not cadherin subtype (Michels et al., 2009; Tinkle et al., 2008). In addition AJ-mediated actin remodelling is necessary for desmoplakin transport to desmosomes (Godsel et al., 2005; Green et al., 1987).

Conversely, AJ maturation is dependent on desmoplakin. Epithelial cells from epidermis-specific desmoplakin knock-out mice fail to reorganise the actin cytoskeleton to support mature AJ (Vasioukhin et al., 2001b). What is more, disruption of keratin IF in cultured cells causes mistargeting of AJ components (Hanada et al., 2005). Additional support that AJ and desmosomes are interdependent comes from in the autoimmune skin blistering diseases pemphigus vulgaris (PV) and pemphigus foliaceus (PF), which are caused by autoantibodies targeting desmosomal cadherins. These autoantibodies not only disrupt desmosomes but also affect AJ and actin organisation by inhibiting RhoA activity (Waschke et al., 2006).

1.10 Mechanosensing

Since cells have to withstand varying mechanical stress, cells needed to develop mechanisms to enable them to adapt to changes in mechanical requirement. Detection and adaptation to mechanical stimuli is called mechanosensing. Recent advances in our understanding of mechanosensing have also highlighted the importance of physical forces during development, where they regulate cell proliferation, polarity and differentiation (Huvneers and de Rooij, 2013; Ingber, 2003; Ingber, 2008).

AJ sense mechanical forces and grow in response to tugging forces or increased actomyosin contraction in order to strengthen cell-cell contacts (le Duc et al., 2010; Liu et al., 2010b; Smutny et al., 2010). Alpha-catenin is a key component of the tension-sensing machinery at AJ (Leckband et al., 2011). It has a masked vinculin-binding site that becomes unmasked as force pulls and stretches α -catenin. Bound vinculin in turn recruits more actin filaments to strengthen the AJ (Huvneers and de Rooij, 2013; Thomas et al., 2013; Yonemura et al., 2010). The actin-bundling protein EPLIN is also bound to α -catenin in an actomyosin contraction-dependent fashion (Taguchi et al.,

2011). Additional, α -catenin independent mechanisms of mechanosensing at AJ probably also exist but have not yet been uncovered (Huveneers and de Rooij, 2013).

The mechanical properties of IF make them ideal mechanosensors and mechanosensing abilities have been demonstrated for IF in different cellular settings (Bordeleau et al., 2012; Gregor et al., 2014; Russell et al., 2004). Since they are involved in cell signalling as scaffold for many different proteins (Kim and Coulombe, 2007; Windoffer et al., 2011), stretch or compression might cause conformational changes, which can alter protein binding and thereby cell responses (Ingber, 2008).

Since desmosomes are mainly found in tissues that are under mechanical stress, desmosomes are good candidates for additional mechanosensors. Especially the unique link between desmosomes and the nuclear envelope provided by IF has the potential to translate mechanical stress detected at the cell periphery into changes of nuclear activity (Bloom et al., 1996; Simpson et al., 2011). Yet, so far research has not addressed the potential of desmosomes as mechanosensors.

1.11 Cadherin-based adhesion and disease

1.11.1 Modulation of cell-cell contacts in cancer

Most tumours are derived from epithelial cells and a hallmark of cancer progression is the loss of the epithelial phenotype in favour of mesenchymal characteristics. This process is referred to as epithelial to mesenchymal transition (EMT) (Craene and Berx, 2013). EMT is linked to the loss of cell-cell contacts and gain of a migratory phenotype, which allows cancer cells to leave the primary tumour site and invade surrounding tissue or reach distant site by entering the blood or lymphatic system. Since cell-cell contacts are signalling hubs, their loss also results in significant changes in cell behaviour and function (Vasioukhin, 2012). Disassembly of AJ for example frees β -catenin, which, if it escapes degradation, can then upregulate Wnt signalling leading to increased tumour cell proliferation (Heuberger and Birchmeier, 2010).

Loss of cell-cell contacts can be caused by genetic mutations of their components that lead to protein deletions or truncations. Mutations can similarly alter the protein's interactions with other proteins or their ability to be modified (Berox and van Roy, 2009). Altered protein modification can also result from aberrant activity of modifying enzymes. Phosphorylation of E-cadherin juxamembrane domain by receptor tyrosine or Src kinases can increase E-cadherin ubiquitination and degradation (Fujita et al.,

2002). Alternatively, junctional components can be downregulated epigenetically or transcriptionally (Berx and van Roy, 2009). Several transcriptional inhibitors are known for E-cadherin and are upregulated by Wnt signalling (Valenta et al., 2012). However, cancer is not always linked to decreased levels of junctional proteins. Instead, changes in protein isoforms or switches to alternative subtypes have been observed (Table 1.1). E-cadherin is exchanged for N-cadherin in several cancer types, which allows interaction of tumour cells with N-cadherin-positive tissues such as stroma and endothelium and therefore promotes tumour dissemination (Wheelock et al., 2008).

1.11.2 Skin diseases and defects in cell-cell contacts

Defects in desmosomes are often linked to skin diseases. Desmosomes in skin disease have been reviewed in (Brooke et al., 2012). To name some examples, the autoimmune skin blistering diseases pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are caused by autoantibodies targeting the desmosomal cadherins Dsg3 or Dsg1, respectively (Amagai et al., 1991; Koulou et al., 1984). PV autoantibodies cause increased internalisation and degradation of Dsg3 and thereby compromise epithelial integrity and result in blister formation (Calkins et al., 2006). However, several changes in cell signalling caused by PV and PF autoantibodies have also been detected (Amagai and Stanley, 2012; Waschke et al., 2006). Another skin disease caused by desmosomal defects is skin fragility ectodermal dysplasia syndrome. The first patient described with this syndrome presented with thickened skin on palms and soles, abnormal nails and blistered skin and carried mutations in both plakophilin 1 alleles leading to premature stop codons (McGrath et al., 1997).

Not just defects in desmosomal core components lead to skin diseases, but mutations in keratin IF have also been identified as triggers. Keratin 14 or 5 mutations cause epidermolysis bullosa (EB), a disease characterised by the development of blisters in response to mechanical trauma due to rupture of the epidermis (Coulombe et al., 2009). Keratin mutations alter the mechanical properties of the keratin cytoskeleton with filaments being less elastic and less resistant to breakage resulting in mechanically softer epithelial cells (Gu and Coulombe, 2005; Ma et al., 2001). Some mutations also cause the collapse of keratin IF and cytoplasmic aggregates (Coulombe et al., 2009).

Table 1.1: Cancer-associated isoform and subtype switches in adhesive receptors and growth factor receptors described in the literature. All switches were observed in human patient samples.

Protein	Switch	Associated disease	Reference
Adhesion			
Cadherin	E- to N-cadherin	Prostate cancer, breast cancer, pancreatic cancer, oral squamous cell carcinoma	(Gravdal et al., 2007; Nagi et al., 2005; Nakajima et al., 2004; Pyo et al., 2007; Tomita et al., 2000)
	E- to T-cadherin	Hepatocellular carcinoma	(Riou et al., 2006)
	E- to P-cadherin	Prostate cancer, colon cancer	(Gravdal et al., 2007; Sun et al., 2011)
	E-cadherin to cadherin 11	Prostate cancer	(Tomita et al., 2000)
	E- and P- to N-cadherin	Oral squamous cell carcinoma, Merkel cell carcinomas	(Islam et al., 1996; Werling et al., 2011)
	E- to P-cadherin	Ovarian cancer	(Patel et al., 2003)
	N- and M- to R-cadherin	Rhabdomyosarcoma	(Charrasse et al., 2003)
Desmoglein	DSG3 to DSG2	Oral squamous cell carcinoma	(Teh et al., 2011)
Neural cell adhesion molecule (NCAM)	NCAM120kDa to NCAM140kDa	Multiple myeloma, acute myeloid leukemia, malignant T-NHL & NK/T cell lymphoma, small lung cell carcinoma, maligma melanoma, rhabdomyosarcoma	(Gattenlöhner et al., 2009)
p120 ^{CTN}	Isoform 3A to Isoform 1A	Clear cell renal cell carcinoma	(Yanagisawa et al., 2008)
Signalling			
Fibroblast growth factor receptor 2 (FGFR2)	IIIb to IIIc	Clear cell renal cell carcinoma	(Zhao et al., 2013)
Insulin receptor	IR-B to IR-A	Breat cancer, colon cancer, myotonic dystrophy type 1 and type 2	(Frasca et al., 1999; Savkur et al., 2001; Savkur et al., 2004; Sciacca et al., 1999)

1.11.3 Intercalated disc components and the cytoskeleton in cardiomyopathies

1.11.3.1 Cardiomyopathies

Cardiovascular disease is one of the leading causes of death in the world and especially in Europe (Nichols et al., 2014). The term summarizes all pathological conditions that impair the normal function of the heart such as dilated or hypertrophic cardiomyopathy, myocardial infarct and arrhythmogenic right ventricular cardiomyopathy (ARVC).

Hypertrophic cardiomyopathy (HCM) is characterized by increased left ventricle (LV) muscle mass without chamber dilation resulting in a reduced chamber volume, a phenotype which has been termed LV hypertrophy (Cecchi et al., 2012). Often, the LV wall thickness is not evenly increased, but hypertrophy is asymmetrically distributed affecting only parts of the wall most often involving the interventricular septum (Cecchi et al., 2012; Seidman and Seidman, 2001). In most patients, increased wall thickness leads to partial obstruction of the LV outflow tract (Cecchi et al., 2012). At the cellular level, myocardial hypertrophy is caused by increased myocyte size, which leads to distortion of the usually highly regular alignment of cardiomyocytes (myocyte disarray) (Seidman and Seidman, 2001). Because the lifetime of hypertrophic cardiomyocytes is reduced, HCM is also linked to increased interstitial fibrosis. Although, the contractile (systolic) function of hypertrophic hearts is usually unaffected, interstitial fibrosis augments cardiac stiffness, which negatively affects diastolic ventricular relaxation and thus impairs atrial emptying. The resulting atrial overload can lead to the development of atrial dilation, a feature often associated with HCM (Seidman and Seidman, 2001). Besides the strong phenotypical alterations of the hypertrophic heart, mortality rate and risk of sudden cardiac death of HCM patients are relatively low (Cecchi et al., 2012). Most patients have mild to moderate shortness of breath and chest pain (Seidman and Seidman, 2001). In about 50% of patient the disease progresses, but only about 5% of patients develop end-stage HCM (Cecchi et al., 2012).

Dilated cardiomyopathy (DCM) is defined by LV dilation and systolic dysfunction with ejection fractions below 50% (Cecchi et al., 2012). DCM patients usually have a poor prognosis because the disease is commonly diagnoses at later disease stages, when contractile function further deteriorates and the disease progresses to heart failure, atrial and ventricular arrhythmias, stroke and sudden cardiac death (Cecchi et al., 2012; Seidman and Seidman, 2001). Early detection of DCM is hindered by mild symptoms (easy fatigue, shortness of breath, palpitations) in early disease stages and the lack of clear anatomical or histological changes (Seidman and Seidman, 2001).

Cardiac mass is increased, but ventricular walls are only little hypertrophic, while atrial and ventricular chambers can be mildly or strongly enlarged (Seidman and Seidman, 2001). Myocytes are not usually hypertrophic or degenerated and their alignment is not disorganised (Seidman and Seidman, 2001). Fibrosis is also not commonly observed in DCM.

Restrictive cardiomyopathy (RCM) is a rare cardiomyopathy with a poor prognosis (Cecchi et al., 2012). It is characterized by extreme diastolic dysfunction due to wall stiffening. Wall thickness and systolic function are usually unaffected. The impaired relaxation hinders ventricular filling, which leads to increased atrial pressure and causes dilation of both atria (Hancock, 2001). The diastolic/systolic volumes can be normal, but are more often reduced (Cecchi et al., 2012). There are several triggers known for increased wall stiffness in RCM, but the most common is amyloidosis. Other causes include idiopathic myocardial fibrosis or diseases of the endocardium (Nihoyannopoulos and Dawson, 2009).

ARVC is an inherited heart muscle disease, where degenerating cardiomyocytes are progressively replaced by fibrofatty tissue which is accompanied by wall thinning (Gemayel et al., 2001). Arrhythmogenic cardiomyopathies usually affect the right ventricle (ARVC), but other forms have been observed where both or mainly the left ventricle are affected (Basso et al., 2012). The patches of fibrofatty tissue form reentry electrical circuits, which results in the generation of cardiac arrhythmias and can lead to sudden cardiac death (Gemayel et al., 2001). Beside ventricular arrhythmias, some patients present with atrial fibrillation (Cecchi et al., 2012). Patients with ARVC might develop right heart failure or occasionally biventricular heart failure due to the thinning of the right ventricle wall, RV dilation and the progressive loss of contractile function associated with cardiomyocyte degeneration (Gemayel et al., 2001).

1.11.3.2 Changes in intercalated discs and the cytoskeleton in cardiomyopathies

Early electron microscopic studies described ultrastructural changes of the intercalated disc and sarcomere arrangement in a variety of patients with different heart diseases as well as in animal models of exercise and cardiomyopathies. These studies observed increased intercellular spaces in the intercalated disc of patients with congestive cardiomyopathy (Ferrans et al., 1973) and patients with valvular heart disease and atrial septal defect (Burch and Sohal, 1969).

Dilations of the intercalated disc and retraction of sarcomeres from it have also been observed in hearts of rats that had undergone extensive swimming exercises or developed cardiac hypertrophy induced by treatment with the catecholamine isoproterenol (to mimic sustained adrenergic stimulation) (Burch and Sohal, 1969; Csapó et al., 1974). Sarcomeres detached from intercalated discs seemed to dissolve, while the undifferentiated disc areas expand and form sacs, which extend into neighbouring cells (Csapó et al., 1974). These sacs were later filled with filamentous material, which was interpreted as repolymerized myofilaments. Similar changes have been reported in Cocksackie Virus B4- or ethanol drinking-induced cardiomyopathies in monkeys or mice, respectively (Burch and Sohal, 1969).

Since then, the molecular composition of intercalated discs and cardiac cytoskeleton has been largely uncovered and specific disease-causing mutations identified in patients (Table 1.2). Specific cardiomyopathies are commonly linked to particular components of the cardiomyocytes. ARVC is mainly linked to defects in desmosomal proteins. Mutation of any desmosomal component leads to destabilisation of the transmembrane complex and loss of plakoglobin from intercalated discs (References in Table 1.2 and (Saffitz, 2011)). In fact, testing plakoglobin location at intercalated discs has been proposed as a method to diagnose ARVC (Asimaki et al., 2009). Loss of desmosomal integrity results in destabilisation of gap junctions observed as decreased connexin43 staining at intercalated discs, which then leads to conductive defects (Relevant references in Table 1.2 and (Saffitz, 2011)). Recent studies also identified ARVC-causing mutations in desmin and titin (Table 1.2). Considering that desmin is linked to desmosomal complexes and required for their stability, this is not surprising. The effect of titin mutations on ARVC has been attributed to decreased titin stability and increased degradation especially in terminal sarcomeres that are inserted into intercalated discs and experience the largest strain (Taylor et al., 2011).

Defects in AJ components would also be expected to be involved in manifestation of ARVC as AJ complexes in intercalated discs are also required for stability of gap junctions (Hertig et al., 1996b; Luo and Radice, 2003). So far, no mutations in AJ core components have been identified in heart disease patients. However, mutations in the AJ-desmosome bridging protein α T-catenin have recently been associated with ARVC (van Hengel et al., 2013). In addition, a mouse model with cardiac-specific N-cadherin deletion shows abnormal conduction and decreased levels of active and total connexin 43 levels, which leads to sudden death at about 2 months of age (Kostetskii et al., 2005; Li et al., 2005).

However, there is not always a strong correlation between perturbed structures in cardiomyocytes and disease. Depending on the amino acid and protein domain affected, mutations might strongly or weakly disrupt different cellular processes with impact on disease manifestation, severity and penetrance. Some mutations in desmosomal proteins have recently been associated with dilated cardiomyopathy (DCM), but DCM and especially hypertrophic cardiomyopathy (HCM) are more often caused by defects in sarcomeric proteins (Table 1.2). There is a strong association of HCM with mutations in sarcomeric proteins involved in force production such as cardiac actin and the myosin components. Hypertrophy is thus probably a compensatory mechanism to counteract sarcomere dysfunction (Fatkin and Graham, 2002).

DCM is caused by defects in a large number of proteins including sarcomeric and desmosomal proteins, ion channels and regulators of a variety of cellular processes (Hershberger et al., 2013). Mutations in titin are particularly associated with DCM, but the mechanism via which titin mutations promote DCM is unclear. A mouse model of DCM carrying titin with deletion of the cardiac specific N2B region suggests that titin is required to balance cardiac growth and regulate elasticity (Radke et al., 2007). Many variants of titin are also found in control populations without signs of heart disease. Therefore, it is likely that certain titin mutants predispose patients for DCM, but only lead to heart disease in combination with other mutations (Herman et al., 2012; Roncarati et al., 2013).

Desminopathies are marked by muscle weakness and heart disease (most commonly DCM), often in conjunction with conductive defects. Desminopathies are caused by mutations in desmin that usually interfere with filament formation (Table 1.2 and (Clemen et al., 2013)). The disease is characterised by desmin aggregates in muscle cells and most commonly affects cardiac, skeletal and smooth muscle simultaneously. Since desmin is involved in aligning myofibrils and linking them to the nucleus and to the extracellular matrix, it is thought that defects in force transmission play a role in the manifestation of desmin-related myopathies and cardiomyopathies (Fatkin and Graham, 2002). In addition, because desmin is a scaffold for many proteins, it is feasible that cell signalling and transcription are also affected by desmin mutations (Clemen et al., 2013).

Instead of mutations, changes in protein levels or localisation can be associated with cardiomyopathies. In HCM, N-cadherin- and desmoplakin-positive junctions appear at

longitudinal parts of the sarcolemma, and intercalated discs are irregular (Masuda et al., 2005). Another patient with HCM had increased levels of N-cadherin and β -catenin at enlarged and disorganised intercalated discs (Masuelli et al., 2003). In myocardial infarct, α E-catenin is lost from the infarcted site and downregulated in remote sides, without mutations in the α E-catenin gene being detectable (van den Borne et al., 2008). The loss of α E-catenin is associated with infarct rupture and the connection between α E-catenin level and rupture were confirmed in mouse models expressing heterozygously a α E-catenin loss-of-function protein. Total cellular levels of tubulin and desmin can be upregulated and cytoskeleton remodelled in end-stage DCM heart failure. These changes probably lead to increased stiffness of cardiomyocytes and promote heart failure (Heling et al., 2000).

However, often total levels of proteins do not necessary change. Instead, the function of proteins is regulated by changes in the expression ratios of protein isoforms or subtypes. Such isoform and subtype switches have frequently been observed in cardiomyopathies (Table 1.3). Different isoforms and subtypes can have distinct structural properties or may alter cell signalling by interacting with different proteins. Titin isoform switches between the N2B isoform, containing only the N2B extendible element, and N2AB, containing 2 extendible elements N2A and N2B, are observed in a variety of different heart diseases (Table 1.3) (Cazorla et al., 2000). The ratio between the two isoforms can significantly alter cardiomyocyte stiffness and contraction force.

Changes in cadherin subtypes have been observed in different types of cancer and correlate with changes in invasiveness and EMT (Table 1.1). No such changes have to date been detected in human cardiomyopathies. However, a switch from N- to E-cadherin has been identified in the heart of the spontaneously hypertensive stroke-prone rat (SHRSP) and the expressed E-cadherin is located at the intercalated disc (Craig et al., 2010). The increase in AJ complexes in the intercalated disc might lead to increased myocardial wall stiffness and left-ventricular diastolic pressure and thereby promotes the development of hypertrophic cardiomyopathy in this animal model. However, the switch might also alter Wnt signalling by sequestering β -catenin at intercalated discs away from the nucleus.

Table 1.2: Mutations in intercalated disc and cytoskeletal proteins associated with human cardiomyopathies

Protein	Human cardiomyopathy	Reference
Intercalated disc		
α T-catenin	ARVC	(van Hengel et al., 2013)
Plakoglobin	ARVC	(Asimaki et al., 2007; Bao et al., 2013; Christensen et al., 2010; den Haan et al., 2009)
	Naxos disease	(McKoy et al., 2000)
Desmoglein2	ARVC	(Bao et al., 2013; Bhuiyan et al., 2009; Christensen et al., 2010; den Haan et al., 2009; Gehmlich et al., 2010; Gehmlich et al., 2012; Lahtinen et al., 2011; Pilichou et al., 2006; Syrris et al., 2007; Yu et al., 2008)
	DCM	(Posch et al., 2008)
Desmocollin 2	ARVC	(Bao et al., 2013; Beffagna et al., 2007; Bhuiyan et al., 2009; Christensen et al., 2010; Gehmlich et al., 2011; Heuser et al., 2006; Simpson et al., 2009; Syrris et al., 2006b)
Desmoplakin	ARVC	(Alcalai et al., 2003; Bao et al., 2013; Bauce et al., 2005; Bauce et al., 2010; Christensen et al., 2010; den Haan et al., 2009; Lahtinen et al., 2011; Norman et al., 2005; Rampazzo et al., 2002; Sen-Chowdhry et al., 2007; Yang et al., 2006; Yu et al., 2008)
	DCM	(Elliott et al., 2010)
	Carvajal syndrome	(Norgett et al., 2000)
	Heart failure	(Uzumcu et al., 2006)
Plakophilin 2	ARVC	(Antoniades et al., 2006; Bao et al., 2013; Bauce et al., 2010; den Haan et al., 2009; Fidler et al., 2009; Gerull et al., 2004; Kannankeril et al., 2006; Lahtinen et al., 2008; Syrris et al., 2006a; Xu et al., 2010)
	DCM	(Elliott et al., 2010)

Cytoskeleton		
Cardiac actin	DCM	(Olson et al., 1998)
	HCM	(D'Amico et al., 2006; Kim et al., 2011b; Mogensen et al., 1999; Mogensen et al., 2004; Olson et al., 2000; Van Driest et al., 2003)
	RCM	(Kaski et al., 2008)
	Atrial septal defect	(Matsson et al., 2008)
Desmin	ARVC	(Brodehl et al., 2013; Klauke et al., 2010; Lorenzon et al., 2013)
	DCM	(Li et al., 1999; Taylor et al., 2007)
	Desmin myopathy	(Bär et al., 2007; Bergman et al., 2007; Dalakas et al., 2000; Goldfarb et al., 1998; Goudeau et al., 2001; Goudeau et al., 2006; Hong et al., 2011; Kaminska et al., 2004; McLaughlin et al., 2013; Park et al., 2000; Piñol-Ripoll et al., 2009; Sugawara et al., 2000; Vernengo et al., 2010)
	RCM	(Arbustini et al., 2006; Pruszczyk et al., 2007)
α -MHC	Atrial septal defect & other congenital heart defects	(Ching et al., 2005; Granados-Riveron et al., 2010; Posch et al., 2011)
	DCM	(Carniel et al., 2005; Hershberger et al., 2010)
	HCM	(Carniel et al., 2005; Niimura et al., 2002)
β -MHC	DCM	(Boda et al., 2009; Daehmlow et al., 2002; Kamisago et al., 2000)
	HCM	(Arad et al., 2005; Chiou et al.; Geisterfer-Lowrance et al., 1990; Mörner et al., 2003; Nanni et al., 2003; Richard et al., 2000; Watkins et al., 1992; Woo et al., 2003)
Essential MLC	HCM	(Chiou et al., 2014; Poetter et al., 1996)
Regulatory MLC	HCM	(Flavigny et al., 1998; Poetter et al., 1996)
Cardiac MyBP-C	DCM	(Daehmlow et al., 2002; Ehlermann et al., 2008; Hershberger et al., 2010)

	HCM	(Bonne et al., 1995; Chiou et al., 2014; Ehlermann et al., 2008; Hirota et al., 2010; Hitomi et al., 2010; Konno et al., 2006; Konno et al., 2003; Mörner et al., 2003; Nanni et al., 2003; Niimura et al., 1998; Niimura et al., 2002; Watkins et al., 1995a)
Titin	ARVC	(Taylor et al., 2011)
	DCM	(Carmignac et al., 2007; Gerull et al., 2006; Gerull et al., 2002; Herman et al., 2012; Itoh-Satoh et al., 2002; Matsumoto et al., 2005; Norton et al., 2013; Roncarati et al., 2013; van Spaendonck-Zwarts et al., 2014; Yoskovitz et al., 2012)
	HCM	(Herman et al., 2012; Satoh et al., 1999)
	RCM	(Peled et al., 2014)
α -Tropomyosin	DCM	(Hershberger et al., 2010; Olson et al., 2001; van de Meerakker et al., 2013)
	HCM	(Thierfelder et al., 1994; Van Driest et al., 2003)
Cardiac troponin-C	DCM	(Hershberger et al., 2010; Lim et al., 2008)
	HCM	(Hoffmann et al., 2001; Landstrom et al., 2008)
Cardiac troponin-I	DCM	(Boda et al., 2009; Carballo et al., 2009; Hershberger et al., 2010)
	HCM	(Arad et al., 2005; Chiou et al., 2014; Kimura et al., 1997; Mörner et al., 2000; Niimura et al., 2002; Van Driest et al., 2003)
	RCM	(Kaski et al., 2008; Kostareva et al., 2009)
Cardiac troponin-T	DCM	(Kamisago et al., 2000)
	HCM	(Ho et al., 2000; Moolman et al., 1997; Thierfelder et al., 1994; Van Driest et al., 2003; Watkins et al., 1995b)
	RCM	(Kaski et al., 2008; Pinto et al., 2011)

ARVC – Arrhythmogenic right ventricular cardiomyopathy, DCM – Dilated cardiomyopathy, HCM – Hypertrophic cardiomyopathy, RCM – Restrictive cardiomyopathy, MLC – Myosin light chain, MHC – Myosin heavy chain, MyBP-C – Myosin binding protein C

Table 1.3: Cardiovascular disease-associated isoform and subtype switches in cytoskeletal proteins described in the literature. All switches were observed in human patient samples unless stated otherwise.

Protein	Switch	Associated disease	Reference
Tropomyosin	β -TM ² to TPM1 κ	Dilated cardiomyopathy, heart failure	(Rajan et al., 2010)
Titin	N2B to N2BA	Heart failure from non-ischemic dilated cardiomyopathy	(Makarenko et al., 2004; Nagueh et al., 2004)
	N2BA to N2B	Diastolic heart failure	(van Heerebeek et al., 2006)
	N2B to N2BA	Ischemic cardiomyopathy	(Neagoe et al., 2002)
	N2BA to N2B	Hypertension induced hypertrophy (rat)	(Warren et al., 2003)
	N2BA to N2B	Aortic stenosis	(Williams et al., 2009)
α -actin	Cardiac α -actin to skeletal muscle α -actin	Dilated cardiomyopathy, hypertrophic obstructive cardiomyopathy, heart failure	(Copeland et al., 2010)
Myosin heavy chain (MHC)	α -MHC to β -MHC	Primary pulmonary hypertension, idiopathic dilated cardiomyopathy, heart failure	(Lowes et al., 1997; Nakao et al., 1997)

1.12 RNA interference

Sequence-specific gene silencing in response to double-stranded RNA (dsRNA) was first discovered in *Caenorhabditis elegans*, and the concept was termed RNA interference (RNAi) (Fire et al., 1998). In the study, it was also shown that double-stranded RNA is much more effective in gene silencing than sense or anti-sense RNA alone. RNAi acts at the post-transcriptional level and leads to the loss of messenger RNA (mRNA). Thus, dsRNA sequences targeting gene promoters or intronic sequences are mostly ineffective for gene silencing. Since this landmark study, the mechanism of RNAi has been largely uncovered.

Most of the initial work on RNAi mechanisms has been done in plants, *C. elegans* and *Drosophila*. There, long dsRNAs are cleaved into 21-25 nucleotide fragments called small interfering RNAs (siRNAs) by an RNase III enzyme family called Dicer (Bernstein et al., 2001; Hannon, 2002). siRNAs are double-stranded and possess two-nucleotide 3' overhangs and 5'-phosphate termini (Elbashir et al., 2001b; Zamore et al., 2000). This conformation is essential to incorporate into the RNA-induced silencing complex (RISC), an endonuclease complex which silences gene expression by mRNA degradation (Elbashir et al., 2001b; Hammond et al., 2000; Nykänen et al., 2001). The complex becomes activated in an ATP-dependent manner and an helicase in the RISC complex unwinds the double-stranded siRNA, which improves target recognition by the single-stranded siRNA (Hannon, 2002; Nykänen et al., 2001). The process of siRNA-mediated gene regulation by mRNA degradation has been termed post-translational gene silencing (PTGS). Evolutionary, it probably developed as an antiviral defence mechanism, where the virus dsRNA is recognized and used to recognize and eliminate further viral RNA copies (Virus-induced gene silencing (VIGS)) (Mourrain et al., 2000; Voinnet et al., 2000)

In vertebrates, similar gene silencing systems exist. However, the antiviral response to long dsRNA differs and in most cell types triggers the complex interferon response involving global inhibition of translation, unspecific RNA degradation and apoptosis, instead of specific mRNA degradation (Chalupnikova et al., 2013; Gantier and Williams, 2007). The RNAi machinery is nevertheless available and functional in mammalian cells as has been shown by experimental gene silencing using siRNAs or short hairpin RNAs (shRNAs) that are too short to induce the interferon response (Brummelkamp et al., 2002; Elbashir et al., 2001a; Paddison et al., 2002; Sui et al., 2002).

Today, a variety of biological functions of small RNAs have been established in addition to PTGS, which differ in the downstream response to target recognition. PIWI-interacting RNAs (piRNAs) are used to control endogenous transposons through transcriptional regulation (Castel and Martienssen, 2013; Ketting et al., 1999; Tabara et al., 1999). Micro RNAs (miRNAs) are natural hairpin dsRNAs with imperfect complementarity to target sequences and mediate translational repression (Castel and Martienssen, 2013; Lee et al., 1993; Reinhart et al., 2000). Nuclear siRNAs recruit the RNA-induced transcriptional silencing complex to epigenetically silenced loci and reinforce silencing through histone H3K9 methylation or establish chromatin modification during DNA replication (Castel and Martienssen, 2013; Volpe et al., 2002). Recent evidence also suggests functions for RNAi pathways in chromosome condensation, telomere maintenance, inhibition of recombination and DNA damage response (Castel and Martienssen, 2013).

Since its discovery, RNAi made its way into the standard molecular biology experimental tool box. SiRNA can be delivered into cells by microinjection, cationic lipid-based transfection reagents and a variety of other nucleic acid delivery systems (McNaughton et al., 2009). The choice of transfection reagent depends on the specific cell type, since different delivery platforms show cell type-specific differences in transfection efficiency and cytotoxicity. After successful delivery, the efficacy of mRNA depletion is dependent on the number of siRNA molecules delivered and the potency of the siRNA sequence (Hannon and Rossi, 2004). A drawback of the use of siRNA is potential off-target effects, which can occasionally be caused by triggering of the interferon response or by off-target recognition through non-contiguous base pairing (Jackson et al., 2003; Persengiev et al., 2004; Sledz et al., 2003). Aside these potential problems, the success of RNAi as experimental tool has allowed the use of directed or genome-wide libraries of siRNAs or shRNAs in large-scale screens to study a diverse range of cellular processes.

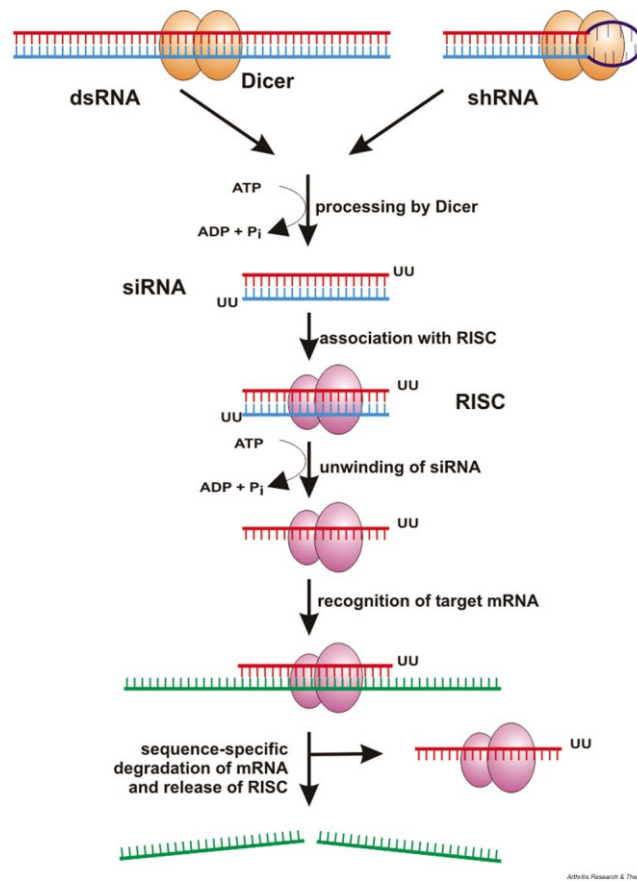


Figure 1.8: The RNA interference pathway. Long double-stranded RNA (dsRNA) or small hairpin RNA (shRNA) are cleaved into 21-25 nucleotide long small interfering RNAs (siRNAs) by the endonuclease Dicer. The siRNA associates with RNA-induced silencing protein complex (RISC) and is unwound in an ATP-dependent manner by a helicase in the complex. The single-stranded siRNA mediates sequence-specific target sequence recognition for subsequent mRNA cleavage. Figure from (Rutz and Scheffold, 2004)

1.13 The use of RNAi screens for the identification of AJ regulators

Although our understanding of epithelial biogenesis and AJ has been growing exponentially, given the complexity of the processes there is still much for us to learn. In recent years, systematic, medium- and large-scale approaches have become popular to study diverse cellular processes, including the use of microarrays to study gene regulation or manifold setups to scan protein-protein interactions. RNAi-mediated protein depletion has been used as medium- and large throughput assays in combination with diverse outputs to observe phenotypic changes (Boutros et al., 2004; Nybakken et al., 2005; Simpson et al., 2008; Toret et al., 2014). Robust screens rely on quantifiable observations and several recent studies have developed workflows to quantify images visually (D'Ambrosio and Vale, 2010; Fuchs et al., 2010). Many screens rely on only one read-out such as remaining wounded area after scratch assay and wound healing to assess siRNA effects on migration (Simpson et al., 2008). However, assessment of multiple parameters allows studying cellular processes in greater detail at high-throughput scale (D'Ambrosio and Vale, 2010; Fuchs et al., 2010). Analysis of complex phenotypes depends on the use of specialised software to aid their measurement and classification (D'Ambrosio and Vale, 2010).

Epithelial biogenesis is characterised by readily visible changes in a short period of time, such as the accumulation of E-cadherin and actin along cell-cell contacts and the compaction of actin bundles towards cell borders, the latter clearly observed within 30 minutes of induction (Adams et al., 1998; Adams et al., 1996; Zhang et al., 2005). Different screens have been performed to assess cell-cell adhesion in different species to identify novel regulators (Elbediwy et al., 2012; Lynch et al., 2012; Omelchenko and Hall, 2012; Toret et al., 2014). All these screens relied on manual assessment of phenotypes. To improve throughput and objectiveness of phenotype analysis, computational methods to analyse junctional defects would be of advantage. Currently, no sufficiently good methods for segmenting confluent monolayers of epithelial cells are available (Dima et al., 2011). To circumvent the problem, thresholding-based methods can be applied based on the assumption that the staining intensity of junctional proteins is highest at cell-cell contacts. Although this method only yields an approximation of junctional area, it can produce reliable results when using a large number of images and comparing multiple siRNA oligos targeting the same protein.

In our lab, we developed a semi-automated workflow to segment and quantify the pool of junctional E-cadherin, junctional actin and circumferential actin bundles. We use this workflow to analyse two independent medium-throughput RNAi screens performed by members of the lab, in order to identify novel regulators of adherens junctions in keratinocytes. Based on the importance of cytoskeletal remodelling for adherens junction formation and the significance of Rho GTPase signalling for actin remodelling, one screen targeted actin-binding proteins (Ann Wheeler, Jennifer Erasmus, manuscript submitted) and a second screen addressed Rho GTPases, Rho GAPs, Rho GEFs and Rho effectors (Jennifer Erasmus, Jessica McCormack, Natalie Welsh, Susann Bruche).

The actin-binding protein screen targeted 327 proteins and yielded 156 potential novel regulators of adherens junctions as result of the primary screen with siRNA pools. For a validation screen with 4 individual siRNA oligos against each protein, 49 of the 156 candidate proteins were chosen. In this secondary screen 31 proteins were validated as having an effect on adherens junctions and 16 of these proteins had not previously been linked to epithelia biogenesis.

The primary Rho GTPase screen targeted 206 proteins comprising all members of the Rho GTPase family and their regulators (GAPs, GEFs) and effectors. The primary screen returned 101 targets with strong effects on AJ formation. Three independent secondary screens were performed for Rho GAPs (Jessica McCormack), Rho GEFs (Natalie Welsh) and Rho GTPases and effectors (Susann Bruche). These screens resulted in overall 69 validated proteins with effects on AJ formation, of which 58 have not yet been linked to AJ regulation.

Yet, in spite of the strong capability of RNAi screens to identify many novel mediators of particular cellular processes, their results have to be considered solely indicative. Conditions used in screens are chosen to maximise efficiency across many proteins, but are not ideal for every single protein. This means that proteins with very short or long half-life might be overlooked, because they are not efficiently depleted. At the same time, RNAi approaches also warrant the possibility to falsely identify proteins as regulators because of off-target effects.

1.14 Hypotheses

AJ formation and maintenance are highly complex processes that are partially governed by GTPase signalling and actin remodelling. Despite the different cellular processes involved in AJ formation, only a few GTPases and actin-binding proteins have been shown to be involved in AJ regulation. I hypothesise that:

- Different types of event are mediated through engagement of different GTPase effectors and actin-binding proteins not previously linked to AJ regulation;
- Multiple pathways cooperate to coordinate AJ assembly.

Epidermal cells and cardiomyocytes both have to withstand constant mechanical stress and therefore contain similar adhesive components to ensure tissue integrity. I hypothesise that:

- Regulators of AJ stability in skin also mediate intercalated disc integrity in cardiomyocytes;
- Regulators of tissue integrity are deregulated in heart diseases.

1.15 Aims

I aim to:

- Verify the involvement of some GTPase effectors and actin-binding proteins identified in RNAi screens;
- Characterise the cellular function of a validated protein in detail;
- Elucidate whether the AJ regulatory function of the validated protein is deregulated in cancer cells and in heart diseases.

Chapter 2 Methods

2.1 Cell culture

2.1.1 Keratinocyte culture

Mouse J2 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% donor calf serum (Sera Laboratories International Ltd, West Sussex, UK) and 5 mM L-glutamine (Sigma-Aldrich). Confluent J2 fibroblasts were treated with 4 µg/ml mitomycin-C (Sigma-Aldrich) two hours before trypsinising and seeding alongside keratinocytes as feeder layer. Human keratinocytes isolated from neonatal foreskin (strain SF, passages 3-6) were cultured on this feeder layer and maintained in FAD medium (DMEM:F12, BioWittaker, Lonza, Germany), supplemented with 10% fetal calf serum (FCS) (Sera Laboratories International Ltd, West Sussex, UK), 1.8 mM CaCl₂, 5 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 10 ng/ml epidermal growth factor (EGF), 0.5 µg/ml hydrocortisone (all Sigma-Aldrich) and 0.1 nM cholera toxin (Quadrant Diagnostics Ltd, Surrey, UK) at 37°C and 5% CO₂ (Rheinwald and Green, 1975).

For experiments initiated in cells without calcium-dependent cell-cell contacts, cells were seeded in standard medium and changed to low calcium medium when about 30-40% confluent. Low calcium medium had a similar composition as standard medium but contained only 0.1 mM CaCl₂ and 10% FCS depleted of divalent ions by treatment with Chelex-100 resin (BioRad, Hertfordshire, UK). Experiments were performed on confluent monolayers and cell-cell contacts were induced by adding 1.8 mM CaCl₂ to the medium (Hodivala and Watt, 1994).

For treatment with insulin-like growth factor 1 (IGF1), FAD medium was replaced with 2-3 days old medium containing 200 ng/ml IGF1 (Merck Millipore, Darmstadt, Germany) for 3-24 hours.

2.1.2 HaCaT and Head and Neck Squamous Carcinoma cell culture

Head and Neck Squamous Carcinoma cell lines HN4, HN12, HN30 and HN31 (Cardinali et al., 1995) and HaCaT cell lines were cultured in DMEM supplemented with 10% FCS and 5 mM glutamine at 37°C and 5% CO₂.

For EGF scattering, HaCaT cells were seeded at a density of 2×10^4 . A day after seeding, cells were transfected with pCS2-eGFP-LZTFL1 or empty vector control cDNA as described below. After 4 hours, the medium was replaced with DMEM with

1% FCS, 5 mM glutamine with or without 100 mM EGF and 0.1% BSA. Cells were fixed 24 hours after EGF addition.

2.2 DNA constructs and cloning

2.2.1 DNA constructs

Mammalian and bacterial expression plasmids used are displayed in Table 2.1 or Table 2.2, respectively. All constructs were sequenced through Beckman-Coulter Ltd (High Wycombe, UK).

2.2.2 Cloning

Primers were designed to clone LZTFL1 full-length coding sequence and the LZTFL1 fragments LZTFL1 (1-142), LZTFL1 (95-299) and LZTFL1 (142-299) into pCS2-eGFP, pCS2-mRFP, pCS2-6myc or pRK5-myc (Table 2.3). All cloned products had their sequence verified.

Polymerase chain reaction (PCR) was carried out using keratinocyte cDNA or subsequently pRK5-myc-LZTFL1 as template. CDNA (1 µl) or vector template (100 ng) were combined with 1x Phusion polymerase buffer (New England Biolabs (NEB), Hitchin, UK), 1 µl (2.5 U) Phusion DNA polymerase (NEB), 1 µM forward and reverse primer (Invitrogen), 0.25 µM dNTPs (Invitrogen), 1 µl dimethyl sulphoxide (DMSO) (Sigma-Aldrich) and 1.5 mM MgCl₂ (NEB) in a total volume of 50 µl. PCR was executed with the following cycle setup: 95°C for 30 seconds, followed by 40 cycles of 95°C for 30 seconds, primer annealing temperature for 30 seconds and 72°C for 1.5 minutes and a subsequent final extension step at 72°C for 10 minutes. The samples were run on a 1% agarose (Invitrogen) gel, visualized with GelRed (Biotium Inc., Hayward California, USA) and the product band cut out. DNA was isolated from the gel using the StrataPrep DNA Gel Extraction kit (Agilent Technologies, Inc., Santa Clara, California, USA) according to manufacturer's instructions before digestion of the purified PCR product and the empty vector with appropriate restriction enzymes (NEB) at 37°C for 1-2 hours. The success of the digestion was checked by running 5 µl of the samples on a 1% agarose gel. The remaining 45 µl of the samples were purified with a StrataPrep PCR Purification kit (Agilent Technologies) according to manufacturer's instructions. Linearized vector (50 ng) and insert were combined at a ratio of 1:3. Quick-Stick ligase and 1x Quick-Stick ligase buffer (Bioline USA Inc, Taunton, Massachusetts, USA) were added to a final volume of 20 µl and the ligation reaction was allowed to proceed for 15

minutes at room temperature. The ligation samples were transformed into DH5 α (NEB) and plated on agarose plates containing the appropriate selection antibiotics.

Table 2.1: Mammalian expression vectors

Coded protein	Species	Plasmid	Product size (kDa)	Source
mRFP	n.a.	pCS2	26.4	S. Bruche, Imperial College London
eGFP	n.a.	pCS2	29.3	S. Bruche, Imperial College London
6myc	n.a.	pCS2	11.1	G. Longmore, Washington University, USA
myc	n.a.	pRK5	4.4	A. Hall, Memorial Sloan-Kettering Cancer Centre, USA
LZTFL1	Human	pRK5-myc	35.9	S. Bruche, Imperial College London
LZTFL1	Human	pCS2-eGFP	62.1	S. Bruche, Imperial College London
LZTFL1	Human	pCS2-mRFP	60.0	S. Bruche, Imperial College London
LZTFL1	Human	pCS-6myc	46.3	S. Bruche, Imperial College London
LZTFL1 (1-142)	Human	pCS2-6myc	28.1	S. Bruche, Imperial College London
LZTFL1 (1-142)	Human	pCS2-mRFP	42.1	S. Bruche, Imperial College London
LZTFL1 (95-299)	Human	pCS2-mRFP	49.5	S. Bruche, Imperial College London
LZTFL1 (142-299)	Human	pCS2-mRFP	44.1	S. Bruche, Imperial College London

Table 2.2: Bacterial expression vectors

Coded protein	Species	Plasmid	Product size (kDa)	Source
His-GST	n.a.	pMSCG10	29.3	R. Nagem, Universidade Federal de Minas Gerais, Brasil
LZTFL1	Human	pMSCG10	63.5	S. Bruche, Imperial College London
GST	n.a.	pGEX-4T1	27.0	GE Healthcare
CIP4	Human	pGEX-2T	88.9	P. Aspenström, Karolinska Institute, Sweden
E-cadherin tail	Mouse	pGEX-6P	44.8	Y. Fujita, University College London, UK
α -catenin	Human	pGEX-2T	127	D. Rimm, Yale University, USA
β -catenin	Human	pGEX-2T	113	D. Rimm, Yale University, USA
p120-catenin	Mouse	pTEX-GST	132	A. Bershadsky, The Weizmann Institute of Science, Israel
α -catenin (1-262)	Mouse	pGEX-2T	55.0	B. Weis, Stanford University, USA
α -catenin (273-906)	Mouse	pGEX-KG-TEV	96.6	B. Weis, Stanford University, USA
α -catenin (385-651)	Mouse	pGEX-2T	56.0	B. Weis, Stanford University, USA
α -catenin (632-906)	Mouse	pGEX-4T	57.0	B. Weis, Stanford University, USA

Table 2.3: Primers used for cloning LZTFL1 into different vectors

Primer name	Sequence
LZTFL1 (142-163)_XhoI fw_new ORF	5'-TATTCTCGAGGGCAGAGTTGGGTCTAAATGAGC-3'
LZTFL1 (142-163)_BspEI fw	5'-ATTATATCCGGAGCAGAGTTGGGTCTAAATGAGC-3'
LZTFL1 (1089-1070)_XbaI rv	5'-CGCTTCTAGAGAGACTGCTTGTATGTTTGC-3'
LZTFL1 (142-163)_BamHI fw	5'-TATTGGATCCGCAGAGTTGGGTCTAAATGAGC-3'
LZTFL1 (1089-1070) rv	5'-CGCTGAATTCGAGACTGCTTGTATGTTTGC-3'
LZTFL1 (1-25)_BamHI fw	5'-TATCGGATCCATGGCAGAGTTGGGTCTAAATGAGC-3'
LZTFL1 no stop-(405-426)_ClaI rv	5'-GGTTATCGATGATTTAGTGGAGCAAGTTTTGGC-3'
LZTFL1 no stop-(897-874)_ClaI rv	5'-GGAAATCGATGATCTTCAGGTTTCATATTGTGC-3'
LZTFL1 (142-163)_XhoI fw	5'-TATTCTCGAGTGGCAGAGTTGGGTCTAAATGAGC-3'
LZTFL1 (283-303)_XhoI fw mRFP ORF	5'-ACGGCTCGAGATCTTAAACTACAGACAGAC-3'
LZTFL1 (424-442)_XhoI fw mRFP ORF	5'-TTGACTCGAGATGAAGGTGGAACAG -3'
LZTFL1 (405-426)_EcoRI rv	5'-CGACGAATTCTCAATTTAGTGGAGCAAGTTTTGGC-3'
LZTFL1_LIC_fw	5'-TACTTCCAATCCAATGCCGCAGAGTTGGGTCTAAATGAGC-3'
LZTFL1_LIC_rv	5'-TTATCCACTTCCAATGTTAGAGACTGCTTGTATGTTTGC-3'

For cloning of LZTFL1 into pMSCG10, the ligation independent cloning (LIC) approach was used (Eschenfeldt et al., 2009). PCR and gel purification were performed as described above. The purified PCR product was treated with T4 polymerase by

combining 20 ng PCR product with 1x T4 polymerase reaction buffer, 1 µl T4 DNA polymerase (NEB), 2.5 mM dCTP (Invitrogen) and 5 mM dithiothreitol (DTT) (Sigma-Aldrich) in a total volume of 40 µl. The reaction mix was incubated at room temperature for 30 minutes and followed by inactivation of the enzyme at 75°C for 20 minutes. The pMSCG10 vector was prepared for LIC by digesting 3 µg vector DNA with 2 µl SspI in a total volume of 60 µl 1x SspI reaction buffer at 37°C for 2 hours. Digestion was checked on a 1% agarose gel and the rest of the reaction was cleaned up with the StrataPrep PCR Purification kit (Agilent Technologies). The linearized vector was treated with T4 polymerase by combining 200 ng vector DNA with 1x T4 polymerase reaction buffer, 1 µl T4 DNA polymerase (NEB), 2.5 mM dGTP (Invitrogen) and 5 mM DTT (Sigma-Aldrich) in a total volume of 40 µl. The reaction mix was incubated at room temperature for 30 minutes, followed by inactivation of the enzyme at 75°C for 20 minutes. Digested vector DNA (15 ng) and PCR product (45 ng) were combined in an Eppendorf tube and incubated on ice for 30 minutes before transformation into DH5α (NEB). The transformation was plated on an agarose plate containing 100 µg/ml ampicillin.

2.3 Transfection techniques

2.3.1 cDNA transfection

For cDNA transfections, 0.25-0.5 µg plasmid DNA per coverslip were incubated at a ratio of 1:2 with JetPrime transfection reagent in JetPrime transfection buffer (Polyplus transfection, Illkirch, France) in a total volume of 50 µl. Tubes were vortexed for 10 seconds, centrifuged briefly and incubated at room temperature for 10 minutes. The old medium was removed from the cells and replaced with 350µl fresh medium per coverslip. The transfection mixture was added to the cells and incubated for 4 hours before replacing it with fresh medium. DNA expression was allowed to continue for 16-24 hours.

2.3.2 siRNA transfection

Oligonucleotides were dissolved in 1x siRNA buffer (Dharmacon, Thermo Scientific, Colorado, USA) in RNase-free diethylpyrocarbonate (DEPC)-treated water (Sigma-Aldrich). Keratinocytes were grown till near confluence and then transfected with a non-targeting oligonucleotide or oligonucleotides targeting the protein of interest (Table 2.4) (Dharmacon, Invitrogen, Sigma-Aldrich). The oligonucleotides targeting MTSS1 and VAV2 were previously published (Basquin et al., 2013; Dawson et al., 2012a; Thalappilly et al., 2010). To prepare the transfection mix, 50 nM final concentration

oligonucleotide were mixed with 3 µl Interferin transfection reagent (Polyplus transfection) in 100 µl serum-free medium for a coverslip. The mix was briefly vortexed and spun down and then incubated for 10 minutes at room temperature. Alternatively, 50 nM oligonucleotide were placed in 50 µl serum-free medium for a coverslip and 3 µl METAFECTENE transfection reagent (Biontex Laboratories GmbH, München, Germany) were added to 50 µl serum-free medium in a second tube. The DNA mix was added to the transfection reagent solution and mixed by careful pipetting. The transfection mix was incubated at room temperature for 15 minutes. The old medium on the cells was replaced with 300 µl of fresh medium per coverslip. The transfection mix was added to the cells and incubated for 4 hours before exchanging the transfection mix-containing medium for fresh medium. The siRNA-induced protein depletion was continued for 48 hours (CIP4, LZTFL1, VAV2) or 72 hours (EF1α, MTSS1).

Table 2.4: siRNA oligonucleotides used in this study

Oligonucleotide	Sequence
CIP4 #1	5'-GAACGGCUAGACCAGGAUAAU-3'
CIP4 #2	5'-CCAUUUACACGGAGUUUGAUU-3'
EF1α #1	5'-GAUGGAAAGUCACCCGUAAUU-3'
EF1α #2	5'-UAGCAUUUGUGCCAAUUUCUU-3'
LZTFL1 #1	5'-GCAAACAGCAGCUUAUCGA-3'
LZTFL1 #2	5'-GCUGAGAAGUGGUAUCUUA-3'
LZTFL1 #3	5'-CUACAAAUGCACUGGAUGA[dT][dT]-3'
MTSS1 #1	5'-CCAGUUGUCUAACGGGUUU-3'
MTSS1 #2	5'-CAAGUGAACAGGUGAUUCU-3'
VAV2 #1	5'-GAAUGACGAUGACGUCUAC[dT][dT]-3'
VAV2 #2	5'-AGUCCGGUCCAUAAGUCAAC[dT][dT]-3'
Non-targeting control	5'-AUGAACGUAAUUGCUCAA-3'

Table 2.5: Primary antibodies used for Western blotting

Antibody	Clone name	Supplier	Source	Dilution
Actin	C4	Sigma-Aldrich	Mouse	1:100,000
α -catenin	VB1	(Braga et al., 1995)	Rabbit	1:1000
α -tubulin	DM1A	Sigma-Aldrich	Mouse	1:1000
β -catenin	VB2	(Braga et al., 1995)	Rabbit	1:1000
β -tubulin	Tub2.1	Sigma-Aldrich	Mouse	1:1000
CIP4	F-10	Santa Cruz	Mouse	1:10,000
Desmoplakin	11 5F	Gift from D. Garrod, The Univeristy of Manchester, UK	Mouse	Neat
E-cadherin	HECD1	Gift from M. Takeichi, Keio University, Japan	Mouse	1:3000
EF1 α		Cell Signalling	Rabbit	1:100
GAPDH	6c5	Abcam	Mouse	1:100,000
GFP	3E1	Abcam	Mouse	1:5000
Keratin14	D1002	Abcam	Mouse	1:1000
LZTFL1	7F6	Abnova	Mouse	1:2500
LZTFL1	Protein-Atlas HPA043466	Sigma-Aldrich	Rabbit	1:1000
MTSS1	2G9	Sigma-Aldrich	Mouse	1:100
Myc	9E10	Sigma-Aldrich	Mouse	1:1000
p120-catenin		Gift from J. Staddon, EISAI	Mouse	1:3000
Plakoglobin	VB3	(Braga et al., 1995)	Rabbit	1:1000
RFP		Abcam	Rabbit	1:1000
VAV2	EP1067Y	Abcam	Rabbit	1:20,000

2.4 Electrophoresis and Western blot

Keratinocytes were lysed in lysis buffer (0.0625 M Tris-HCl pH6.8, 0.5% SDS, 10% glycerol) and 1x sample buffer (5x stock: 0.2 mM Tris-HCl pH6.8, 50% glycerol, 5% SDS, 0.25 M DTT, 250 mg bromo-phenyl Blue dye) was added. For pull-down assays, 1x sample buffer was added directly to the beads. The samples were heated for 5 minutes at 95°C. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) by wet transfer (transfer buffer: 50 mM Tris-HCl, 380 mM glycine and 20% methanol) or semi-dry transfer (transfer buffer: 48 mM Tris-HCl, 40 mM glycine and 0.037% SDS). Proteins on the membrane were visualised by amido black staining (Sigma-Aldrich).

Subsequently, membranes were blocked with 5% non-fat dry milk (Marvel) in Tris buffered saline (TBS) pH 7.5 containing 0.1% Tween-20 (Sigma-Aldrich) (TBS-T) or with Western Blocking Solution (GE Healthcare) for 60 minutes while shaking. Primary antibodies (Table 2.5) were diluted in milk/TBS-T or TBS-T and incubated with the membrane for 1 hour at room temperature or overnight at 4°C. Loading controls (actin, GAPDH, β -tubulin, HSP90) were chosen based on the size of the protein of interest, to allow parallel probing of the blot. Membranes were washed three times 5 minutes in TBS-T before adding the appropriate conjugated secondary antibody (Table 2.6) in milk/TBS-T or TBS-T for 1 hour with agitation. After three washes in TBS-T, blots were developed with ECL (GE Healthcare) and exposed to Hyperfilm ECL (Amersham Biosciences). Films were developed with a Compact X4 Automatic Processor (Xograph Healthcare, Gloucestershire, UK). Alternatively, blots incubated with fluorescent secondary antibodies were scanned using the Ettan DIGE Imager (GE Healthcare). Western blots were quantified by densitometry using ImageJ (Rasband, NIH, USA).

Table 2.6: Secondary conjugated antibodies used for Western blotting

Conjugate	Supplier	Source	Dilution
Indocarbocyanine (Cy3)-conjugated anti-rabbit IgG	Jackson Immuno Research Laboratories	Donkey	1:3000
Indodicarbocyanine(Cy5)-conjugated anti-mouse IgG	Jackson Immuno Research Laboratories	Donkey	1:1000
HRP-conjugated anti-mouse IgG	Pierce	Goat	1:10,000
HRP-conjugated anti-rabbit IgG	Pierce	Goat	1:5000

2.5 Quantitative Real-Time PCR

RNA from mice hearts was provided by Dr Tommaso Poggioli (Imperial College London, UK) and RNA from hearts from human patients was provided by Prof Ralph Knöll and Prof Steven Marston (Imperial College London, UK). Clinical data of some of the samples has been previously described (Bayliss et al., 2013; Hoskins et al., 2010; Marston et al., 2009; Marston et al., 2013). RNA was isolated from keratinocytes using the Absolutely RNA Miniprep Kit (Agilent) according to manufacturer's instructions. RNA amount and quality were measured using a Nanodrop1000 (Thermo Scientific). If necessary, RNA was further purified by RNA precipitation. For this purpose, 0.1 volumes of 3 M sodium acetate (pH 5.2), 2.2 volumes ice-cold 100% ethanol and 50 µg/ml GlycoBlue (Applied Biosystems) were added to the RNA. The RNA was precipitated over night at -20°C and then spun down at maximum speed for 10 minutes. The supernatant was removed and the pellet was washed with 70% ice-cold ethanol. After centrifugation at maximum speed for 5 minutes, the ethanol was removed and the pellet air-dried and then resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA).

RNA was transcribed to cDNA by reverse transcription. RNA (100-500 µg) was combined with 0.5 mM dNTPs, 1.5 µg random primers and water to a total volume of 15.7 µl and heated at 65°C for 5 minutes. Samples were spun down at 14,000 rpm for 1 minute and 2 µl 10x reverse transcriptase buffer and 200 U M-MuLV Reverse Transcriptase (NEB) added. After incubation for 5 minutes at 25°C, samples were kept at 42°C for 1 hour. The enzyme was inactivated by 5 minute incubation at 80°C before addition of 2 U RNaseH (NEB). RNA was digested for 20 minutes at 37°C and the enzyme then inactivated at 65°C for 20 minutes.

Primers used for quantitative real-time PCR (qPCR) are listed in Table 2.7. Primers against LZTFL1 were designed using Primer3Plus (Untergasser et al., 2012). Most other primers were chosen from the literature. Primers against mouse α T-catenin (CTNNA3) were obtained from the PCR primer database PrimerBank (Spandidos et al., 2008; Spandidos et al., 2010; Wang and Seed, 2003). Primers against E-cadherin were designed and manufactured by PrimerDesign Ltd (Southampton, UK). Sequences for primers against human GAPDH and mouse Hprt and Rplp0 were provided by Dr Paul Kemp (Imperial College London, UK).

For qPCR, cDNA was diluted 10-30 times in water. A qPCR reaction was set-up by combining 10 µl 2x QuantiFast SYBR Green (Qiagen, Hilden, Germany), 5 µl water, 3 µl diluted cDNA and 2 µl of 5 µM qPCR primers in MicroAmp® Optical 96-Well Reaction Plates (Applied Biosystems). All reactions were done in duplicate. QPCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction consisted of an initial activation step of 5 minutes at 95°C, followed by 40-45 cycles of denaturation (10 seconds, 95°C) and annealing/amplification (30 seconds, 60°C). PCR products were verified by melting curve analysis. The obtained C_T -values were normalised to the mean of two housekeeping genes (GAPDH, HPRT or RPLP0) and represented either as $2^{-\Delta C_T}$ or relative to a control as $2^{-\Delta\Delta C_T}$.

Table 2.7: Primers used for qPCR

Gene	Primer sequence	Product size (bp)	Species	Reference
Acta1	Fw: 5'-CCGGGAGAAGATGACTCAAA-3' Rv: 5'-CTCATAGATGGGCACGTTGT-3'	157	Mouse	(Buitrago et al., 2005)
CDH1	Fw: 5'-CATGAGTGTCCCCCGGTATC-3' Rv: 5'-CAGTATCAGCCGCTTTCAGA-3'	89	Human	n/a
Cdh1	Fw: 5'-GTGACACAGCCTCTGGATAG-3' Rv: 5'-ACCGCTTCCCCATTTGATG-3'	80	Mouse	n/a
CDH2	Fw: 5'-TGTTTGACTATGAAGGCAGTGG-3' Rv: 5'-TCAGTCATCACCTCCACCAT-3'	152	Human	(Alexander et al., 2006)
Cdh2	Fw: 5'-GCGAGCCAGCAGATTTCAAG-3' Rv: 5'-TTGCCTGCTCTGCAGTGAGA-3'	81	Mouse	(Chauvet et al., 2009)
CDH13	Fw: 5'-TGCTGATAACCCTGGAGGAC-3' Rv: 5'-ATGGGCAGGTTGTAGTTTGC-3'	265	Human	(Ordelleide et al., 2011)
Cdh13	Fw: 5'-AAGCCCACACTTCAAGGTGAA-3' Rv: 5'-GCCCACCGCAGTGATGTT-3'	67	Mouse	(Chauvet et al., 2009)
CTNNA1	Fw: 5'-ACTTGGCCTGTTCCATCTCAAA-3' Rv: 5'-TGCTAAAGCCAGTGCAGCATTA-3'	113	Human	(Bertrand, 2010)
Ctnna1	Fw: 5'-AAGCTTGTCCGAATGTCTGC-3' Rv: 5'-AGCCAAGAAGTCATCGATGG-3'	198	Mouse	(Schimmer et al., 2011)

CTNNA3	Fw: 5'-AGAGAAGATTGCCCAGGAAGCTA-3' Rv: 5'-TTTCAGAGCTTCACTTTCTTTGCGA-3'	88	Human	(Bertrand, 2010)
Ctnna3	Fw: 5'-ACGCCAATAACCCTGAATATGG-3' Rv: 5'-TTGGAAGGATTCTGCGGACAA-3'	125	Mouse	PrimerBank 29243986a1
GAPDH	Fw: 5'-GGTGGTCTCCTCTGACTTCAACA-3' Rv: 5'-GTTGCTGTAGCCAAATTCGTTGT-3'	127	Human	P. Kemp, IC, UK
HPRT1	Fw: 5'-GCTATAAATTCTTTGCTGACCTGCTG-3' Rv: 5'-AATTACTTTTATGTCCCCTGTTGACTGG-3'	140	Human	(Ge et al., 2007)
Hprt	Fw: 5'-GCAGTACAGCCCCAAAATGG-3' Rv: 5'-AACAAAGTCTGGCCTGTATCCAA-3'	85	Mouse	P. Kemp, IC, UK
LZTFL1	Fw: 5'-TTGAGGGCTTCATTCTCACC-3' Rv: 5'-TCTTGGTCCACAGACAATGG-3'	86	Human	n/a
LZTFL1 isoform1	Fw: 5'-TCTCCCGTTCCTTTAGGCTG-3' Rv: 5'-AGCTGTGCGCAGAAGTAACAC-3'	298	Human	n/a
LZTFL1 isoform2	Fw: 5'-CCACCCTTTGGTGACTTTTC-3' Rv: 5'-AGGTCTTGGAAGCAGGAATC-3'	167	Human	n/a
LZTFL1 isoform3/X1	Fw: 5'-GCACATGGCTGAAAAGATATGAAC-3' Rv: 5'-GTGGGATGACCAGACAGAATC-3'	234	Human	n/a
Lztfl1	Fw: 5'-ACCTACGGAAAAGACTGGCA-3' Rv: 5'-ACATGCCTGAGGTGAATGGA-3'	111	Mouse	n/a
MYH6	Fw: 5'-CCAGATGGCTGACTTTGGGG-3' Rv: 5'-TCGGGCACGAAGCACTCAGT-3'	111	Human	(Tran et al., 2009)
NPPA	Fw: 5'-CACAAGTGCTCAGTGAGCCGA-3' Rv: 5'-ATCTCTCTGGGCTGGGCTGA-3'	101	Human	(Tran et al., 2009)
Nppa	Fw: 5'-GTGCGGTGTCCAACACAGA-3' Rv: 5'-TTCTACCGGCATCTTCTCCTC-3'	74	Mouse	(Dingar et al., 2010)
Rplp0	Fw: 5'-GGACCCGAGAAGACCTCCTT-3' Rv: 5'-TGCTGCCGTTGTCAAACACC-3'	202	Mouse	P. Kemp, IC, UK

2.6 Immunofluorescence and microscopy

2.6.1 Immunofluorescence staining

Cells were fixed either with 3% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes at room temperature or with ice-cold methanol for 5 minutes at -20°C. Alternatively, cells were pre-permeabilised with CSK buffer (10 mM PIPES pH6.8, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose and 0.5% Triton X-100) for 10 minutes at room temperature prior to fixation in 3% PFA. Cells were blocked and permeabilised in 10% FCS with 0.1% Triton X-100 for 10 minutes at room temperature. Coverslips were then incubated with primary antibodies (Table 2.8) diluted in 10% FCS in PBS for 30 minutes. After washing the coverslips in PBS, secondary antibodies (Table 2.9) in 10% FCS in PBS were added onto the coverslips for 30 minutes. For double or triple staining, these steps were repeated for the subsequent antibodies. Coverslips were subjected to final nine washes in PBS and three washes in water and were mounted on glass slides (Fisher Scientific UK Ltd, Loughborough, UK) with Mowiol (Calbiochem, California, USA). Images were acquired on an Olympus Provis BX51 microscope coupled to a SPOT RT monochrome camera using SimplePCI 6 software (Hamamatsu, Japan) or with a Zeiss LSM-510 inverted confocal microscope using Zeiss ZEN 2012 (Blue Edition) (Carl Zeiss AG, Oberkochen, Germany) software. Pictures were processed with Zeiss ZEN 2012 (Blue Edition) software or ImageJ.

To test the specificity of the LZTFL1 antibodies (Abnova and ProteinAtlas), the antibodies were diluted as appropriate and added to GST beads or GST-LZTFL1 beads. The beads were rotated at 4°C overnight, spun down and the supernatant was used for immunofluorescence staining or Western blot.

2.6.2 Image analysis

E-cadherin and desmoplakin levels at cell-cell contacts were measured by thresholding of the E-cadherin/desmoplakin channel using ImageJ. For experiments performed in standard calcium medium, images were first cropped to 300x300 pixels to remove areas not covered by keratinocytes. Images were placed in an image stack. The stack was thresholded looking at the control images. The threshold was chosen in a way to maximise the amount of junctional E-cadherin/desmoplakin highlighted and minimise the amount of cytoplasmic staining highlighted. The thresholded area was measured in all images and calculated as percentage of the area of the whole image (% area).

To quantify the amount of CIP4 at junctions, E-cadherin images were individually thresholded in ImageJ to minimize contribution of cytoplasmic staining and maximise junction coverage. The thresholded images were converted to binary images and binary E-cadherin images were dilated by one pixel. These images were used as a mask to segment the junctional CIP4 pool from the corresponding CIP4 image using image calculator in ImageJ. A global threshold across all experimental conditions was applied to the resulting segmented CIP4 images to measure the CIP4 junction coverage and calculate % thresholded area (% area).

To quantify the effect of LZTFL1 depletion on Keratin 14, images were thresholded in the nuclei channel using ImageJ, followed by particle analysis. This step assigned a number to every nucleus. A random number generator (random.org) in combination with visual quality control was used to pick 5 focused cells on each image. Using the segmented line tool, a line was drawn along the midline of the shortest and the longest junction of every chosen cell. The mean grey value was measured along the line as well as along a line in 1 pixel distance to either side of the line. The average of the three values was calculated for every junction and values compared between conditions.

Cellular localisation of overexpressed LZTFL1 constructs was assessed semi-quantitatively. Junctions were considered positive for LZTFL1, if there were at least 2 patches of higher intensity than cytoplasm in the area of the junction in the LZTFL1 channel. Nuclei were counted as positive for LZTFL1, if the area of the nucleus showed intensity similar or stronger to the cytoplasm in the LZTFL1 channel.

Scattering was quantified with a custom-made program (Dr Chris Tomlinson, Bioinformatics Support Service, Imperial College London), which was used to measure the internuclear distance of a transfected cell to its closest direct neighbours, with a maximum of 5 neighbours per analysed cell. The cell area of transfected cells was measured using ImageJ. For this, transfected cells were isolated by cropping. The channel showing the overexpressed protein was thresholded so that background would be excluded, but the whole cell area would be highlighted and then the thresholded area was measured.

Table 2.8: Primary antibodies used for immunofluorescence staining

Antibody	Clone name	Supplier	Source	Dilution
α -catenin	25B1	Abcam	Mouse	1:200
β -tubulin	Tub2.1	Sigma-Aldrich	Mouse	1:1000
CIP4	F-10	Santa Cruz	Mouse	1:200
Desmoplakin	11 5F	Gift from D. Garrod, The University of Manchester, UK	Mouse	1:10
E-cadherin	ECED2	Invitrogen	Rat	1:750
E-cadherin	HECD1	Gift from M. Takeichi, Keio University, Japan	Mouse	1:1000
Keratin14	D1002	Abcam	Mouse	1:100, oN
LZTFL1	Protein-Atlas HPA043466	Sigma-Aldrich	Rabbit	1:200
Myc	9E10	Sigma-Aldrich	Mouse	1:200
RFP		Abcam	Rabbit	1:500

Table 2.9: Secondary antibodies and conjugates used for immunofluorescence staining

Conjugate	Supplier	Source	Dilution
Alexa Fluor 488 (AF488)-conjugated anti-mouse IgG	Jackson Immuno Research Laboratories	Goat	1:1000
Alexa Fluor 488 (AF488)-conjugated phalloidin	Molecular Probes		1:1000
Cyanine (Cy2)-conjugated anti-mouse IgG	Jackson Immuno Research Laboratories	Goat	1:1000
DAPI	Sigma-Aldrich		1:3000
Indocarbocyanine (Cy3)-conjugated anti-mouse IgG	Jackson Immuno Research Laboratories	Donkey	1:3000
Indocarbocyanine (Cy3)-conjugated anti-rabbit IgG	Jackson Immuno Research Laboratories	Donkey	1:3000
Indodicarbocyanine(Cy5)-conjugated anti-rat IgG	Jackson Immuno Research Laboratories	Donkey	1:400

2.7 Protein production and purification

2.7.1 Bacterial fusion protein production

GST-fusion proteins were produced in the Rosetta (DE3) *Escherichia coli* (*E. coli*) (Merck KGaA, Darmstadt, Germany) or BL21 competent *E. coli*. Cultures of 100 ml were grown at 37°C in LB medium with 100 µg/ml ampicillin and were diluted with fresh LB with ampicillin to 1 litre the next morning. These cultures were grown at 37°C to an optical density at 600 nm of 0.6-1.0 (Jenway 6300 Spectrophotometer, Bibby Scientific Limited, Staffordshire, UK). Protein production was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at the concentration and for the time stated in Table 2.10. A sample of 1 ml was taken before and after induction. The bacteria were pelleted and re-suspended in 1x sample buffer for analysis by SDS-PAGE. At the end of induction, bacteria were centrifuged at 6000 rpm for 15 minutes (Sorvall RC-5B, GSA rotor (Sorvall Products, L.P., Newtown, Connecticut, USA)) and re-suspended in 5 ml ice-cold lysis buffer (see Table 2.10). The re-suspension was sonicated four times with amplitudes of 7 microns for 20 seconds with 20 seconds breaks on ice (Soniprep 150 (MSE (UK) Ltd, London, UK)). The samples were then centrifuged at 14,000 rpm for 20 minutes at 4°C (Sorvall RC-5B, SS-14 rotor). A small sample was taken of the supernatant and the remaining supernatant was incubated with glutathione sepharose 4B beads (Pharmacia), which had been washed 2 times in lysis buffer. After 1 hour of incubation, beads were washed 3 times in wash buffer (Table 2.10) and re-suspended in 60% glycerol in wash buffer. A sample of the beads were taken and analysed along the other samples taken during protein production. Samples were analysed by SDS-PAGE, staining of the gel with Coomassie Blue (0.1% Brilliant Blue R250 (Sigma-Aldrich), 45% methanol (VWR), 10% acidic acid (VWR) in water) and destaining in Coomassie destain (20% methanol, 15% acidic acid in water). Protein concentrations were estimated relative to BSA (Fluka) standards of known concentration. Production of GST-CIP4 and α-catenin fragments were described previously (Pokutta et al., 2002; Pokutta and Weis, 2000; Yan et al., 2013).

GST-LZTFL1 fusion protein was produced in ArcticExpress (DE3) strain of *E. coli* (Agilent). An overnight culture in 20 ml LB broth containing 20 µg/ml gentamycin and 100 µg/ml ampicillin was diluted to 1 litre with fresh LB without antibiotics. The culture was incubated for 3 hours at 30°C before the culture was moved to 12°C. After temperature equilibration, protein production was induced for 24 hours by addition of 1 mM IPTG. Protein extraction, assessment and storage was done as described above, except that beads were blocked in 10% FCS in lysis buffer for 1 hour at 4°C

and washed once in lysis buffer. The supernatant containing GST-LZTFL1 was added to 1 ml of the blocked beads and the suspension was incubated over night at 4°C. The beads were collected by centrifugation (800xg, 4°C) and washed three times with wash buffer (Table 2.10).

To cleave the GST tag from α -catenin, beads with immobilised GST-protein were washed once with thrombin resuspension buffer (50mM Tris.HCl pH 7.5, 150mM NaCl, 0.1mM DTT) and were then resuspended in 1 ml buffer. Thrombin (20 Units) (Sigma-Aldrich) were added and beads rotated for 30 minutes at room temperature. Beads were centrifuged at 800xg and the supernatant was collected. The beads were washed once in same buffer and the supernatant combined with the initial supernatant. The thrombin was removed by incubation with 80 μ l of p-aminobenzamidine-agarose (Sigma-Aldrich) under rotation for 30 minutes at 4°C. After centrifugation and the supernatant was collected. To cleave LZTFL1 off the beads, beads were washed once with TEV cleavage buffer (50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 1 mM DTT). Beads were resuspended in 10 ml TEV cleavage buffer and TEV enzyme (Promega, Madison, WI, USA) was added (1 U for 2-5 μ g fusion protein). The suspension was incubated at room temperature overnight and then centrifuged as above. The supernatant was collected and the beads washed once with 5 ml TEV cleavage buffer. Both supernatants were combined and the cleaved protein was concentrated using an Amicon Ultra-15 Centrifugation Filter Unit with Ultracel-30 membrane (Millipore) at room temperature. A sample of the sepharose beads after cleavage was analysed together with the supernatant by SDS-PAGE to check cleavage efficiency.

Table 2.10: Protein production conditions

Protein	IPTG (mM)	Temp induction (°C)	Length of induction (hours)	Lysis buffer	Wash buffer
α -catenin A907	0.3	16	o/N	D	B
α -catenin (1-262)	1	37	4	E	G
α -catenin (273-906)	1	37	4	F	A
α -catenin (385-651)	1	37	4	F	A
α -catenin (632-906)	1	37	4	F	A
β -catenin	0.3	30	4	A	A
CIP4	0.5	37	4	I	I
E-cadherin tail	0.25	37	4	C	C
GST	0.3	30	3	C	A
LZTFL1	1	12	24	H	H
p120 catenin	0.05	15	o/N	J	K

A = 50 mM Tris pH7.5, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF),
5 μ g/ml LPP (5 μ g/ml leupeptin, 5 μ g/ml pefabloc, 5 μ g/ml pepstatin)

B = 50 mM Tris pH7.5, 100 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 mM LPP

C = 50 mM Tris pH7.5, 100 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM LPP

D = 10 mM Tris pH 8.0, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 1 mM PMSF, 5 μ g/ml
LPP

E = 50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 5 μ g/ml LPP

F = 50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF,
5 μ g/ml LPP

G = PBS with 1 M NaCl, 0.05% Tween 20, 5 mM DTT

H = 50 mM Tris pH7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM PMSF, 5 μ g/ml LPP

I = 50 mM Tris/HCl pH 8.0, 500 mM NaCl, 1 mM DTT, 1 mM PMSF, 5 μ g/ml LPP

J = 50 mM Tris pH 8.0, 150 mM NaCl, 10 mg/ml lysosyme, 1 mM PMSF, 5 μ g/ml LPP

K = 50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, 5 μ g/ml LPP

2.8 Binding assays and co-immunoprecipitation

2.8.1 Pull-down assays with cell lysates

For pull-down assay experiments, GST-tagged fusion protein-coated glutathione sepharose beads were blocked with 10% heat-denatured BSA for 1 hour at 4°C. Keratinocytes were lysed in lysis buffer (0.5% Triton X-100, 10% glycerol, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 µg/ml LPP (5 µg/ml leupeptin, 5 µg/ml pefabloc, 5 µg/ml pepstatin), 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride, 1 mM sodium vanadate). After centrifugation of the lysates at 14,000 rpm at 4°C for 5 minutes, a small sample was taken for later analysis and the remaining lysate was added to either GST-tagged fusion protein beads or GST alone beads for two hours at 4°C. Beads were washed three times in wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl). SDS-PAGE sample buffer was added to the beads and boiled at 95°C. The total volume of bead suspension was loaded for SDS-PAGE and pre-pull-down assay lysate was run alongside to compare protein input.

2.8.2 Pull-down assays with purified proteins

GST-tagged proteins or GST alone immobilised on beads were incubated with thrombin-cleaved α -catenin or TEV-cleaved LZTFL1 in a total volume of 100 µl *in vitro* pull-down assay buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1% Triton X-100). Samples were mixed 1 hour at 4°C and subsequently beads were washed three times in 500 µl *in vitro* pull-down assay buffer. SDS-PAGE sample buffer was added to the beads and total volume of bead suspension and samples of the cleaved fusion proteins were subjected to electrophoresis and Western blotting.

2.8.3 Co-immunoprecipitation

For co-immunoprecipitation experiments, keratinocytes were lysed (0.5% Triton X-100, 10% glycerol, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 µg/ml LPP, 1 mM PMSF, 50 mM sodium fluoride, 1 mM sodium vanadate) and lysates subjected to centrifugation at 14,000 rpm at 4°C for 5 minutes. The supernatants were pre-cleared using 50 µl protein A/G-Sepharose 4B beads (Sigma-Aldrich) for two hours. Lysates were centrifuged at 4,000 rpm at 4°C for 1 minute and the supernatant subjected to immunoprecipitation over night with 3 µg of CIP4 or 0.85 µg of VAV2 antibody. The antibody was precipitated with 50 µl protein A/G-Sepharose 4B bead slurry for 2 hours at 4°C. Beads were washed three times in wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl), SDS-PAGE sample buffer was added to the beads and beads boiled.

For SDS-PAGE and Western blotting, the total volume of bead suspension was loaded, alongside lysate samples to estimate protein input.

2.8.4 RFP-trap assay

RFP-Trap_A agarose beads (Chromotek, Martinsried, Germany) (15 µl) were washed twice with wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl₂). Keratinocytes over-expressing mRFP or mRFP-LZTFL1 constructs were lysed as above and lysates cleared by centrifugation. Lysates were diluted 1:2 with wash buffer and added to the RFP-Trap_A beads. Samples were rotated for 1 hour at 4°C. Beads were washed three times with 500 µl wash buffer and resuspended in SDS-PAGE sample buffer. For SDS-PAGE and Western blotting, the total volume of bead suspension was loaded, alongside lysate samples to estimate protein input.

2.9 Surface biotinylation

After junction assembly for 0, 5, 15 and 30 minutes, cells were washed once in PBS containing 1 mM MgCl₂ and 1 mM CaCl₂ (PBS-Mg²⁺-Ca²⁺) and then incubated on ice for 30 minutes with 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Rockford, IL, USA) dissolved in PBS-Mg²⁺-Ca²⁺. Cells were washed once and the biotinylation reaction quenched by incubating twice for 5 minutes with 15 mM glycine/PBS-Mg²⁺-Ca²⁺. Cells were washed once in PBS-Mg²⁺-Ca²⁺ and lysed in 500µl lysis buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1 mM DTT, 5 µg/ml LPP, 1 mM PMSF, 50 mM sodium fluoride, 1 mM sodium vanadate). Lysates were cleared by centrifugation at 14,000 rpm for 2 minutes. Streptavidin-immobilised agarose beads (Fluka Analytical, Buchs, Germany) were equilibrated with lysis buffer, before lysates were added to 70 µl 1:1 beads slurry and incubation for 1 hour at 4°C. Beads were washed three times in lysis buffer and then SDS-PAGE sample buffer was added. The total volume of bead suspension was loaded alongside lysate samples to measure input for analysis by SDS-PAGE and Western blotting.

2.10 Dispase assay

Dispase assay was performed as adapted from (Hudson et al., 2004). Cell-cell contact formation was induced for 30 minutes by addition of 1.8 mM CaCl₂ to confluent monolayers of keratinocytes grown in low calcium medium in 2 cm² wells. Subsequently, cells were washed once with D-PBS (PBS, 50 mM HEPES pH7.4,

1.8 mM CaCl_2 , 0.5 mM MgCl_2) and then detached by incubation with 300 μl dispase II (2.4U/ml, Roche Diagnostics Ltd, West Sussex, UK) in D-PBS. Detached monolayers were transferred to a new well containing 500 μl D-PBS and imaged using a 2x objective on an EVOS XI Core transmitted light microscope (Advanced Microscopy Group (AMG), Life Technologies) with EVOS XI software. Monolayers were put under mechanical stress by agitation at 220 rpm on an orbital shaker for 10 minutes at room temperature and were then re-imaged.

2.11 Aggregation assay

Confluent monolayers of keratinocytes maintained in low calcium medium were detached with trypsin in 500 μl trypsinisation buffer (60% versene (v/v), 0.1% trypsin (w/v), 1 mM CaCl_2). The cell suspension was then gently pipetted to disrupt clusters. Cells were counted and pelleted by centrifugation at 800 rpm for 5 minutes. The cell pellet was resuspended in standard calcium medium to a density of 5×10^4 cells/ml. A humid chamber was created by adding PBS to the bottom of a 6 cm^2 dish and six drops of 20 μl resuspended cells were pipetted onto the inside of the lid of the dish. The lid was placed back on the dish and the drops were photographed at 0, 60 and 120 minutes after standard calcium medium addition. After 120 minutes drops were pipetted up and down six times to disaggregate the formed cell aggregates and images of the resulting cell disaggregates were taken. All images were taken on an Axiovert 135M inverted microscope with a 10x phase contrast objective (Zeiss). The assay was analysed using ImageJ as described in (Nola et al., 2012).

2.12 Mouse models

Protein samples of different mouse models of physiological or pathological cardiac hypertrophy and of myocardial infarct (MI) were provided by Dr Lorna Fiedler (Imperial College London). RNA samples of control or isoproterenol-treated mice were provided by Dr Tommaso Poggioli (Imperial College London). Mice were housed in the College's animal facilities according to animal welfare regulations and all experiments had ethical approval.

To induce MI, the chest of anaesthetised mice was surgically opened and a needle was passed through the left ventricle under the coronary artery and a suture was tied around it. The same procedure was performed for sham-operated mice with the difference that no suture was tied around the coronary artery. When extracting the tissue 24 hours after procedure, MI was confirmed visually. Every heart was separated

into two parts, the left ventricle apex, containing the infarcted area and border zone in MI mice, and a second part consisting of the remaining heart comprising atria, right ventricle and some left ventricle.

Transverse aortic constriction (TAC) was performed similarly as described above. However, instead of ligation of the coronary artery, the suture was tied around the transverse aorta between the innominate and left carotid arteries. The knots were tied against a blunt needle and the needle was removed to yield the constriction of the aorta. For sham-operated mice the aorta was not ligated. Successful ligation-induced hypertrophy was confirmed by echocardiography a week after the operation. Mice were sacrificed and hearts harvested 2 or 4 weeks after operation.

For the model of exercise-mediated hypertrophy, 8-week old mice were housed in a cage with a Fast-Trac Wheel with a Cateye Velo Wireless Counter for 4 weeks. The sedentary control group did not have a wheel in their cage. After four weeks mice were sacrificed and hypertrophy confirmed by measuring heart weight over tibia length.

To measure the effect of mouse IGF1 (mIGF1) on cardiomyocytes, protein samples were harvested from hearts of wild type mice and mice over-expressing mIGF1 specifically in cardiomyocytes. Mice were sacrificed when they were 7 months of age or older.

For all models, heart tissue was perfused with PBS prior to extraction. Protein was extracted from tissue in lysis buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1% Triton X-100, protease and phosphatase inhibitor cocktail (Pierce)) by mechanical disruption with metal beads in a TissueLyser LT machine (Qiagen).

Isoproterenol-induced cardiac hypertrophy was investigated in 8-9 weeks old immunosuppressed mice of the strain NOD.CB17 Prkdcscid/JHliHSD. Hearts were injected daily for 7 days with saline (control) or 1 mg.kg⁻¹.d⁻¹ isoproterenol. A third group was injected with HEK293 cells transiently overexpressing IGF1-Ea 24 hours prior to the first injection with isoproterenol. To this end, a total of 10⁶ cells were injected in 5 locations of the left myocardium. In order to be able to verify the presence of the HEK cells in the heart later, HEK293 nuclei were labelled with DAPI prior to injection. After 7 days of isoproterenol or saline injection, stroke volume and end diastolic volume were measured by magnetic resonance imaging and left ventricular

mass was determined to confirm hypertrophy. RNA was isolated from the whole heart tissue.

2.13 Computational analysis

Amino acid sequence identity across species was evaluated using the SIAS server (<http://imed.med.ucm.es/Tools/sias.html>). Amino acid conservation was analysed with ConSurf (<http://consurf.tau.ac.il/> (Ashkenazy et al., 2010)) and JalView (<http://www.jalview.org/> (Waterhouse et al., 2009)). Nucleotide and amino acid sequences were aligned with BioEdit (T. Hall, Ibis Biosciences, Carlsbad, CA, USA). Nucleotide sequences were translated into protein sequences and molecular weights calculated using the ExPASy translate (<http://web.expasy.org/translate/>) and compute tools (http://web.expasy.org/compute_pi/). Motifs and post-translational modifications were predicted with PredictProtein (<https://www.predictprotein.org/> (Yachdav et al., 2014)). Secondary structure of LZTFL1 was predicted using PredictProtein, Robetta (<http://www.rosetta.org/submit.jsp> (Kim et al., 2004)), BioInfoBank Meta Server (<http://meta.bioinfo.pl/> (Bujnicki et al., 2001)), I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/> (Zhang, 2008)), HHPred (<http://toolkit.tuebingen.mpg.de/hhpred> (Söding et al., 2005)), Phyre² (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index> (Kelley and Sternberg, 2009)) and YASPIN (<http://www.ibi.vu.nl/programs/yaspinwww/> (Lin et al., 2005b)) and a consensus sequence was manually curated. Microarray data sets were accessed through NCBI GEO DataSets server (<http://www.ncbi.nlm.nih.gov/gds/>). Image processing and image analysis was performed with ImageJ (Rasband, NIH, USA).

2.14 Statistics

Data was statistically analysed using GraphPad Prism v5.04 (GraphPad Software, Inc., La Jolla, CA, USA) and MatLab R2014a (The MathWorks, Inc., Cambridge, UK). Data generally appeared normally distributed or data sets were too small to warrant the use of non-parametric test statistics. Therefore, parametric tests were used through this thesis. Data originating from repeated experiments were analysed by 2-way ANOVA followed by Tukey's HSD (honest significant difference) post-hoc test to compare all experimental groups to each other. Alternatively, when comparing groups to a control condition, Dunnett post-hoc test was used. Data originating from a single experiment or from repeated experiments but producing only a single experimental value for each replicate, were analysed by t-test or 1-way ANOVA. One-way ANOVA was followed by Tukey's HSD or Dunnett post-hoc analysis.

Chapter 3 Novel adherens junction regulators identified in an RNAi screen

3.1 Introduction

3.1.1 RNAi screens as tools to unravel cellular pathways

This chapter will deal with the further validation of some of the hit proteins identified during the actin-binding protein screen and Rho GTPase and effector screen performed in our laboratory. Proteins were chosen based on (i) their novelty as adherens junction (AJ) regulators, (ii) their expression in keratinocytes, (iii) availability of some molecular and functional information in the literature and (iv) the availability of tools such as antibodies. MTSS1, EF1 α and VAV2 were selected from the actin-binding protein screen. CIP4 was a hit protein, both in the actin-binding protein screen and the Rho GTPase screen. Finally, LZTFL1 was chosen from the Rho GTPase screen.

3.1.2 MTSS1

Metastasis Suppressor 1 (MTSS1), also called Missing in Metastasis (MIM), as the name suggests, is a tumour suppressor and is differentially expressed in tumour cells (Lee et al., 2002; Mertz et al., 2014). Decreased MTSS1 expression has been correlated with poor prognosis in many tumours (Liu et al., 2010a; Parr and Jiang, 2009; Xie et al., 2011). On the other hand, in colorectal cancer and hepatocellular carcinoma high MTSS1 expression is associated with early stage disease and reduced 5-year survival, respectively (Ma et al., 2007; Wang et al., 2011). Dawson *et al.* found an interesting explanation to this contradiction. They demonstrate that during early stages of tumour development in head and neck squamous cell carcinoma MTSS1 promotes proliferative signals, by increasing levels of epidermal growth factor (EGF) receptor at the cell surface (Dawson et al., 2012b). During later stages when cell density is high, increased levels of MTSS1 do not promote EGF signalling but rather cell differentiation (Dawson et al., 2012b).

MTSS1 function as tumour suppressor can further be explained with its inhibitory effects on migration through regulation of actin, GTPases and endocytosis. MTSS1 possesses an N-terminal I-BAR (Bin/amphiphysin/Rvs) domain, a proline-rich domain and a C-terminal WASP-homology 2 (WH2)-domain (Lee et al., 2002; Yamagishi et al., 2004). MTSS1 affects directional migration in *Drosophila* border cells through negative regulation of endocytosis by sequestering cortactin, a cortical actin-organising protein, away from the pro-endocytic complex endophilin/CD2AP/cortactin (Quinones et al., 2010). On the other hand, in mouse embryonic fibroblasts MTSS1 depletion decreases endocytic uptake (Yu et al., 2011). MTSS1 can dimerize via its I-BAR domain and as

homodimer is able to bind to the membrane and form membrane protrusions, which are required for MTSS1-mediated endocytosis (Cao et al., 2012; Mattila et al., 2007). MTSS1 is a regulator of the actin cytoskeleton and its I-BAR domain is able to bundle F-actin (Gonzalez-Quevedo et al., 2005; Yamagishi et al., 2004). Its proline-rich domain binds cortactin thereby promoting cortactin-mediated actin polymerisation (Lin et al., 2005a). On the other hand, MTSS1 sequesters G-actin via its WH2-domain, which inhibits Arp2/3-mediated actin polymerisation probably through competition for G-actin binding with N-WASP (Wiskott–Aldrich syndrome protein) (Lin et al., 2005a; Mattila et al., 2003).

MTSS1 also indirectly influences actin cytoskeleton through regulation of Rho small GTPases. It can bind to Rac1 via its I-BAR domain and mediate Rac1 activation, which is required for lamellipodia formation (Bompard et al., 2005). Furthermore, MTSS1 negatively regulates RhoA activity and stress fibre formation in breast cancer cells, while it is a positive regulator of both processes in mouse embryonic fibroblasts (Lei et al., 2014; Yu et al., 2011). A recent study by Saarikangas *et al.* showed that MTSS1 localises to AJ in Madin-Darby canine kidney (MDCK) cells and that it plays a role in junctional actin formation (Saarikangas et al., 2011). They also demonstrate that MTSS1 knockout mice suffer severe urinary concentration effect as a result of reduced integrity of kidney epithelia (Saarikangas et al., 2011). In collaboration with Prof Laura Machesky from the University of Glasgow we further studied the role of MTSS1 at AJ.

3.1.3 EF1 α

Transcriptional elongation factor 1 alpha (EF1 α) is ubiquitously expressed in human tissues, although different isoforms are prevalent in specific tissues (Knudsen et al., 1993). EF1 α is best known for its role in translation, where it mediates the binding of aminoacyl-tRNA to ribosomes in a GTP-dependent manner (Moldave, 1985; Reed et al., 1994). However, EF1 α has since been shown to have regulatory functions in a variety of different processes independent of translation. It is a regulator of apoptosis (Blanch et al., 2013; Duttaroy et al., 1998; Kato et al., 1997), protein degradation (Gonen et al., 1994), mRNA stability (Yan et al., 2008) and transformation (Gopalkrishnan et al., 1999; Tatsuka et al., 1992).

Moreover, EF1 α was shown to be an important regulator of the cytoskeleton being able to bind and regulate both actin and tubulin. EF1 α can directly bind and bundle microtubules (MT) but also has MT severing functions (Durso and Cyr, 1994; Shiina et al., 1994). Similarly it can bind and stabilise F-actin via two actin-binding sites in the N-

(amino acid 1-49) and C-terminus (amino acids 403-456) and is able to bundle F-actin in a unique square-packed fashion, which excludes other actin binding proteins (Demma et al., 1990; Murray et al., 1996; Owen et al., 1992). Actin- and aminoacyl-tRNA-binding are mutually exclusive and distribution between either task is regulated by several mechanisms including cellular pH and post-translational modifications such as phosphorylation by Rho-associated kinase (Izawa et al., 2000; Liu et al., 1996). This allows for precise spatial and temporal regulation of EF1 α function.

Strikingly, EF1 α was found enriched at specific actin structures at the leading edge of rat mammary adenocarcinoma and lamellipodia of chicken embryo fibroblasts, where it co-localises with β -actin mRNA (Edmonds et al., 1996; Liu et al., 2002). EF1 α is required to anchor β -actin mRNA to F-actin in these locations and this localisation is necessary for cell polarity and directional migration (Liu et al., 2002; Shestakova et al., 1999). Interestingly, EF1 α expression is increased in rat mammary adenocarcinoma, while at the same time EF1 α affinity for F-actin is reduced (Edmonds et al., 1996). The increased metastatic potential of these cells might be explained with (i) changes in actin dynamics due to the loss of tight control by EF1 α leading to random, undirected migration and/or (ii) by increased amounts of free, not-actin bound EF1 α available for protein synthesis. Its important effects on the cytoskeleton make EF1 α a prime candidate for a novel regulator of AJ.

3.1.4 VAV2

VAV2 is a member of the VAV family of Rho GEFs, comprising VAV1, VAV2 and VAV3. While VAV1 expression is restricted mainly to haematopoietic cells, VAV2 and VAV3 are expressed in a wide range of tissues (Katzav et al., 1989; Movilla and Bustelo, 1999; Schuebel et al., 1996). VAV2 is a Rho GEF for the three best characterised Rho GTPases Cdc42, Rac1 and RhoA (Abe et al., 2000; Liu and Burridge, 2000) as well as RhoB, RhoC and RhoG (Arthur et al., 2002; Schuebel et al., 1998) and has thus been implicated in many processes involving cytoskeleton remodelling including cell spreading (Marignani and Carpenter, 2001), migration (Jones et al., 2013; Liu and Burridge, 2000; Sastry et al., 2006) and integrin-mediated adhesion (Marcoux and Vuori, 2003). Furthermore, VAV2 is recruited and activated upon cadherin *trans*-interaction in EL cells (Fukuyama et al., 2006).

VAV2 is activated downstream of integrin, cadherin and receptor tyrosine kinases (RTK) such as EGFR, platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR) (Fukuyama et al., 2006; Garrett et al.,

2007; Marignani and Carpenter, 2001; Pandey et al., 2000). VAV2 activation requires phosphorylation, usually through RTK or c-Src (Fukuyama et al., 2006; Garrett et al., 2007; Tamás et al., 2003). Phosphorylated VAV2 then activates Rho GTPases, which leads to remodelling of the cytoskeleton. These signalling pathways have to be tightly controlled and temporally limited and it has been shown in mammary epithelial cells that EGF-induced VAV2 signalling is abrogated by ubiquitination and degradation of VAV2 (Duan et al., 2011). VAV2 is important for Rho GTPase signalling and cytoskeletal remodelling and is a target of the oncogene c-Src. Therefore, it comes as no surprise that hyperphosphorylated, overactive VAV2 has been linked to increased invasiveness in head and neck squamous cell carcinoma (Patel et al., 2007). Although VAV2 has previously been shown to be activated downstream of cadherin *trans*-interaction, it has not been tested whether VAV2 is required for AJ formation. I therefore decided to further study its mechanism downstream of cadherin and to test whether RNAi screens can predict novel signalling pathways.

3.1.5 CIP4

Three different groups identified CIP4 in different contexts. A partial CIP4 sequence was first isolated by Lee *et al.* as a thyroid hormone receptor-interacting protein and was therefore called TRIP10 (Lee et al., 1995). A year later Tsuji *et al.*, identified the full length rat homologue as a protein that can transfer salt-tolerance when transfected into salt-intolerant yeast mutants and they thus call it salt-tolerant protein (STP) (Tsuji et al., 1996). In 1997, the human full-length protein was identified by Aspenström as a Cdc42-interacting protein and consequently he called it CIP4 (Aspenström, 1997). CIP4 is present as several different isoforms in human, some of which have been linked to cancer (Tsuji et al., 2006; Wang et al., 2002).

CIP4 possesses an N-terminal F-BAR (Fer-CIP4 homology Bin/amphiphysin/Rvs) domain, a central feline sarcoma-related (FER)-homologous domain and a C-terminal SH3 domain (Aspenström, 1997; Tsujita et al., 2006). CIP4a, the most abundant CIP4 isoform, is an important regulator of endocytosis and participates in different steps of the process. Its F-BAR domain is thought to aid the formation of membrane invaginations (Tsujita et al., 2006) and furthermore, CIP4a can promote vesicle scission by recruiting dynamin-2 to endocytic vesicles (Tsujita et al., 2006). Importantly, CIP4a integrates cytoskeletal remodelling with endocytosis by recruiting the actin regulator N-WASP, which in turn activates the actin-nucleating protein Arp2/3 to promote actin polymerization and aid vesicle transportation (Fricke et al., 2009; Tian et al., 2000; Tsujita et al., 2006).

Different targets for CIP4-mediated endocytosis have been identified over the years. Importantly, CIP4 is involved in the endocytosis of DE-cadherin, the *Drosophila* homologue of E-cadherin (Leibfried et al., 2008). During this process a complex of Cdc42 and the polarity complex components Partitioning defective 6 (Par6) and atypical protein kinase C (aPKC) recruits CIP4, which recruits the vesicle-scissoring GTPase dynamin and N-WASP (Leibfried et al., 2008). CIP4a also plays a role in late stages of EGFR endocytosis specifically in the transportation of the endocytotic vesicles to the lysosome (Hu et al., 2009) and in the endocytosis of the growth factor receptor PDGFR β (Toguchi et al., 2010). A fourth well-established CIP4-dependent trafficking event is glucose transporter type 4 (GLUT4) translocation. The CIP4 isoform CIP4h binds to the active Rho GTPase TC10 (RhoQ) and insulin stimulation causes TC10-mediated translocation of CIP4h to the membrane (Chang et al., 2002). There, CIP4h sequesters the Rab GTPase GEF Gapex-5 to reduce Rab31 activity to change vesicle transport routes and allow GLUT4 transport to the membrane (Chang et al., 2002; Lodhi et al., 2007). Later, when blood glucose levels decline, CIP4a is involved in GLUT4 endocytosis (Hartig et al., 2009). The physiological importance of CIP4 for endocytosis and GLUT4 regulation has been confirmed with a CIP4 knockout mouse. The mouse strain showed increased glucose uptake due to higher GLUT4 levels at the muscle membrane as well as other defects in endocytotic pathways (Feng et al., 2010).

However, CIP4 not only regulates actin dynamics downstream of endocytosis, but was also shown to be required for lamellipodia formation and directional migration in chronic lymphocytic leukaemia (Malet-Engra et al., 2013) and invadopodia formation and migration of breast cancer cells (Pichot et al., 2010). In contrast to Pichot *et al.*, Hu *et al.* reports an inhibitory effect of CIP4 on invasion of breast cancer cells by promoting the internalisation of transmembrane type I matrix metalloprotease from invadopodia (Hu et al., 2011). CIP4 also inhibits neurite formation by conveying actin rips formation inside lamellipodia at the protruding edge membrane of neurons (Saengsawang et al., 2012; Saengsawang et al., 2013). In this process CIP4 acts downstream of Rac1 and through the actin-regulatory proteins Wiskott-Aldrich syndrome protein family member 1 (WASF1), Dishevelled-associated activator of morphogenesis 1 (DAAM1) and Ena/VASP (vasodilator-stimulated phosphoprotein-like), which mediate the formation of unbranched actin filaments (Saengsawang et al., 2013). Together, CIP4 involvement in actin remodelling and in DE-cadherin endocytosis in *Drosophila* suggests that CIP4 is a promising candidate for a regulator of AJ in human keratinocytes.

3.1.6 LZTFL1

Very little is known about leucine zipper transcription factor like 1 (LZTFL1). The protein was named in reference to its predicted secondary structure and motifs comprising a leucine zipper motif and a coiled-coil domain, which are typical features of transcription factors (Kiss et al., 2001). The LZTFL1 gene is located on chromosome 3 at position 3p21.31, which is a region frequently lost in solid tumours (Petursdottir et al., 2004). In addition, the LZTFL1 gene lies within the minimum common region of loss of the 3p21.31 region in acute lymphoblastic leukaemia patients (Tsuzuki et al., 2007) and is therefore thought to be a tumour suppressor. First direct evidence for the tumour suppressive function of LZTFL1 came from a study by Wei *et al.* (Wei et al., 2010). Whereas intense LZTFL1 staining was observed in normal tissues, weak staining was detected in corresponding tumour tissue. Patients with stomach cancer have an inverse correlation between LZTFL1 levels and Tumour-Node-Metastasis (TNM) stage as well as a significant correlation between LZTFL1 expression and survival. In addition, LZTFL1 overexpression in HeLa cells decreases colony formation and migration *in vitro* and reduces tumour growth in nude mice (Wei et al., 2010). Immunofluorescence staining showed co-localisation of E-cadherin and LZTFL1 at the membrane of the human intestinal epithelial cell line HT-29 (Wei et al., 2010). LZTFL1 will be the focus of chapter 4 and will there be introduced in greater detail. However, the knowledge so far suggests that LZTFL1 is a tumour suppressor that localises to membranes. Therefore, I postulated that LZTFL1 might be involved in AJ regulation.

3.2 Hypothesis and aims

I hypothesise, that an RNAi screen can reliably predict novel regulators of AJ and that similar phenotypes can suggest interacting partners in a signalling pathway. I aim to (i) validate the effect of depletion of MTSS1, EF1 α , CIP4, VAV2 and LZTFL1 on newly forming AJ as indicated by previous RNAi screens and (ii) to test whether EF1 α , CIP4 and VAV2 can interact, based on their similar screen phenotypes.

3.3 Results

I optimised the siRNA-mediated depletion of the proteins of interest with the four single oligonucleotides from the RNAi screen, by identifying the optimal time point for depletion and testing different siRNA concentrations (data not shown). Protein depletion was confirmed by Western blot. The most efficient two oligonucleotides were used for experiments in low calcium keratinocytes.

3.3.1 MTSS1 is required to stabilise adherens junctions

MTSS1 was selected from the actin-binding protein RNAi screen. The phenotype of its depletion was a negative effect on E-cadherin as well as junctional actin. However, the effect on junctional actin was not strong enough for MTSS1 to be considered a candidate protein on this parameter (data not shown). At the time that my work on MTSS1 was done, the study by Saarikangas *et al.* was not published and in collaboration with Laura Machesky (University of Glasgow) we aimed to show a function of MTSS1 downstream of AJ. I used aggregation assays with the aim to test whether MTSS1 depletion destabilises cell-cell contacts. For this purpose, confluent layers of keratinocytes maintained in low calcium medium were treated with control siRNA or siRNA targeting MTSS1 (Figure 3.1 B). Cells were detached with trypsin in the presence of calcium to protect E-cadherin from being clipped by trypsin. Cells were counted, pelleted and resuspended in standard calcium medium to contain the same number of cells per millilitre. Single cell suspensions were allowed to aggregate for two hours before aggregates were disrupted by pipetting (Figure 3.1 A). Quantification of the size of the resulting fragments, showed that MTSS1 depletion caused a significant decrease in disaggregate size as compared to control-treated cells, indicating weaker cell-cell contacts (Figure 3.1 C).

3.3.2 EF1 α modulates soluble E-cadherin levels at junctions

EF1 α has been implicated in the regulation of a variety of actin-driven processes. However, so far no study has addressed a role for EF1 α in actin remodelling underlying AJ formation. In our actin-binding protein RNAi screen, EF1 α depletion strongly decreased junctional actin formation and to a lesser extent E-cadherin (data not shown). In fact, the effect on E-cadherin was not strong enough for EF1 α to be considered a candidate protein on this parameter. In order to test EF1 α function at AJ, I depleted EF1 α in monolayers of keratinocytes grown in low calcium medium. The formation of adherens junctions was induced by the addition of calcium to the medium and cells were stained for E-cadherin (Figure 3.2 A+B).

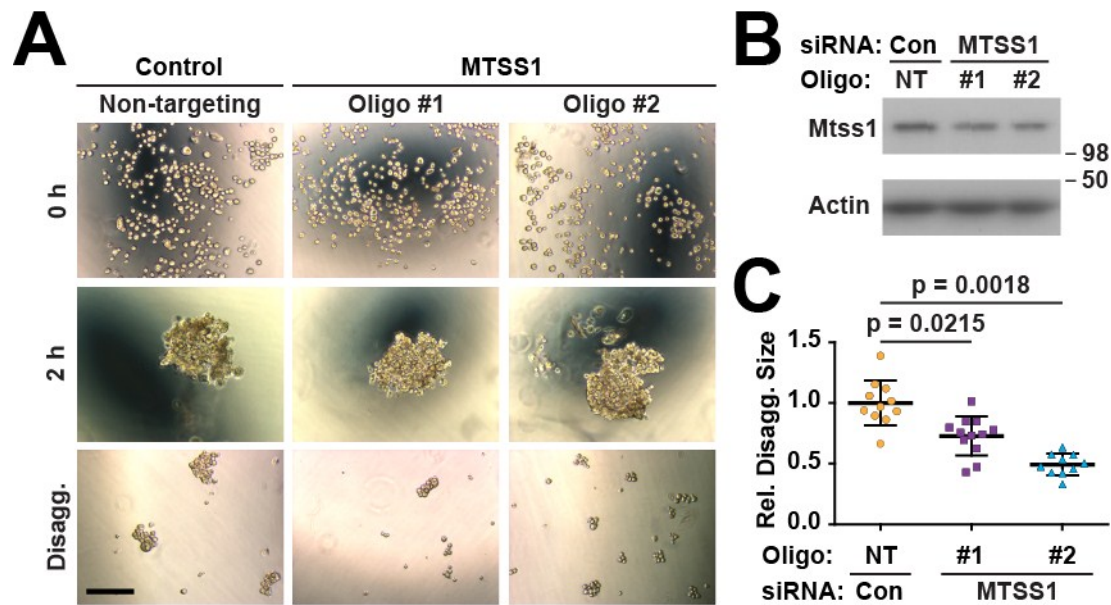


Figure 3.1: MTSS1 depletion weakens cell-cell contacts. Keratinocytes treated with non-targeting control (NT) or MTSS1 (oligos #1 or #2) siRNA were made into a single cell suspension (0 h) and were allowed to aggregate in hanging drops (2 h). Clusters were then disrupted by trituration (Disagg.). A) Representative images of an aggregation assay in control and MTSS1 depleted keratinocytes. B) Western blot of treated samples probed with antibodies against MTSS1 or actin as loading control. Molecular weight markers are shown on the right. Average depletion of MTSS1 with oligo #1 $29 \pm 1\%$ and with oligo #2 $38 \pm 10\%$. C) Quantification of the disaggregate size relative to the original aggregate (2 h). Graph represents mean $\pm$ SD. Data was analysed statistically by two-way ANOVA and Dunnett post-hoc test. N = 11 for NT, N = 13 for MTSS1 #1, N = 9 for MTSS1 #2, from two experiments. Scale bar = 200 μ m.

The amount of junctional E-cadherin was measured by thresholding and determining the percentage thresholded area. Surprisingly, EF1 α did not reduce E-cadherin levels as in the RNAi screen but instead significantly increased the amount found at the junction (Figure 3.2 C). The change is only significant for oligonucleotide #1, because only one repeat with oligonucleotide #2 could be included in the analysis.

My hypothesis was that EF1 α is required to remodel actin underneath AJ. I therefore tested whether the increased levels of E-cadherin at contacts are functional and linked to the actin cytoskeleton. The experiment was performed as above, but before fixation, cells were pre-extracted with CSK buffer containing 0.5% Triton X-100 to remove soluble proteins and retain insoluble, cytoskeleton-bound proteins. Cells were stained for E-cadherin (Figure 3.3 A) and the amount of junctional E-cadherin measured. In contrast to experiments without pre-extraction, EF1 α did not significantly alter E-cadherin levels at junctions (Figure 3.3 C). Instead, EF1 α depletion with oligonucleotide #2 showed a trend to decreased E-cadherin levels, although the change was not significant based on the two repeats evaluated. In conclusion this indicated, that EF1 α is required to limit the amount of E-cadherin at newly formed AJ and that a role for EF1 α in E-cadherin anchoring could not be proven.

3.3.3 CIP4 is a regulator of E-cadherin surface levels

CIP4 is a regulator of vesicular trafficking of different transmembrane proteins including DE-cadherin in *Drosophila* (Leibfried et al., 2008). I thought to investigate whether CIP4 could play a similar role in human keratinocytes. Results from our RNAi screens were controversial (data not shown). CIP4 was identified as a candidate protein for the E-cadherin parameter in both screens, but showed a negative effect in the actin-binding protein screen but increased junctional E-cadherin levels in the Rho GTPase screen.

CIP4 was depleted in keratinocytes grown in low calcium, junction formation was induced and cells fixed and stained for E-cadherin (Figure 3.4 A+B). Quantification of junctional levels showed a partial loss of E-cadherin from AJ similar to the phenotype observed for CIP4 depletion in the actin-binding protein RNAi screen (Figure 3.4 C). Total levels of E-cadherin were not affected by CIP4 depletion, and neither were the levels of α - or β -catenin or plakoglobin (Figure 3.4 B).

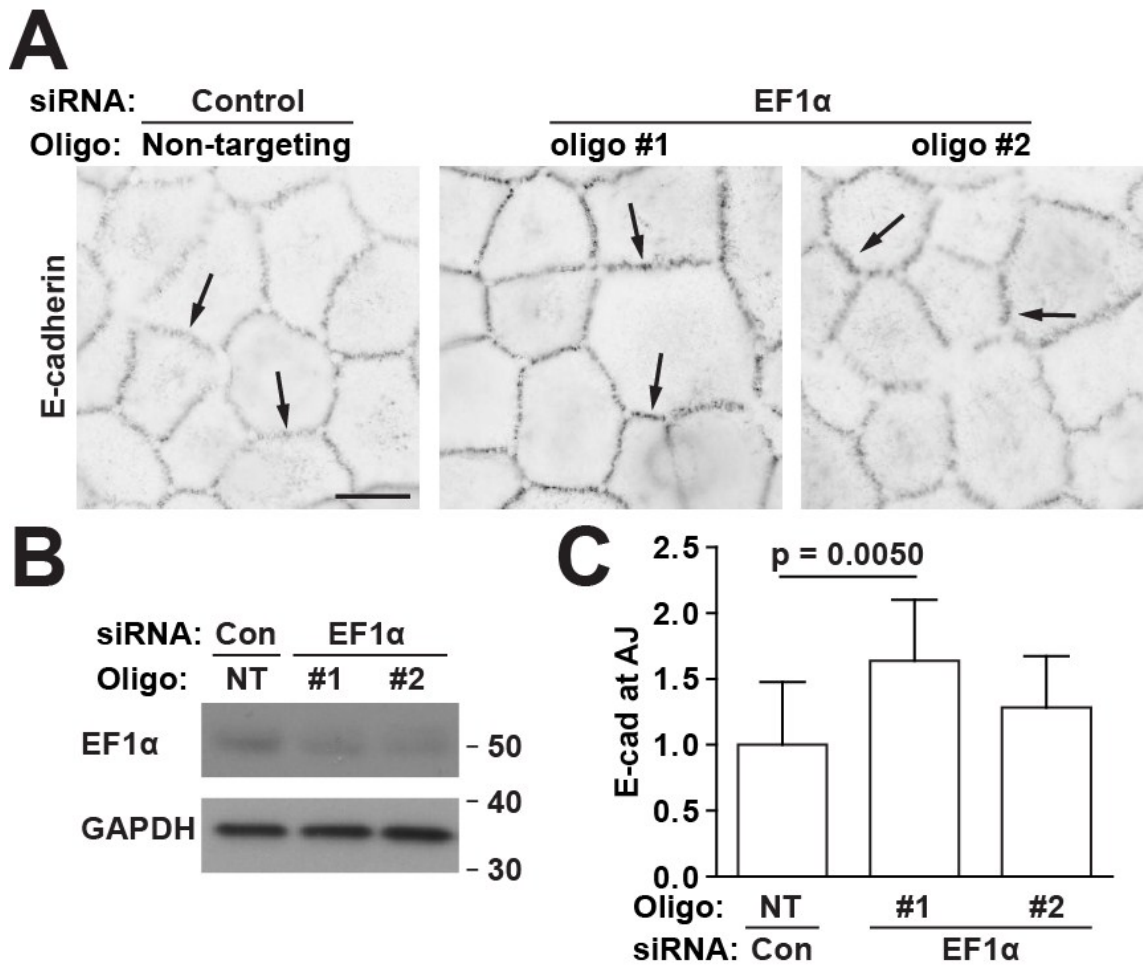


Figure 3.2: EF1α depletion increases levels of junctional E-cadherin. Monolayers of keratinocytes in low calcium medium and treated with non-targeting control (NT) or EF1α (oligos #1 or #2) siRNA were switched to standard calcium medium for 30 minutes before fixation and staining for E-cadherin. A) Inverted immunofluorescence images of E-cadherin staining. Arrows point at representative junctions. B) Western blot of treated samples probed with antibodies against EF1α or GAPDH as loading control. Molecular weight markers are shown on the right. Average depletion of EF1α with oligo #1 $44 \pm 2\%$ and with oligo #2 49% . C) Quantification of E-cadherin at junctions represented as thresholded E-cadherin-stained area over total image area and relative to control cells. Graph shows mean $\pm$ SD. Data was analysed statistically by two-way ANOVA and Dunnett post-hoc test. N = 13 for NT, N = 12 for EF1α oligo #1 (both from 2 experiments), N = 5 for EF1α oligo #2 (from 1 experiment). Scale bar = 20 μ m.

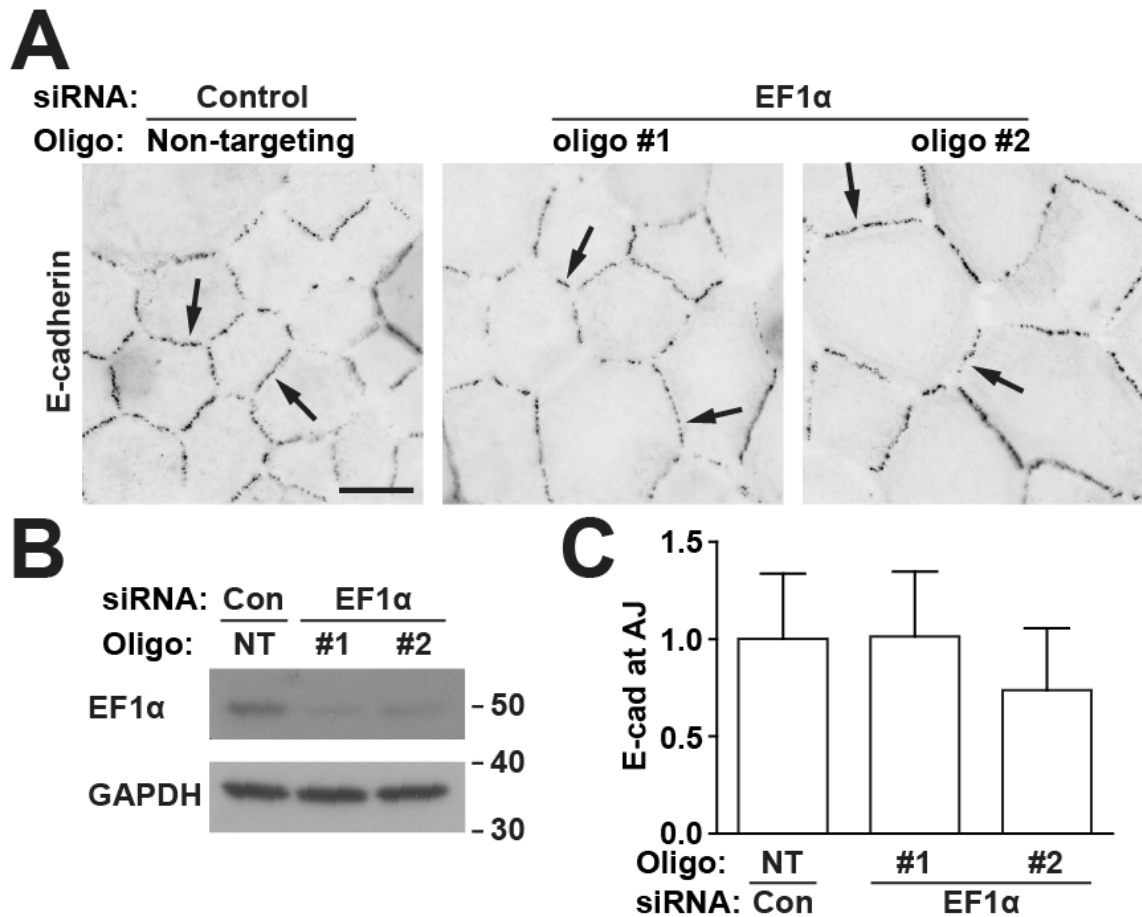


Figure 3.3: The amount of insoluble, junctional E-cadherin does not change when EF1α is depleted. Monolayers of keratinocytes in low calcium medium and treated with non-targeting control (NT) or EF1α (oligos #1 or #2) siRNA were switched to standard calcium medium for 30 minutes. Pre-extracted with 0.5% TritonX-100 buffer, cells were subsequently fixed and stained for E-cadherin. A) Inverted immunofluorescence images of E-cadherin staining of Triton X-100 insoluble proteins. Arrows point at representative junctions. B) Western blot of treated samples probed with antibodies against EF1α or GAPDH as loading control. Molecular weight markers are shown on the right. Average depletion of EF1α with oligo #1 $68 \pm 13\%$ and with oligo #2 $65 \pm 2\%$. C) Quantification of E-cadherin at junctions represented as thresholded E-cadherin-stained area over total image area and relative to control cells. Graph represents mean $\pm$ SD. Two-way ANOVA and Dunnett post-hoc test show no significant difference between treatments. N = 20 for NT, N = 15 for EF1α oligo #1 and N = 14 for oligo #2, from two experiments. Scale bar = 20 μ m.

Levels of E-cadherin at newly formed junctions were reduced, but total E-cadherin levels were not changed by CIP4 depletion. Considering the involvement of CIP4 in receptor transport, it was therefore likely that this change was caused by defects in E-cadherin trafficking. To test this possibility, I determined surface and total levels of E-cadherin during a time course of junction assembly using biotinylation assays (Figure 3.5 A+B).

Unexpectedly and in contrast to the results shown in Figure 3.4 B, total levels of E-cadherin were significantly increased with CIP4 depletion in cells without cell-cell contacts ($p = 0.0034$) (0 min, Figure 3.5 C). This difference might potentially be explained by differences in the lysis buffer composition and sample preparation between both experiments. Protein lysates were normally prepared at room temperature from cells straight out of the incubator. Lysates for biotinylation assays were prepared on ice, after cells had been treated on ice for about 45 minutes. The lysis temperature could affect protein solubility. In addition, the lysis buffer used in the biotinylation assay was optimised for extraction of membrane proteins and contained 0.1% SDS and 1% Triton X-100, while the general lysis buffer used for most Western blots was more stringent containing 0.5% SDS and 10% glycerol. A bias towards preferentially solubilising membrane proteins is supported by comparison of the graphs showing total and cell-surface levels of E-cadherin (Figure 3.5 C+D). The graphs of total and surface E-cadherin show similar trends over time. Upon other reasons, similar protein populations in both sample types could explain this effect. Based on the composition of the lysis buffer, it is likely that total E-cadherin does not correspond to a true total cellular pool of E-cadherin, but rather a total surface pool of E-cadherin containing both biotinylated and unbiotinylated E-cadherin, since biotinylation efficiency is not 100%.

Over the time course of 30 minutes, amounts of total extracted E-cadherin constantly increased in control cells, while levels in CIP4-depleted cells declined (Figure 3.5 C). At 30 minutes, levels were significantly lower in CIP4 depleted as compared to control cells ($p = 0.0238$) (Figure 3.5 C). Similarly to total extracted E-cadherin, biotinylated E-cadherin surface levels of CIP4 siRNA-treated cells were significantly higher compared to control ($p = 0.0106$) when AJ were not in place (Figure 3.5 D). Over the course of a 30 minutes calcium switch, biotinylated E-cadherin amounts at the surface of CIP4-depleted cells constantly decreased, while they increased in control cells. By 30 minutes after junction formation, surface levels were about 25% lower in CIP4-depleted

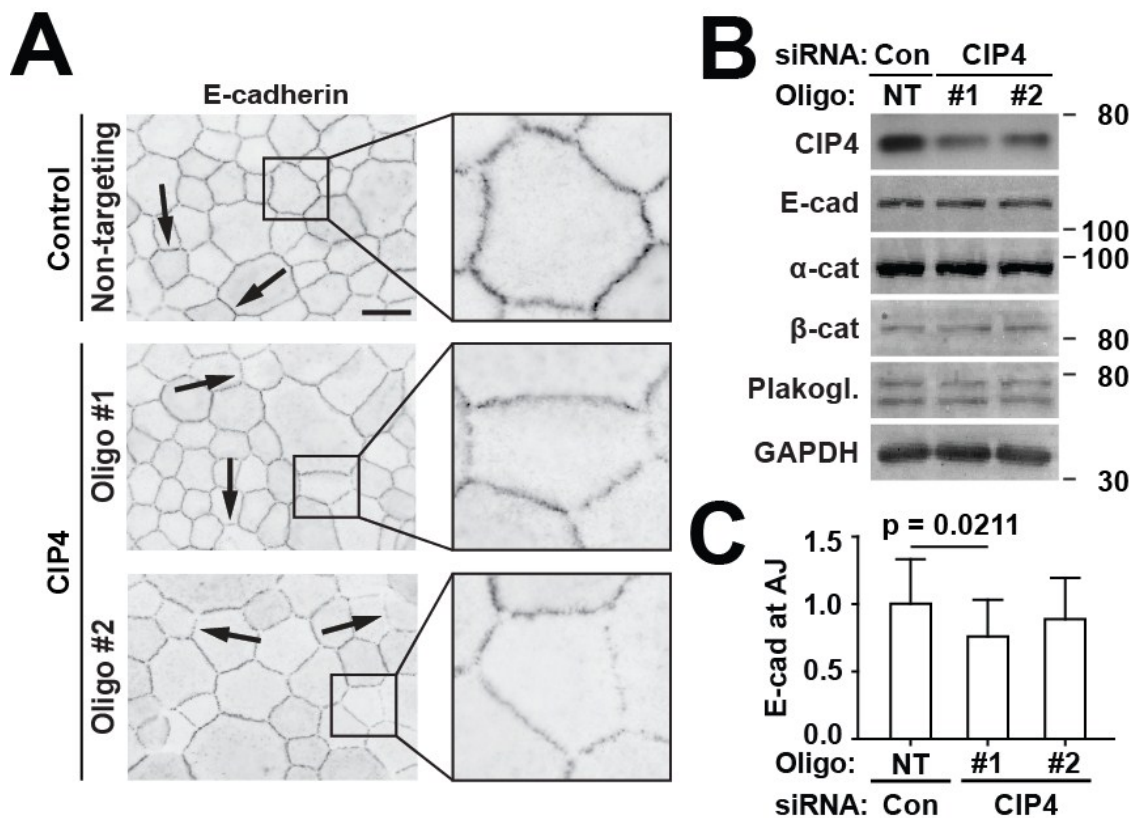


Figure 3.4: RNAi-mediated reduction of CIP4 decreases the amount of E-cadherin at newly formed adherens junctions.

Monolayers of keratinocytes in low calcium medium and treated with non-targeting control (NT) or CIP4 (oligos #1 or #2) siRNA were switched to standard calcium medium for 30 minutes before fixation and staining for E-cadherin. A) Inverted immunofluorescence images of E-cadherin staining. Arrows point at representative junctions and squares on the right show magnified images of the boxed area. B) Western blot of treated samples probed with antibodies against CIP4, E-cadherin, α -catenin, β -catenin, plakoglobin or GAPDH as loading control. Molecular weight markers are shown on the right. Average depletion of CIP4 with oligo #1 $51 \pm 7\%$ and with oligo #2 $72 \pm 8\%$. C) Quantification of E-cadherin at junctions represented as thresholded E-cadherin-stained area over total image area and relative to control cells. The graph shows mean $\pm$ SD. Data was analysed statistically by two-way ANOVA and Dunnett post-hoc test. N = 22 for NT, N = 21 for CIP4 oligo #1 and N = 21 for oligo #2, from 3 experiments. Scale bar = 20 μ m.

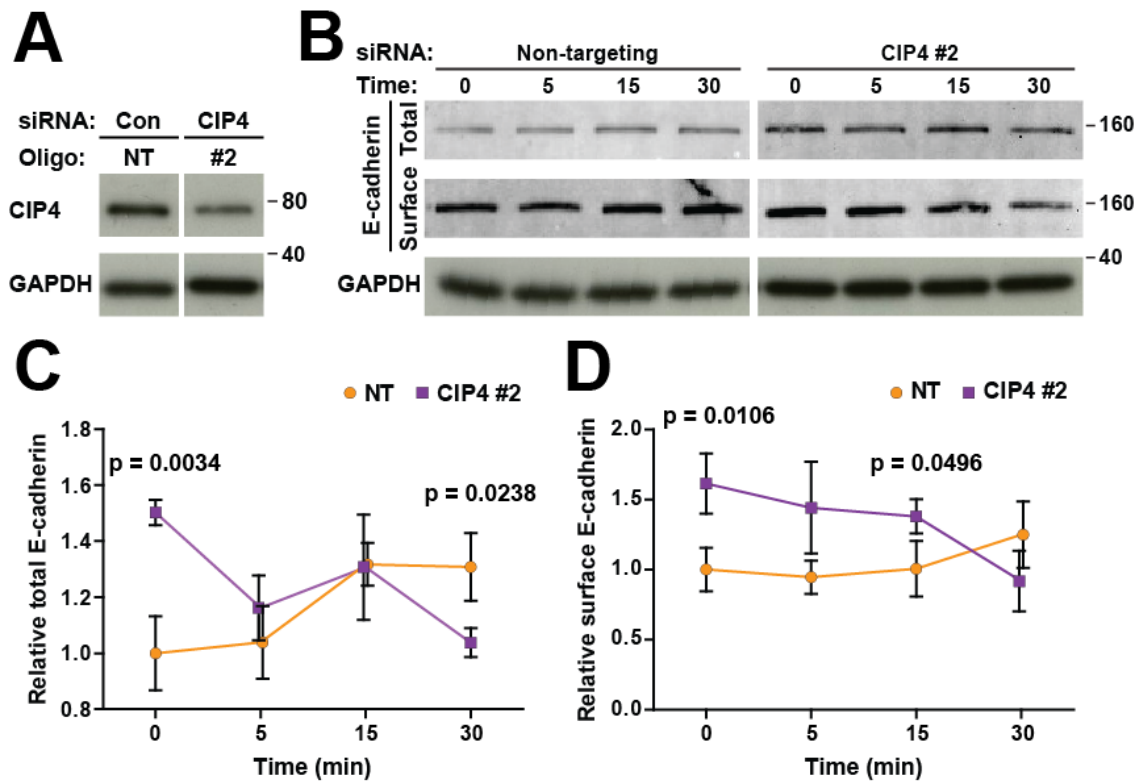


Figure 3.5: CIP4 depletion interferes with E-cadherin levels at the surface.

Keratinocytes grown in the absence of cell-cell contacts were treated with non-targeting control (NT) or CIP4 siRNA oligos and induced to assemble junctions by addition of 1.8mM CaCl_2 for 0, 5, 15 or 30 minutes. Cells were then biotinylated and surface or total pools of E-cadherin collected. A) Western blot of treated samples probed with antibodies against CIP4 or GAPDH as loading control. Molecular weight markers are shown on the right. Average depletion of CIP4 with oligo #2 $42 \pm 12\%$. B) Western blot of treated samples. Ten percent of the total pool were loaded and probed with antibodies against E-cadherin or GAPDH as loading control and 100% of the surface pool was loaded and probed with an antibody against E-cadherin. Molecular weight markers are shown on the right. C-D) Levels of total (C) or surface (D) levels of E-cadherin were quantified, corrected to GAPDH levels and presented relative to time zero of non-targeting controls. Differences between samples at a given time point were assessed using t-test. Graphs show mean $\pm$ SD. N = 3.

cells compared to control, although the difference was not significant based on three experimental repeats (Figure 3.5 D).

The decreased E-cadherin surface levels corresponded well with the reduction in junctional E-cadherin levels observed by immunofluorescence (CIP4 #1: 25%, Figure 3.4 C). I assumed that total cellular E-cadherin levels do not change with CIP4 depletion, as indicated by Western blotting of stringent cell lysates of control and CIP4-depleted cells (Figure 3.4 B). Therefore, the results from the biotinylation assay suggested, that the reduction of junctional E-cadherin observed in CIP4 knock-down cells (Figure 3.4 C) might be caused by defects in E-cadherin trafficking to the cell surface.

3.3.4 CIP4 interacts with the Rho GEF VAV2

In order to further investigate the mechanism through which CIP4 regulates AJ, I aimed to identify novel interaction partners of CIP4. Phenotype similarities in RNAi screens and protein interaction networks based on predicted interactions from online databases can be powerful to infer pathways and specific interactions. Depletion of EF1 α or CIP4 both showed weak defects on junctional E-cadherin in the actin-binding protein RNAi screen, while reduced levels of VAV2 decreased E-cadherin levels at junctions more strongly. I hypothesised, that nodes of a signalling pathway might cause similar effects upon depletion, but that the magnitude of the defect would depend on the hierarchy within the pathway. The further upstream in the signalling pathway a given protein is, the stronger would the effect be, because multiple downstream targets might be affected. I therefore predicted that VAV2 is upstream of EF1 α and/or CIP4 in a potential signalling pathway.

GST-CIP4 was produced by bacterial protein production (Figure 3.6 A). GST-CIP4 on beads was incubated with lysates from keratinocytes grown in standard calcium and tested for the presence of EF1 α and VAV2. VAV2 was found specifically bound to CIP4 beads, but not EF1 α (Figure 3.6 B). The interaction between CIP4 and VAV2 was confirmed by co-immunoprecipitation (Figure 3.6 C), while no interaction was detected between VAV2 and EF1 α (data not shown). Finally, I wanted to test whether the interaction between CIP4 and VAV2 was regulated. Lysates prepared at different time points after induction of cell-cell contacts were used for pull-down assays with CIP4-bound beads. No change in association between both proteins was observed over 60 minutes of calcium switch (Figure 3.6 D). In summary, these experiments identified

VAV2 as a novel partner of CIP4 at steady state and during calcium-induced AJ formation.

Since the association between CIP4 and VAV2 was not modulated during AJ formation, this raised the questions, whether this interaction is at all required for contact formation, or whether the two proteins interact in the regulation of another process. I first confirmed whether VAV2 was required for AJ formation in primary keratinocytes, since so far it has only been shown that VAV2 is activated downstream of cadherin *trans*-interaction in EL-cells, fibroblasts constitutively overexpressing E-cadherin (Fukuyama et al., 2006). VAV2 depletion in low calcium keratinocytes seemed not to influence total levels of AJ components (Figure 3.7 B). AJ formation was induced for 30 minutes (Figure 3.7 A). Quantification showed decreased amounts of junctional E-cadherin in VAV2-depleted cells as compared to control siRNA treated cells (Figure 3.7 C). This result confirmed that VAV2 is required for AJ formation in keratinocytes.

Since a pool of CIP4 seems associated with VAV2 and VAV2 is recruited to clustered E-cadherin (Fukuyama et al., 2006), I hypothesised that CIP4 might be recruited to nascent cell-cell contacts through VAV2. To this end, I first studied the localisation of CIP4 during cell-cell contact formation. In low calcium keratinocytes, CIP4 was uniformly distributed throughout the cytoplasm (0 min, Figure 3.8 A). Upon junction formation, CIP4 was recruited to newly formed cell-cell contacts early on and maintained at junctions at least until one hour after contact induction (Figure 3.8 A).

In order to test whether VAV2 is required for CIP4 localisation to new junctions, VAV2 was depleted in low calcium keratinocytes and cell-cell contacts were induced for 30 minutes before staining for E-cadherin (data not shown) and CIP4 (Figure 3.8 B). Quantification of junctional CIP4 showed that CIP4 recruitment to cell-cell contacts was significantly inhibited by VAV2 depletion (Figure 3.8 C), suggesting that VAV2 is necessary for CIP4 spatial distribution.

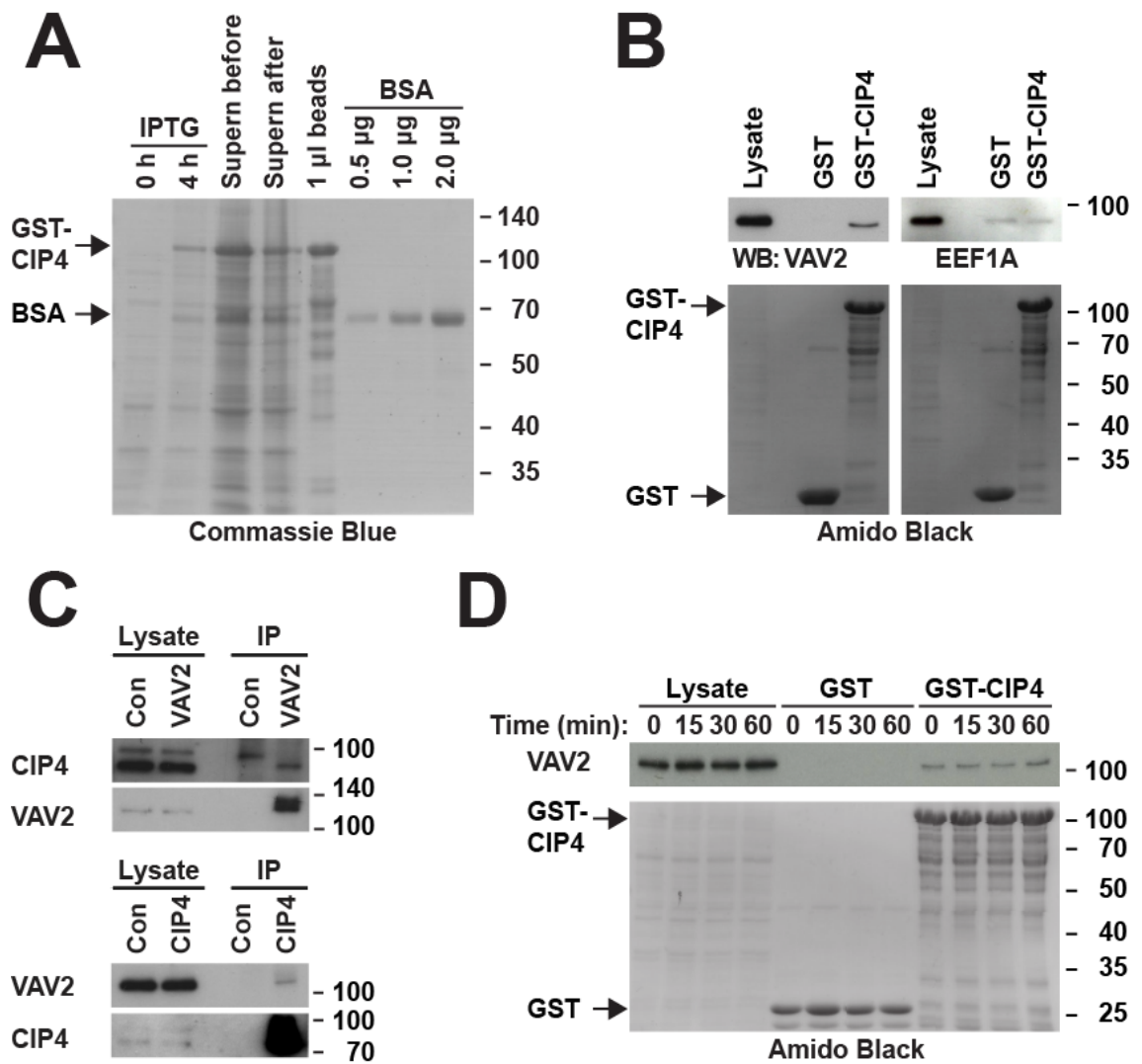


Figure 3.6: CIP4 interacts with VAV2, but not EF1 α , in keratinocytes. A) GST-CIP4 was produced in BL21 *E.coli* by induction with IPTG for 4 hours with lane 1 showing bacterial lysate before induction and lane 2 representing lysate after induction. Cells were lysed (Supern before) and GST-CIP4 was immobilised on glutathione sepharose beads (1 μ l beads). Lane 4 shows bacterial lysate after incubation with glutathione beads. The amount of CIP4 on the beads was estimated by comparison to different amounts of BSA (lanes 6-8). B-C) Protein lysates from keratinocytes maintained in standard Ca²⁺ medium were tested for interaction between VAV2 and CIP4 by pull down (B) or immunoprecipitation (C). Samples were then processed for western blot and probed for proteins as noted on the blots. B) N = 3; C) N = 2. D) Beads coated with GST alone or GST-CIP4 were incubated with lysates from keratinocyte monolayers maintained in low calcium medium (0 minutes), or after the addition of 1.8 mM CaCl₂ for different lengths of time. Bound protein complexes were probed for VAV2. N = 3

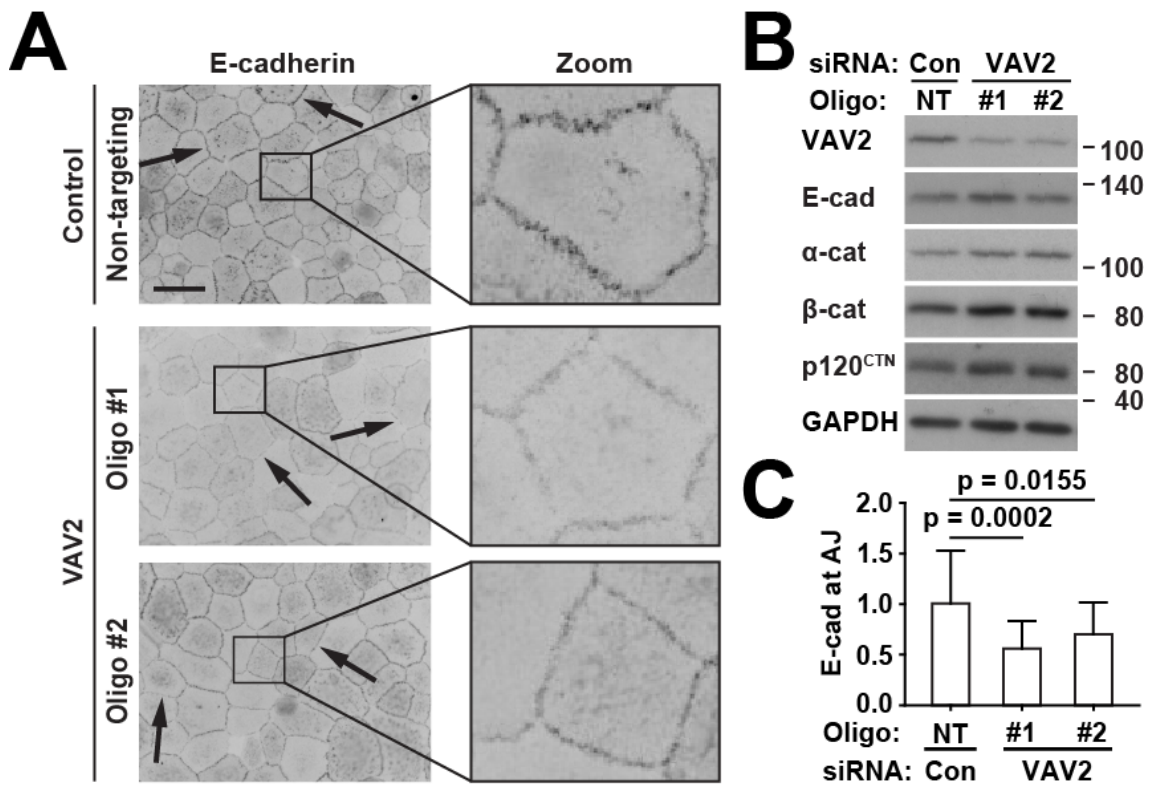


Figure 3.7: VAV2 depletion reduces E-cadherin levels in nascent adherens junctions in keratinocytes. Monolayers of keratinocytes in low calcium medium and treated with non-targeting control (NT) or VAV2 (oligos #1 or #2) siRNA were switched to standard calcium medium for 30 minutes before fixation and staining for E-cadherin. A) Inverted immunofluorescence images of E-cadherin staining. Arrows point at representative junctions and squares on the right show magnified images of the boxed area. B) Western blot of treated samples probed with antibodies against VAV2, E-cadherin, α-catenin, β-catenin, p120^{CTN} or GAPDH as loading control. Molecular weight markers are shown on the right. Average depletion of VAV2 with oligo #1 $52 \pm 20\%$ and with oligo #2 $45 \pm 9\%$. C) Quantification of E-cadherin at junctions represented as thresholded E-cadherin-stained area over total image area and relative to control cells. The graph shows mean $\pm$ SD. Data was analysed statistically by two-way ANOVA and Dunnett post-hoc test. N = 24 for NT, N = 28 for VAV2 oligo #1 and N = 24 for oligo #2, from 3 experiments. Scale bar = 20 μ m.

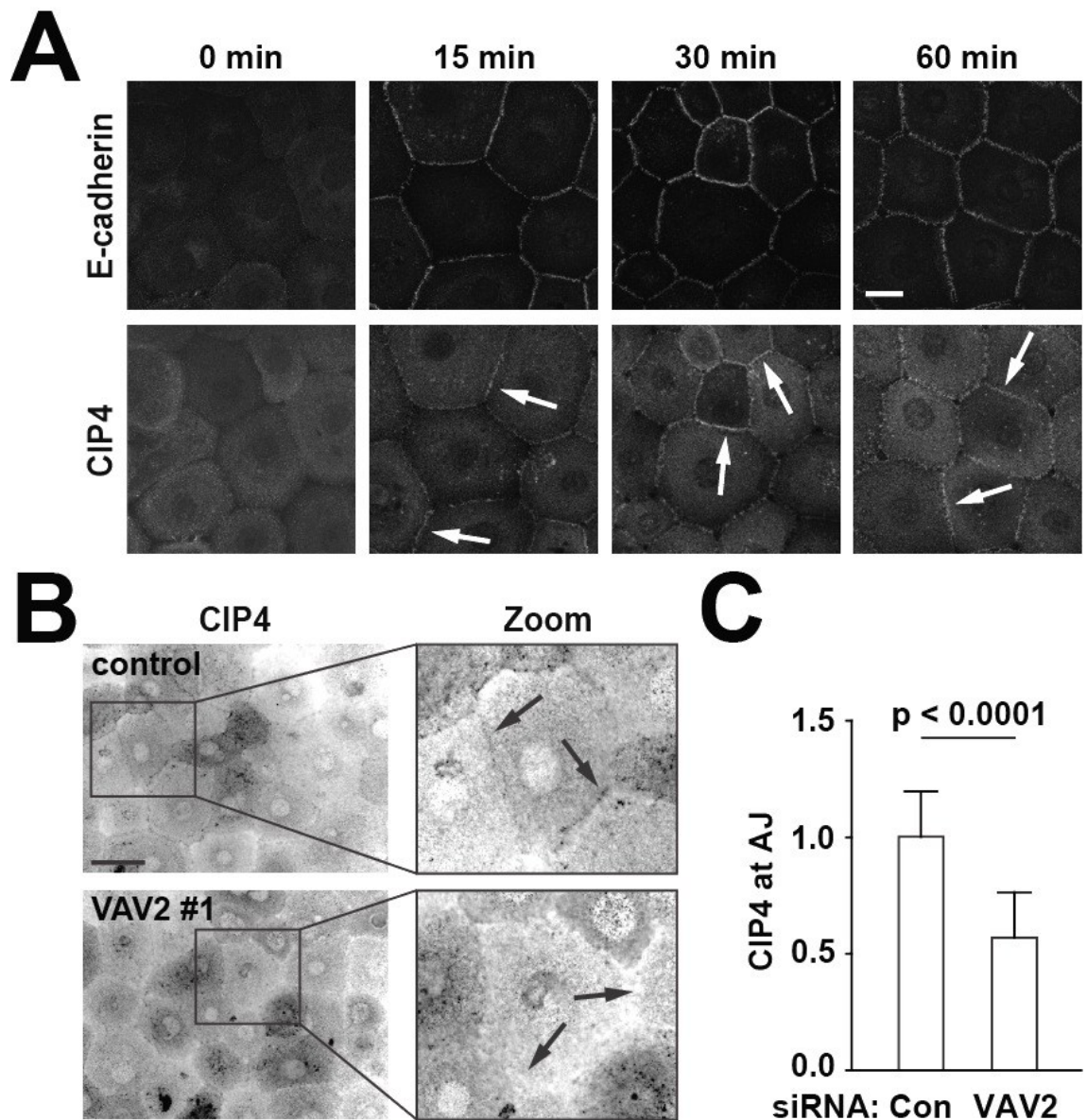


Figure 3.8: CIP4 is recruited to newly forming cell-cell contacts in a VAV2-dependent manner. A) Monolayers of keratinocytes in low calcium medium were switched to standard calcium medium for a 0 to 60 minutes before fixation and staining for CIP4 and E-cadherin. Confocal images through the zonula adherens were taken at different time points of a calcium switch. If more than one confocal slice was passing through the zonula adherens the relevant slices were summed using ImageJ. Arrows point at CIP4 at cell-cell contacts. B) Monolayers of keratinocytes in low calcium medium and treated with non-targeting control (NT) or VAV2 siRNA (average VAV2 depletion $60 \pm 17\%$) were switched to standard calcium medium for 30 minutes before fixation and staining for CIP4. Shown are representative inverted widefield immunofluorescence images and magnifications of the boxed area. Arrows point at adherens junctions. C) Quantification of CIP4 at junctions represented as thresholded CIP4-stained area over total adherens junction area (E-cadherin staining not shown) and relative to control cells. The graph shows mean $\pm$ SD. Data was analysed statistically by two-way ANOVA and Dunnett post-hoc test. N = 30 for NT and N = 18 for VAV2 oligo #1, from 3 experiments. Scale bar = 20 μ m.

3.3.5 LZTFL1 disturbs nascent AJ and is required for cell-cell contact stability

A final screen candidate protein studied in this chapter is LZTFL1, a widely uncharacterised protein. In the Rho GTPase and effector screen, LZTFL1 had a moderate negative effect on E-cadherin and junctional actin as well as a moderate positive effect on actin thin bundles (data not shown).

Keratinocytes grown to confluence in the absence of cell-cell contacts were treated with control or LZTFL1 siRNA before induction of junctions for 30 minutes and staining for E-cadherin and actin (Figure 3.9). Overall levels of AJ components did not change with LZTFL1 depletion (Figure 3.9 A). LZTFL1 knock-down produced a mild but distinct phenotype on both E-cadherin and actin staining. In LZTFL1-depleted cells, E-cadherin staining appeared less regular along junctions both in terms of width of staining as well as intensity. Furthermore, small gaps could be regularly observed in the E-cadherin staining and more so in the junctional actin (Figure 3.9 B). Because of the subtleness of the effect, a quantification of the phenotype proved difficult.

Therefore, I decided to test the strength of the newly formed cell-cell contacts in order to confirm an effect of LZTFL1 depletion on AJ. To test contact strength, a dispase assay was employed. Confluent keratinocytes were treated with control or LZTFL1 siRNA before induction of contact formation by calcium addition (Figure 3.10 B). Cell sheets were released from the dish with dispase and placed under mechanical stress to provoke disruption (Figure 3.10 A). Quantification of the number of fragments obtained after disruption showed a significant increase in pieces obtained from keratinocyte sheets deprived of LZTFL1 (Figure 3.10 C). This indicated that mild disturbance of AJ observed by immunofluorescence staining does indeed correlate with weaker cell-cell contacts. Together these results suggested that LZTFL1 might be required for junction stabilisation rather than de novo junction formation.

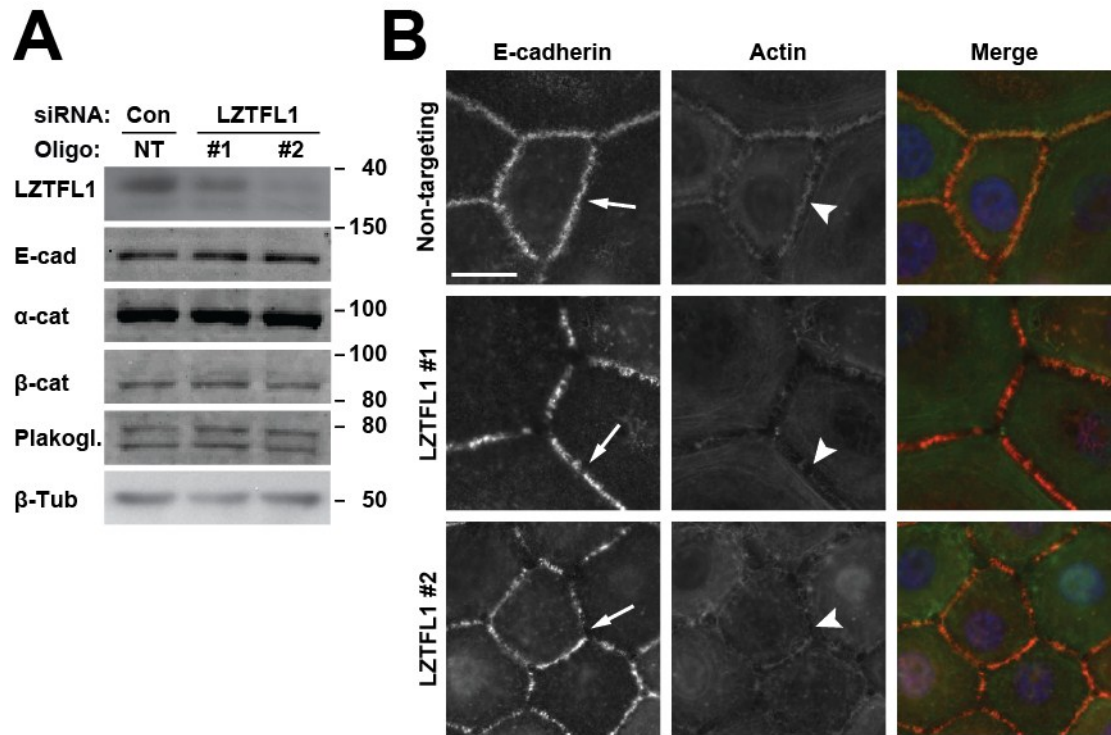


Figure 3.9: LZTFL1 depletion disturbs nascent adherens junctions. Monolayers of keratinocytes in low calcium medium and treated with non-targeting control (NT) or LZTFL1 (oligos #1 or #2) siRNA were switched to standard calcium medium for 30 minutes before fixation and staining for E-cadherin and actin. A) Western blot of treated samples probed with antibodies against LZTFL1 (clone 7F6), E-cadherin, α -catenin, β -catenin, plakoglobin or β -tubulin as loading control. Molecular weight markers are shown on the right. Average depletion of LZTFL1 with oligo #1 $20 \pm 8\%$ and with oligo #2 $59 \pm 28\%$. B) Immunofluorescence images of E-cadherin and actin staining. Arrows point at E-cadherin at representative junctions and arrow heads at gaps in junctional actin. N = 2. Scale bar = 20 μ m.

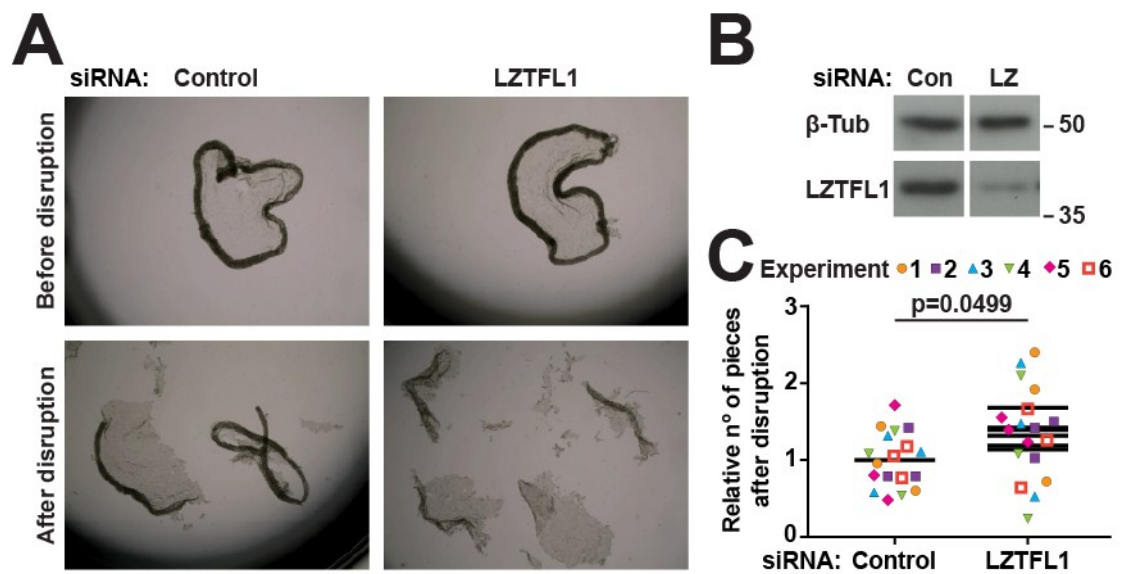


Figure 3.10: LZTFL1 depletion weakens newly formed cell-cell contacts in keratinocytes. Cell-cell contact formation was induced for 30 minutes in monolayers of keratinocytes treated with control or LZTFL1 siRNA. Subsequently, dispase was added until the cells detached as a sheet. The cell sheet was placed under mechanical stress on an orbital shaker for 10 minutes. A) Representative images of control and LZTFL1 siRNA-treated keratinocyte cell sheets before and after mechanical stress. B) Western blot of siRNA-treated cell lysates probed for LZTFL1 (clone 7F6) and β -tubulin as loading control. Molecular weight markers are shown on the right. Average depletion of LZTFL1 with oligo #1 $70 \pm 17\%$. C) Quantification of number of fragments obtained after mechanical disruption. All experiments were performed in triplicate. Horizontal bars represent experiment mean. Data was analysed statistically by two-way ANOVA and Dunnett post-hoc test. N = 6 experiments with 3 data points each for control and LZTFL1 depletion.

3.4 Conclusions

In summary, I identified four novel regulators of AJ in mammalian cells as well as confirmed a function for MTSS1 in junction formation in primary keratinocytes. Depletion of MTSS1 weakens cell-cell contacts as shown by greater disruption of cell clusters with mechanical stress. Similarly, LZTFL1 knock-down also leads to a subtle defect on junctional E-cadherin and actin which is accompanied by increased fragmentation of monolayers upon mechanical stress.

EF1 α reduction, in contrast, increases levels of junctional E-cadherin, which however are not resistant to detergent extraction and therefore not functionally linked to the actin cytoskeleton. This implies that EF1 α might be required to limit E-cadherin at the cell-cell contacts. Another regulator of E-cadherin, CIP4, might regulate E-cadherin trafficking. CIP4 depletion not only reduces levels of E-cadherin at nascent AJ but also influences cell surface levels of E-cadherin. CIP4 is recruited to the membrane during junction formation and this translocation is dependent on VAV2. VAV2 is identified as a novel interaction partner of CIP4 and my data indicates that VAV2 lays upstream of CIP4 in the modulation of E-cadherin stability at contact sites. These results confirm that RNAi screens are a valid and successful way of finding novel regulators of specific cellular processes and that they can give indications of possible cross-talk between proteins by careful comparison of phenotype similarity.

3.5 Discussion

3.5.1 MTSS1 stabilises AJ via Rac1 activation and actin assembly

Several cellular functions have been assigned to the tumour suppressor MTSS1 that make this protein an excellent candidate for an AJ regulator. Our study in collaboration with Dawson and Machesky and the study by Saarikangas *et al.* confirmed MTSS1 as a stabilizer of AJ and uncovered the signalling pathways involved (Dawson *et al.*, 2012a; Saarikangas *et al.*, 2011).

MTSS1-depleted keratinocytes have weaker cell-cell contacts compared to control cells, despite E-cadherin localisation to junctions being widely unaffected (Dawson *et al.*, 2012a). This let us to conclude that MTSS1 is required for AJ stability rather than formation, a conclusion similarly drawn by Saarikangas *et al.* based on the late onset of junctional defects in epithelial kidney cells in *Mtss1* knock-out mice (Saarikangas *et al.*, 2011). Both studies showed a reduction of actin, particularly thin bundles, at cell-cell contacts of MTSS1-deprived cells.

Different MTSS1 functions could mediate its stabilising effect on AJ. A pool of E-cadherin is constantly internalised and recycled both in the absence and presence of AJ, but the rate of E-cadherin trafficking is significantly reduced in mature AJ (Le *et al.*, 1999). The MTSS1 proline-rich domain can negatively affect endocytosis during migration by inhibiting pro-endocytic activity of cortactin (Quinones *et al.*, 2010). Therefore, MTSS1 could stabilise AJ by limiting E-cadherin endocytosis from established junctions. However, since junctional E-cadherin seemed widely unaffected, this explanation is unlikely. At the same time, interaction between MTSS1 and cortactin has been shown to promote actin polymerisation (Lin *et al.*, 2005a). Since actin at AJ was affected by MTSS1 depletion (Dawson *et al.*, 2012a; Saarikangas *et al.*, 2011), regulation of actin polymerisation through cortactin is a further possible mechanism for AJ stabilisation by MTSS1. However, Saarikangas *et al.* demonstrated in their study, that at least in MDCK cells cortactin binding is not required for *Mtss1*-mediated AJ stability (Saarikangas *et al.*, 2011).

Although, MTSS1 does not control actin downstream of AJ through cortactin, other actin-regulatory functions of MTSS1 may be involved. MTSS1 can bundle F-actin with its I-BAR domain, which could be required to stabilise thin bundles beneath AJ (Gonzalez-Quevedo *et al.*, 2005; Yamagishi *et al.*, 2004). Indeed, an intact MTSS1 I-

BAR domain is required for AJ stabilisation, although no direct evidence for an impact on actin bundling has been provided (Dawson et al., 2012a; Saarikangas et al., 2011).

Furthermore, MTSS1 could indirectly control actin through regulation of Rho GTPases. MTSS1 is a regulator of RhoA activity (Lei et al., 2014; Yu et al., 2011) and while RhoA is required for AJ formation, activation has to be spatially and temporarily regulated (Braga et al., 1997; Braga and Yap, 2005; Yamada and Nelson, 2007). In addition, MTSS1 can increase Rac1 activity (Bompard et al., 2005) and Rac1 activation is one of the hallmarks of AJ formation (Betson et al., 2002; Nakagawa et al., 2001; Noren et al., 2001). Indeed, we show that Rac1 is activated and localised to AJ by MTSS1 downstream of E-cadherin (Dawson et al., 2012a). Active Rac1 could then mediate actin polymerization, e.g. through WAVE2-Arp2/3, to stabilise AJ (Verma et al., 2012), a notion which is supported by the finding that Arp2/3 is required for Mtss1-mediated AJ stabilisation (Saarikangas et al., 2011).

In conclusion, MTSS1 is required to stabilise AJ through Rac1 activation and Arp2/3-dependent actin polymerization. RhoA regulation and F-actin bundling may also contribute to MTSS1-dependent AJ stabilisation, but further work is required to test this hypothesis. Stabilising AJ likely contributes to the ability of MTSS1 to prevent EMT and act as a tumour suppressor.

3.5.2 EF1 α is required to maintain E-cadherin levels at AJ

EF1 α is required for protein translation, but many studies have also shown its importance for actin regulation in different cellular processes. However, its function in the actin-dependent process of AJ formation and stabilisation has not been addressed so far. Here I show, that EF1 α depletion leads to increased amounts of E-cadherin at junctions without changes in total cellular E-cadherin levels. The additional junctional E-cadherin however does not represent a pool of receptors in AJ. Detergent insolubility levels are not proportionally increased and keratinocyte monolayers are fragmented more easily upon mechanical stress (J. Erasmus, unpublished data). This suggests that EF1 α is required (i) to limit E-cadherin numbers at AJ and (ii) to stabilise AJ.

There are two possible ways by which EF1 α could restrict E-cadherin levels at AJ: (i) by limiting the transport of E-cadherin to the contacts or (ii) by removing it from AJ. E-cadherin is constantly turned over by endocytosis, even at mature AJ (Le et al., 1999). This process is highly dependent on the local formation of specialised actin structures (Kaksonen et al., 2006). EF1 α has been shown to be enriched at and recruit β -actin

mRNA to specialised actin structures during migration (Liu et al., 2002; Shestakova et al., 1999). However, so far there is no evidence to suggest that EF1 α has similar functions during endocytosis to support a role for EF1 α in E-cadherin removal from AJ.

A mechanism of E-cadherin trafficking is lateral diffusion, which is mainly involved in very early stages of contact formation but it is then slowed down in an F-actin-dependent manner (Cavey et al., 2008; Hong et al., 2010; Kusumi et al., 1993; Sako et al., 1998). Two specialised actin populations are involved in limiting E-cadherin mobility by diffusion. Small actin patches help clustering E-cadherin and a contractile actin network confines the area for diffusion movement (Adams et al., 1998; Cavey et al., 2008; Kusumi et al., 1993; Sako et al., 1998). EF1 α could be involved in the regulation of both populations through actin bundling or regulation of actin polymerization by tethering β -actin mRNA to new contacts.

A recent paper demonstrated that β -actin mRNA targeting to contacts is essential for AJ formation in MDCK cells and is mediated through RhoA-Src kinase signalling (Gutierrez et al., 2014). Preventing β -actin mRNA targeting to contacts strongly disrupted E-cadherin localisation at contacts. EF1 α could be the missing link that enables targeting of β -actin mRNA to nascent junctions. However, the phenotypes caused by β -actin mRNA mistargeting (Gutierrez et al., 2014) and EF1 α depletion differ strongly. Therefore, if EF1 α is involved, it either cannot be the sole responsible for mRNA targeting or other proteins can compensate the loss of EF1 α . Among possible candidates that might co-operate with EF1 α or compensate its loss are Src-associated in mitosis, 68kDa (Sam68) or Zipcode binding protein 1 (ZBP1), which both also bind β -actin mRNA. Furthermore, both proteins are substrates of Src kinase and therefore could regulate β -actin mRNA localisation downstream of RhoA-Src kinase signalling (Fumagalli et al., 1994; Huttelmaier et al., 2005; Itoh et al., 2002a; Ross et al., 1997; Taylor and Shalloway, 1994).

EF1 α depletion and disturbed β -actin mRNA targeting may therefore lead to the untethering of E-cadherin receptors, which would increase diffusion but might also facilitate internalisation of E-cadherin. Since the net effect of EF1 α depletion is an increase of soluble E-cadherin at junctions, diffusion must however outweigh internalisation. Increased diffusion would lead to accumulation of untethered E-cadherin at cell-cell contact sites due to the higher local concentration of E-cadherin molecules, which locally increases the rate of collision with untethered, diffusing E-cadherin molecules. The close proximity upon collision would allow transient *cis*-

binding between tethered and untethered E-cadherin and thereby increase the retention time of diffusible E-cadherin at cell-cell contact sites (Hong et al., 2010). Overall, this would result in increased detectable E-cadherin levels at junctions without an increase in detergent insolubility.

The second question is how EF1 α depletion disturbs junction stability without changing detergent-resistant levels of junctional E-cadherin. One explanation could be a detrimental effect of the increased, unstable E-cadherin levels, observed with EF1 α depletion. Indeed, it has been shown that E-cadherin pools that are uncoupled from the actin cytoskeleton can interfere with the stability of actin-bound E-cadherin structures (Hong et al., 2013). On the other hand, EF1 α could also regulate AJ stability independent of E-cadherin levels for example by an effect on actin contractility. Direct evidence for EF1 α regulation of actin tension has not yet been provided, but EF1 α is thought to be involved in contractile ring formation during cytokinesis (Numata et al., 2000), making it a likely candidate of an actin tension regulator.

3.5.3 CIP4 might be a regulator of E-cadherin trafficking in keratinocytes

CIP4 has been shown to be involved in the maintenance of epithelial polarity in *Drosophila* by mediating DE-cadherin endocytosis at mature AJ (Leibfried et al., 2008). Here I show that CIP4 depletion increases surface levels of E-cadherin in keratinocytes in the absence of AJ, suggesting a role of CIP4 in limiting E-cadherin levels under these conditions, for example by promoting endocytosis. Conversely, as junctions are formed in CIP4-depleted cells, E-cadherin surface level decreases continuously, implying that CIP4 is required to positively influence E-cadherin at the membrane during *de novo* junction formation. I propose that CIP4 might have dual functionality in E-cadherin trafficking in keratinocytes regulating both endo- and exocytosis under different cellular conditions similar to its role in GLUT4 receptor regulation (Chang et al., 2002; Hartig et al., 2009).

E-cadherin turnover is slowed down but not stopped when cell-cell contacts form (Le et al., 1999). The slowing down of E-cadherin turnover following junction assembly could have different reasons. On one hand, it may be caused by increased stability of junctional E-cadherin, which is linked by F-actin remodelling into AJ. On the other hand, it may also be caused by a change in turnover mechanism. E-cadherin can be removed from the cell surface by different mechanisms depending on the cellular signals, including clathrin- or caveolin-dependent endocytosis and macropinocytosis (Delva and Kowalczyk, 2009). Without specific signals such as growth factor

stimulation or over-active GTPases, E-cadherin seems to be internalised by clathrin-mediated endocytosis (de Beco et al., 2012; Delva and Kowalczyk, 2009; Le et al., 1999). In contrast, a study by Hong *et al.* showed that the removal of E-cadherin from mature AJ still occurs when the endocytic motifs in the E-cadherin cytoplasmic tail are inactivated (Hong et al., 2010). This supports the theory that at least in some cell types AJ formation leads to a change in E-cadherin turnover mechanism.

Based on the above, I propose that in the absence of AJ CIP4 might be involved in internalization of E-cadherin. CIP4 depletion would reduce E-cadherin internalization, while recycling would be unaffected, leading to establishment of a new steady state with increased cell surface levels of E-cadherin. Thirty minutes after junction formation, E-cadherin cell surface levels and levels at AJ are reduced in CIP4-depleted cells as compared to control cells. These results suggest a defect in efficient E-cadherin delivery to nascent junctions when CIP4 is depleted, while endocytosis possibly through CIP4-independent mechanisms might be unaffected. A similar switch between endocytosis- and exocytosis promoting function based on cellular context has previously been shown for CIP4-regulated GLUT4 transport (Chang et al., 2002; Hartig et al., 2009). On the other hand, above results could also be explained by an endocytosis inhibiting effect of CIP4 upon AJ formation that is lost in CIP4-depleted cells. A likely mechanism may be CIP4-mediated regulation of actin polymerisation as has been shown during migration and neurite formation (Malet-Engra et al., 2013; Pichot et al., 2010; Saengsawang et al., 2012; Saengsawang et al., 2013).

Consistent with a role for CIP4 in targeted E-cadherin delivery to AJ or in F-actin remodelling at junctions, is its recruitment to nascent AJ early during their formation. CIP4 recruitment is dependent on VAV2. Previous studies on VAV2 downstream of AJ show that it is recruited to cadherin clusters and activated by Src kinase and might be involved in Rac1 activation (Fukuyama et al., 2006). My results suggest that VAV2 is required for AJ formation and that its function downstream of cadherin involves CIP4 recruitment.

An important question that remains to be answered is how CIP4 could switch between roles in E-cadherin endocytosis and junction formation. AJ-dependent activation of GTPases is involved in F-actin remodelling and may also participate in junction-dependent regulation of trafficking (Watanabe et al., 2009). Since VAV2 is a Rho GEF and CIP4 a Rho GTPase effector, CIP4 might be recruited to junctions in response to VAV2-induced Rho GTPase signalling. At junctions, CIP4 could then aid F-actin

remodelling or operate locally in distinct trafficking events instead all over the general cell surface.

VAV2 is a GEF for Cdc42, Rac1 and RhoA, B, C and G (Abe et al., 2000; Arthur et al., 2002; Liu and Burridge, 2000; Schuebel et al., 1998). CIP4 acts downstream of Cdc42 and TC10 (Aspenström, 1997; Chang et al., 2002). The latter GTPase has not been formally investigated as a substrate for VAV2. VAV2 could activate Cdc42, which then recruits CIP4 to new cadherin complexes. However, it has been shown that Cdc42 is not required for AJ formation in primary keratinocytes (Erasmus et al., 2009). This indicates that VAV2 and CIP4 may interact through a distinct, less well-studied Rho GTPase during junction formation.

3.5.4 LZTFL1 is required for cell-cell contact stability

AJ in LZTFL1-depleted cells show only a small degree of disturbance by immunofluorescence staining, but cell-cell contacts are significantly less resistant to mechanical stress. This suggests that LZTFL1 is not essential for AJ formation but rather for AJ maintenance. Further studies are necessary to confirm that weaker junctions observed with LZTFL1 depletion are caused by perturbation of AJ function. The dispase assay used here measures the strength of calcium-dependent adhesion, and therefore the contribution of desmosomal adhesion should also be considered. Chapter 4 will explore AJ regulation by LZTFL1 in greater detail.

3.6 Future work

I have identified five regulators of cell-cell contacts based on the effect that their depletion has on newly forming AJ. Most of the proteins have several well-described functions in the literature that could explain their role downstream of cadherin homophilic interactions. Further work is now needed to test the different hypotheses described above.

3.6.1 Does MTSS1 affect actin at AJ through other mechanisms than Rac1 signalling?

Two independent studies have shown that MTSS1 regulates AJ stability through Rac1 or Arp2/3, respectively. It is likely that all three proteins work in the same pathway with MTSS1 activating Rac1 and Rac1 recruiting and activating Arp2/3, but direct evidence is needed to confirm this hypothesis.

Furthermore, MTSS1 has been shown to regulate actin through other mechanisms than Rac1 and Arp2/3 activation. It will be interesting to test whether MTSS1 regulates these pathways downstream of AJ. MTSS1 depletion leads to defects in actin thin bundles and therefore, it seems feasible that MTSS1 may bundle F-actin at AJ. Electron microscopic studies of actin thin bundles in control and MTSS1-depleted cells could be used to detect bundling defects. To test an effect of MTSS1 on RhoA upon junction induction, RhoA effector pull-down assays could be used to compare activation profiles between control and MTSS1-deleted cells.

3.6.2 How does EF1 α regulate E-cadherin distribution and AJ stability?

Increased E-cadherin levels at the junction could be caused by increased exocytosis, reduced endocytosis or augmented lateral diffusion. To distinguish defects on diffusion or trafficking to or from the membrane, surface biotinylation assays could be performed. Increased diffusion will not alter total surface levels, while vesicular trafficking defects would be expected to increase E-cadherin at the surface. Live-cell imaging techniques such as the use of photoconvertible E-cadherin constructs could also be used to directly observe E-cadherin trafficking.

Immunofluorescence and possibly electron-microscopic studies should be used to detect defects in the actin cytoskeleton beneath AJ. EF1 α depletion reduces junction stability and defects in E-cadherin binding to actin might play a role in this effect. E-cadherin tension as a measure of its connection to the actin cytoskeleton could be

assessed using E-cadherin-TsMod constructs, which possess the FRET pair mTFP/Venus separated by an elastic linker in the cytoplasmic domain of E-cadherin (Borghi et al., 2012). It would also be interesting to test whether β -actin mRNA is targeted to nascent AJ by EF1 α . For this purpose fluorescence *in situ* hybridization (FISH) can be used to detect β -actin mRNA localisation in control and EF1 α -depleted cells.

3.6.3 How does CIP4 switch between different functions in E-cadherin trafficking and AJ formation?

My data suggests that CIP4 might be involved in E-cadherin endo- and possibly exocytosis in different cellular conditions. Further experiments will be needed to confirm this hypothesis. Surface biotinylation assays represent a powerful tool that can be varied in different ways to specifically assess endo- or exocytotic events (Le et al., 1999). Electron-microscopic studies could be another tool to observe defects in vesicular trafficking. These assays would need to be performed in the absence and presence of AJ to test for a switch of function of CIP4 between these two different cellular make-ups.

Immunofluorescence and possibly electron-microscopic studies can also be used to test CIP4 involvement in actin polymerization downstream of AJ. E-cadherin connection to the actin cytoskeleton could be measured using E-cadherin-TsMod constructs. CIP4 full-length cDNA and constructs missing the actin-binding domain could be used to try and rescue the CIP4-depletion phenotype.

The next question to answer would be, which cue switches CIP4 from an endocytic regulator to a facilitator of AJ assembly. Different GTPase signalling upstream of CIP4 is most likely. Cdc42 is not required for AJ formation in primary keratinocytes, but regulates DE-cadherin endocytosis upstream of CIP4 in *Drosophila*. Therefore, a good candidate for the endocytic switch is Cdc42. VAV2 is a GEF for multiple GTPases including Rac1, RhoA, RhoB, RhoC and RhoG. CIP4 does not bind to Rac1 or RhoA, but has not been tested for its ability to act as an effector of RhoB, RhoC and RhoG. At least RhoB and RhoC are transiently activated at 5 minutes after calcium-induced junction formation (N. Welsh, unpublished data). Therefore as a first step, CIP4 binding to RhoB, RhoC and RhoG should be tested through fusion protein pull-down assays. Subsequently, the effect of depletion of any identified Rho-binding partners of CIP4 on E-cadherin trafficking needs to be assessed and phenotypes similar to CIP4 rescued

by CIP4 overexpression. Conversely, it should be tested whether VAV2 can activate TC10 and whether TC10 is required for AJ formation in keratinocytes.

3.6.4 How does LZTFL1 stabilise AJ?

LZTFL1 depletion destabilises cell-cell contacts, with a subtle morphological defect on AJ. A first step in studying LZTFL1 function will be to confirm that LZTFL1 stabilises contacts by influencing AJ, but not desmosomes. Since LZTFL1 does not seem to be required for AJ formation, future studies can be performed on mature junctions. Furthermore, characterising the localisation of LZTFL1 by immunofluorescence should gain further clues about pathways that this protein might be involved in. Considering that LZTFL1 may co-localise with E-cadherin at AJ, it will be interesting to test whether LZTFL1 can bind to any core component of AJ or to the cytoskeleton.

Chapter 4 LZTFL1 is a stabiliser of cell-cell contacts

4.1 Introduction

4.1.1 LZTFL1 – The protein and its expression

The LZTFL1 gene exists in vertebrates and invertebrates and is quite conserved at the amino acid level with human and mouse proteins being nearly 91% identical, human and zebrafish about 69% and human and sponge still 35% identical (Figure 4.1 A).

The human LZTFL1 gene was first described by Kiss *et al.* as being part of the chromosomal region 3p21.3 (Kiss et al., 2001). The gene consists of 10 exons and has a genomic size of 18 kb. In human, the open reading frame (ORF) is 900 bp long and encodes a protein of 299 amino acids with a molecular weight of 34.6 kDa. The paper described a polymorphism (A or G) at position 736 in the ORF sequence, which leads to a non-conservative amino acid substitution from asparagine to aspartic acid. The 3'-untranslated region of the LZTFL1 transcript possesses a regular polyadenylation signal (AATAAA) starting at position 1464 bp and an alternative polyadenylation signal (TATAAA) at position 3361 bp. Accordingly, Kiss *et al.* detected two different transcripts on Northern blots (1.7 and 3.6 kb). The shorter transcript is mainly expressed in testis, but was also detected in lower amounts in heart, skeletal muscle, pancreas, thymus, and fetal kidney. The longer transcript could be found in heart, brain, placenta, lung, skeletal muscle, kidney and spleen at low levels and in pancreas, thymus and testis at higher levels. The mouse protein is also composed of 299 amino acids with a predicted molecular mass of 34.8 kDa. Northern blot hybridization demonstrated that the mouse gene also has a shorter and a longer transcript (approximately 2 and 4.5 kb). The 4.5 kb *Lztf1* transcript is ubiquitous expressed with lower expression in spleen and the shorter *Lztf1* transcript seems not to be expressed in spleen and testis.

The LZTFL1 protein is entirely made up of α -helices and linker regions, with three shorter α -helices at the N-terminus predicted to comprise the amino acids 8 to 42, 51 to 94 and 102 to 123. The C-terminus consists of a coil-coil domain covering the amino acids 146 to 294 and comprises the name giving leucine zipper motif between amino acids 240 and 261, a motif commonly found in transcription factors. LZTFL1 is predicted to form self-dimers via its coil-coil domain and this prediction has been confirmed by co-IP and cross-linking experiments (Seo et al., 2011). The most conserved parts of the LZTFL1 protein are α -helices 2 and 3 as well as both extreme ends of the coil-coil domain (Figure 4.1 B).

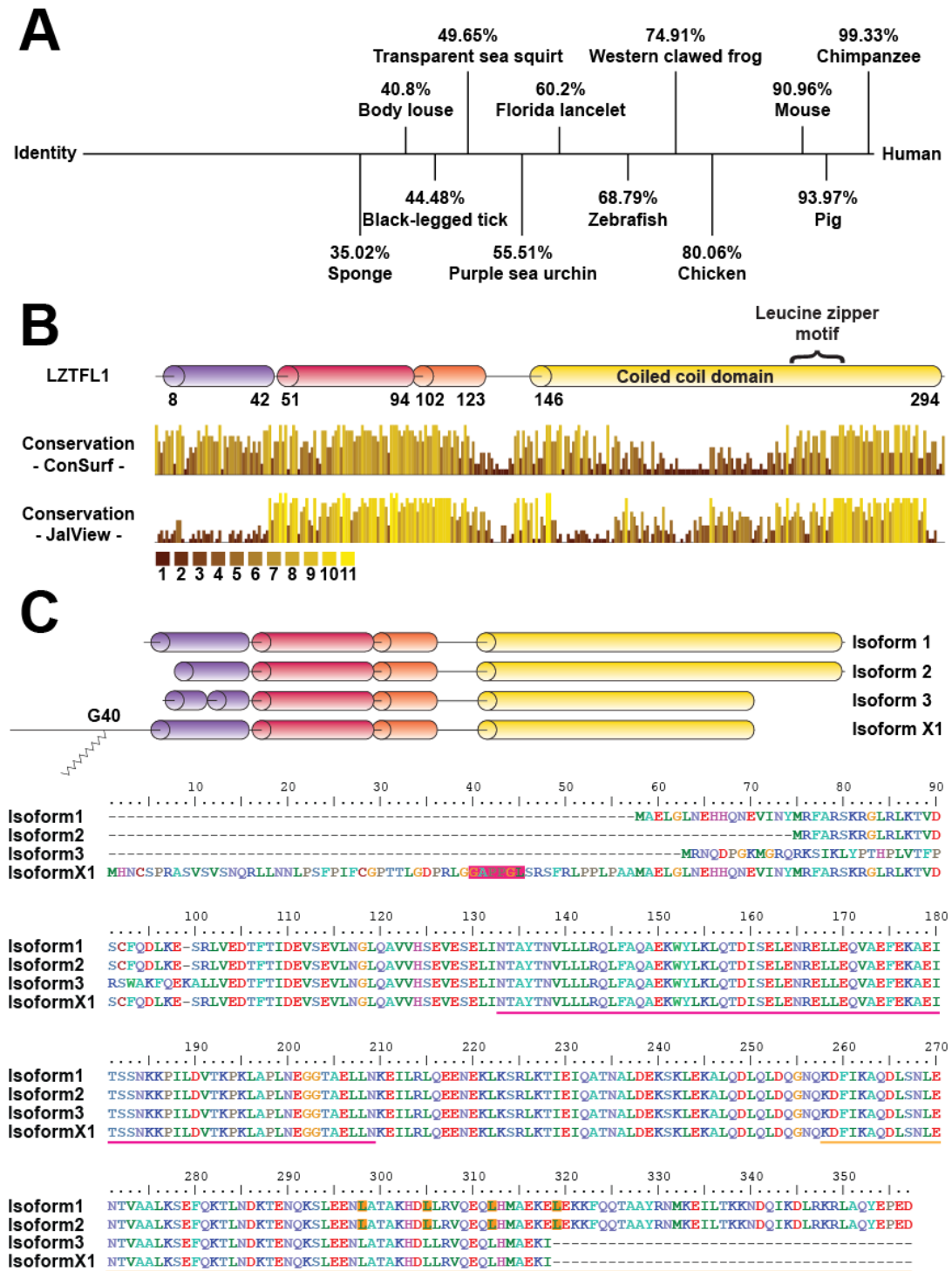


Figure 4.1: LZTFL1 conservation, structure and isoforms. A) Overall amino acid identity of LZTFL1 from different species as compared to human LZTFL1. Identity was calculated as number of identical residues over length of the shortest sequence. B) Secondary structure of LZTFL1 as predicted using multiple online prediction tools. LZTFL1 consists of 3 short α -helices at the N-terminus and a long coil-coil domain containing a leucine zipper motif at the C-terminus. The conservation of individual amino acid positions was calculated with the tools ConSurf and JalView and is represented as column graphs underneath the secondary structure cartoon. 1 = least conserved; 9 or 11 = most conserved, for ConSurf or JalView, respectively. C) Secondary structure and aligned amino acid sequences of the proposed LZTFL1 isoforms. Isoform X1 is predicted to be myristylated at glycine in position 40 (G40). The modification motif is highlighted pink in the alignment. Leucines of the leucine zipper motif are highlighted orange in the amino acid alignment. LZTFL1 antibody recognition sequences are underlined pink (ProteinAtlas) or orange (clone 7F6).

Since its first description, several additional LZTFL1 mRNAs have been identified (Figure 4.1 C). To date, mRNAs coding three LZTFL1 isoforms have been confirmed and a fourth isoform called X1 has been predicted based on automated computational analysis (NCBI eukaryotic genome annotation pipeline) and is supported by several RNA-Seq alignments. LZTFL1 isoform 2 (32.6 kDa) is identical to isoform 1 apart from missing the N-terminal 17 amino acids. Isoforms 3 and X1 have an identical shortened C-terminus and lack the leucine zipper motif. Isoform 3 is the shortest isoform (256 amino acids length; 29.3 kDa) and has a unique N-terminus predicted to comprise two short α -helices. The isoform X1, on the other hand, is the largest isoform (317 amino acids; 35.8 kDa). Compared to isoform 1, it has an additional 57 amino acids at the N-terminus, with a putative myristylation signal, which would cause myristylation at glycine in position 40 and would imply that this isoform might be transiently associated with membranes. However, since myristylation usually occurs at an N-terminal glycine, myristylation of LZTFL1 in cells is unlikely (Martin et al., 2011). All isoforms possess the highly conserved second and third α -helices and start of the coil-coil domain, but only isoforms 1 and 2 comprise the conserved C-terminal part of the coil-coil domain (compare Figure 4.1 B and C).

4.1.2 LZTFL1 in cancer

Genes in the chromosomal region 3p21.3 are thought to encode tumour suppressors, because this chromosomal region is frequently lost in many types of solid tumours and in acute lymphoblastic leukaemia (Petursdottir et al., 2004; Tsuzuki et al., 2007). Therefore, LZTFL1 is predicted to be a tumour suppressor. LZTFL1 levels are significantly lower in tumour samples from breast, oesophagus, pancreas, stomach, ovary, prostate, lung, colon, thyroid, kidney, bladder and liver by immunohistochemistry (IHC) (Wei et al., 2010). In a study with 84 patients with gastric cancer, significant inverse correlations between LZTFL1 levels and Tumour-Node-Metastasis (TNM) stage of the tumour and number of metastasized lymph nodes was found by IHC. Conversely, patients with high LZTFL1 levels had a significantly longer survival time than patients with low expression levels (Wei et al., 2010). IHC profiling of additional 311 patients and qPCR profiling of 50 patients confirmed the difference in LZTFL1 levels between normal and gastric tumour samples (Wang et al., 2014).

Tumour cell line models show that LZTFL1 expression specifically inhibits anchorage-independent growth in HeLa cells, intestinal epithelial carcinoma HT-29 cells and breast cancer MCF-7 cells *in vitro* (Wei et al., 2010). Furthermore, LZTFL1 expression

in HeLa cells reduces tumour growth *in vivo* as well as cell migration in Transwell assays (Wei et al., 2010).

The LZTFL1 gene is not completely lost in all tumours, but the expression of LZTFL1 is downregulated in most tumour samples. These findings suggest that LZTFL1 expression may be regulated epigenetically. Indeed, LZTFL1 expression at least in HT-29 cells is inactivated through the action of histone deacetylase (Wei et al., 2010). Epigenetic silencing of genes is part of the normal differentiation program. A correlation between LZTFL1 expression and cellular differentiation has been shown in gastric epithelial and gastric cancer cells (Wang et al., 2014) and as graded expression in the villus with strongest expression in the apex, which contains the most differentiated intestinal cells (Wei et al., 2010).

4.1.3 Tumour-suppressive function of LZTFL1

As part of its tumour suppressor function, LZTFL1 may be required to stabilise AJ. LZTFL1 co-localises with E-cadherin in normal colon epithelial cells and LZTFL1 overexpression in HT-29 gastric cancer cells reduces phorbol 12-myristate 13-acetate (PMA)-induced cell scattering (Wei et al., 2010). In addition, LZTFL1 is an interaction partner and regulator of β -catenin-shuffling between the cytoplasm and the nucleus (Wang et al., 2014). High levels of LZTFL1 retain β -catenin in the cytoplasm, therefore preventing it from stimulating transcription of EMT-promoting transcription factors such as slug, twist and Zeb1. These transcription factors have been shown to prevent transcription of E-cadherin and the tight junction protein ZO-1 (Craene and Berx, 2013). Accordingly, overexpression of LZTFL1 upregulates E-cadherin and ZO-1 expression and reduced LZTFL1 levels reverse the effect. Since LZTFL1 regulates the expression of EMT-controlling proteins, unsurprisingly, cell characteristics changing during EMT, such as proliferation, migration and invasion, are also affected by LZTFL1 expression or depletion (Wang et al., 2014).

4.1.4 LZTFL1 in ciliary trafficking and ciliopathies

LZTFL1 has been identified as a regulator of ciliary trafficking of the BBSome complex (Seo et al., 2011). Cilia are slim microtubule-based protrusions on the surface of cells that have important signalling functions during development and tissue homeostasis (Goetz and Anderson, 2010). They are particularly linked to the regulation of the hedgehog and Wnt signalling pathways, transcription-regulatory pathways that are especially important during embryonic development. Defects in ciliary proteins have been linked to a variety of diseases that are referred to as ciliopathies (Goetz and

Anderson, 2010). One of these ciliopathies is Bardet-Biedl syndrome, a genetic disorder that is characterised by symptoms such as polydactyly, retinitis pigmentosa, obesity, cognitive impairment and nephropathy. Proteins, that cause Bardet-Biedl syndrome when defective, have therefore been named BBS proteins (Zaghloul and Katsanis, 2009). Seven of these proteins, BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9 and the protein BBIP10 form a complex which is called BBosome and is thought to be required for the transport of certain proteins to and from the cilia (Jin et al., 2010; Loktev et al., 2008; Nachury et al., 2007). BBS6, BBS10 and BBS12 are required for BBosome assembly (Seo et al., 2010) and BBS3 is a member of the Ras superfamily of GTPases and is involved in BBosome recruitment to the membrane and ciliary entry (Jin et al., 2010).

LZTFL1 was identified as a novel interaction partner of the BBosome by tandem affinity purification from mouse testis overexpressing BBS4 (Seo et al., 2011). Additional co-IP experiments identified BBS9 as the interaction partner of LZTFL1 and the interaction site on LZTFL1 was mapped to the coil-coil domain. The interaction between LZTFL1 and the BBosome occurs in the cytoplasm, consistent with the finding that LZTFL1 does not enrich in cilia in human retinal pigment epithelial cells. LZTFL1 is not a constitutive component of the BBosome, but associates with the BBosome to retain the complex in the cytoplasm. LZTFL1 depletion or mutation of the highly conserved lysine and arginine residues at positions 24 and 25 lead to accumulation of the BBosome complex in cilia.

Cilia are important signalling hubs during development and Bardet-Biedl-Syndrome is linked to severe developmental defects. The Sonic Hedgehog (SHH) signalling cascade involves the binding of SHH to its receptor Patched 1 (PTCH1), which activates Smoothened (SMO). SMO then activates the transcriptional regulators GLI1 and GLI2 and represses GLI3, which overall results in changes in transcriptional programs (Villavicencio et al., 2000). Intact BBosome is required for SMO translocation to cilia in response to agonistic signals. LZTFL1 depletion permits SMO to enter the cilia even in the absence of agonistic signals (Seo et al., 2011). This suggests that the BBosome and LZTFL1 are involved in SHH signalling by controlling SMO transport to cilia.

Consistent with a role in SHH signalling and BBosome regulation, patients with LZTFL1 mutations had severe developmental defects and were diagnosed with Bardet-Biedl-Syndrome. The first case described a 10 years old boy of Algerian origin who

presented with retinal degeneration, obesity, cognitive impairment, mesoaxial polydactyly (fusion of third and fourth finger bone), hypogonadism (reduced production of testosterone), polyuria-polydypsia (increased need to urinate and increased thirst) and situs inversus (inverted positioning of inner organs) (Marion et al., 2012). It was found that the chromosomal region containing the LZTFL1 gene was present homozygously and LZTFL1 in the homozygous locus exhibited a five nucleotides deletion (nucleotides 402-406 of the coding region) leading to a premature stop code. LZTFL1 was essentially absent in dermal fibroblasts of the patient on both the mRNA and protein level. The authors also tested the expression levels of proteins of the SHH pathway and found a strong upregulation of SHH, SMO, GLI1 and GLI2 suggesting a strong overactivation of the SHH pathway. They conclude that LZTFL1 is likely involved in body patterning via SHH signalling.

A second case study described twin sisters with Bardet-Biedl-Syndrome (Schaefer et al., 2014), that had postaxial polydactyly of hands and feet and additional mesoaxial polydactyly of the hands. They also had polyuria-polydypsia that led to chronic kidney failure, retinitis pigmentosa, learning difficulties and anosmia (impairment of smell). The LZTFL1 allele was dizygotic, but both alleles were mutated. One allele had a missense mutation at position 260 (T to C), which likely leads to abnormal splicing and no protein translation. The second allele had a nonsense mutation at position 778 (G to T) causing an early stop codon. This protein seems to be expressed in these patients at a lower than normal level, since a protein product of about 30 kDa was detected by Western blot with an LZTFL1 antibody.

LZTFL1 mutations seem to be a rare event among Bardet-Biedl-Syndrome patients (Marion et al., 2012). The presentation of mesoaxial polydactyly in both cases of LZTFL1 mutation-induced Bardet-Biedl-Syndrome is striking, since it has not been described in cases of Bardet-Biedl-Syndrome with other genetic causes. There seems to be a genotype-phenotype correlation between LZTFL1 mutation and mesoaxial polydactyly (Schaefer et al., 2014), but more patients are necessary to confirm this correlation.

4.1.5 LZTFL1 function in the brain

LZTFL1 is found in nearly all organs and cell types, suggesting that it might have a wide variety of functions. Exercising of a mouse strain with early onset ageing and associated learning and memory deficiency leads to a beneficial effect on contextual fear memory (Sakurai et al., 2011). Among genes differentially expressed in

hippocampus in response to exercise, Lztf1 levels are significantly increased in exercised mice both at the mRNA and protein levels. Neuro 2a cells overexpressing Lztf1 showed increased spontaneous and differentiation-induced neurite outgrowth both in terms of neurite number and length. This suggests that Lztf1-driven neurite outgrowth might be involved in exercise-induced improvement of cognitive abilities by promoting neural structural changes.

4.2 Hypothesis and aims

LZTFL1 has multiple functions depending on the cellular context. I hypothesise that LZTFL1 stabilizes cell-cell contacts in primary keratinocytes by influencing the underlying cytoskeleton. I predict that its stabilising effect on junctions is required for its tumour suppressive function.

I aim to study the effect of LZTFL1 depletion on cell-cell contacts and cytoskeletal filaments. Finally, I will study LZTFL1 levels in head and neck squamous carcinoma cell lines and the effect of LZTFL1 expression on EGF-induced cell scattering.

4.3 Results

4.3.1 LZTFL1 depletion effects both AJ and desmosomes

In the previous chapter I have shown that LZTFL1 depletion causes mild visible defects on newly formed AJ, but that these disturbances significantly affect cell-cell contact strength. I wanted to test whether these morphological changes are transient and corrected as AJ mature. Further, since the assay used to test resistance to mechanical stress does not distinguish between effects of AJ and desmosomes, it was necessary to test whether LZTFL1 depletion has an effect on desmosomes.

I depleted LZTFL1 in keratinocytes grown in standard calcium and stained cells for E-cadherin and actin (Figure 4.2 A+B). LZTFL1 depleted cells showed irregularities in E-cadherin staining (Figure 4.2 A) and the effect seemed to be more pronounced than in low calcium cells (compare with Figure 3.9). E-cadherin staining appeared jagged, irregular and interrupted as compared to control siRNA-treated cells (Figure 4.2 A). Reduced levels of E-cadherin at AJ were observed when measured as E-cadherin %thresholded area at the junction (Figure 4.2 C). Circumferential actin bundles also appeared to be affected by LZTFL1 depletion at least with oligonucleotide #2, as bundles seemed less compacted towards cell-cell contacts (Figure 4.2 A). This correlated with an overall apparent decrease of compactness of LZTFL1 depleted cells (compare amount of white intercellular spaces between control and LZTFL1 depleted cells). Due to time constraints these phenotypes were not quantified. Further experiments are needed to confirm defects in cell compaction.

Similarly, LZTFL1-depleted keratinocytes were assessed for defects in desmosomes (Figure 4.2 D+E). Desmoplakin staining appeared slightly weaker in LZTFL1-depleted cells. Junctional desmoplakin levels were quantified as % thresholded area and were significantly reduced in cells depleted of LZTFL1 using oligonucleotide #3. Cells depleted using oligonucleotide #2 also showed a trend to reduced desmoplakin levels at contact sites, but the difference was not significant (Figure 4.2 F). Therefore, further experiments will be necessary to confirm an effect of LZTFL1 depletion on desmosomes.

These results demonstrate that LZTFL1-depletion disturbs AJ and possibly desmosomes at mature cell-cell contact sites. The effect of LZTFL1 depletion appears to be stronger on AJ than on desmosomes and mature AJ seem to be more affected by LZTFL1 depletion than nascent AJ.

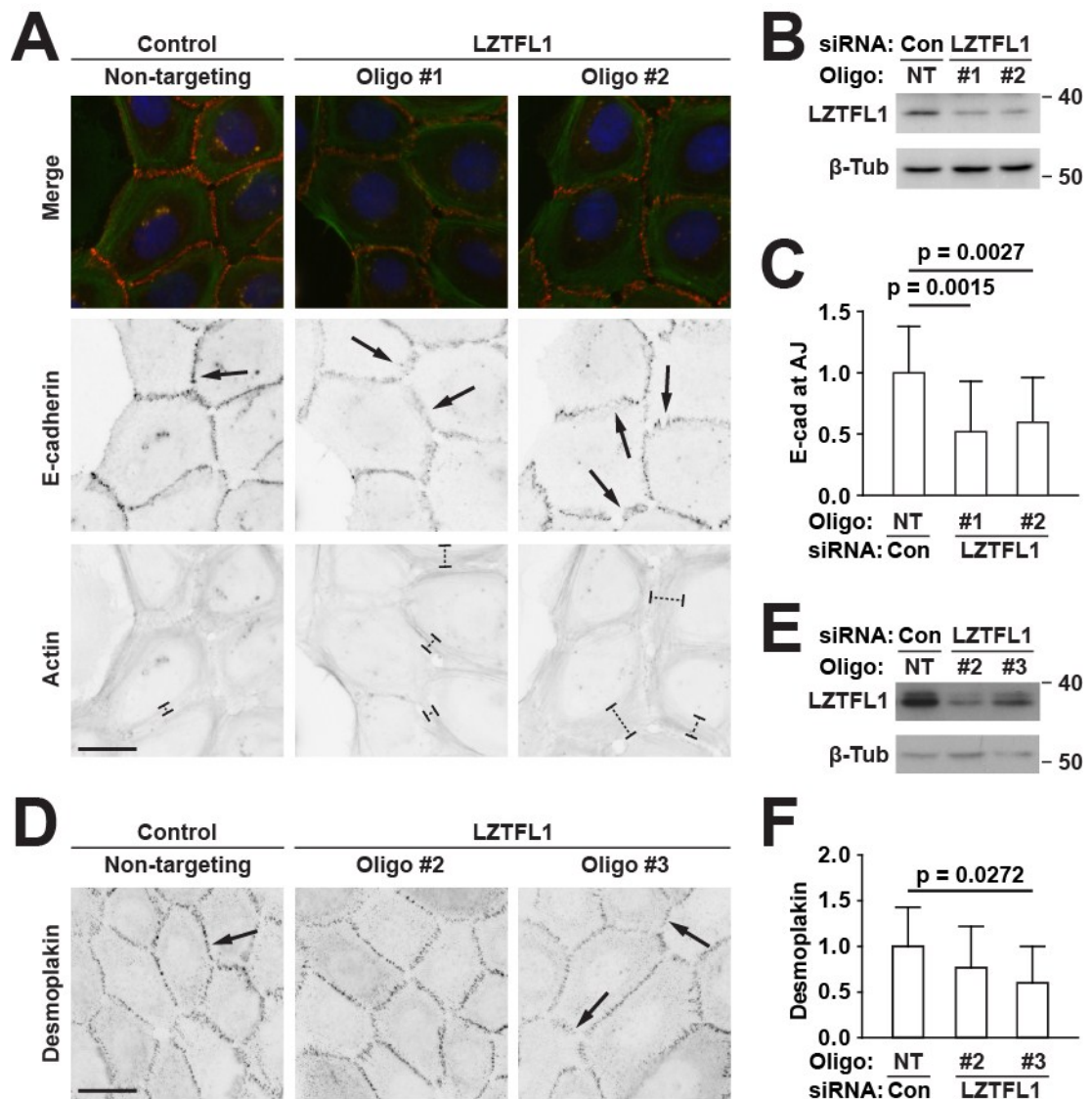


Figure 4.2: LZTFL1 depletion disturbs mature adherens junctions and desmosomes. Keratinocytes grown in standard medium were treated with control and LZTFL1 (oligos #1, #2 and #3) siRNA before fixation and staining. A) Representative inverted and merge immunofluorescence images of E-cadherin and actin staining. Arrows point at representative junctions. Lines cross the width of representative parts of circumferential thin bundles. B + E) Western blot of treated samples probed with antibodies against LZTFL1 (clone 7F6 (B) or Proteinatlas (E)) and β-tubulin. Molecular weight markers are shown on the right. Average depletion of LZTFL1 with oligo #1 $57 \pm 14\%$ and with oligo #2 $65 \pm 9\%$ (B). Average depletion of LZTFL1 with oligo #2 $72 \pm 5\%$ and with oligo #3 $43 \pm 30\%$ (E). C) Quantification of E-cadherin at junctions represented as thresholded E-cadherin-stained area over cropped image area and relative to control cells. N = 19 for NT from 3 experiments, N = 14 for LZTFL1 oligo #1 from 2 experiments, N = 22 for oligo #2 from 3 experiments. D) Representative inverted immunofluorescence images of desmoplakin staining. Arrows point at representative contacts. F) Quantification of desmoplakin at cell-cell contacts represented as thresholded desmoplakin-stained area over cropped image area and relative to control cells. N = 18 for NT from 3 experiments, N = 20 for LZTFL1 oligo #2 from 3 experiments, N = 13 for oligo #3 from 2 experiments. Graphs show mean $\pm$ SD. Data was analysed statistically by two-way ANOVA and Dunnett post-hoc test. Scale bar = 20 μ m.

4.3.2 Cellular localisation of overexpressed and endogenous LZTFL1

To obtain further insights through which mechanisms LZTFL1 regulates cell-cell contact stability, I tested where LZTFL1 localises in primary keratinocytes. I cloned LZTFL1 isoform 1 into mammalian expression vectors with mRFP- or 6myc-tags and also fragments of LZTFL1 based on the predicted secondary structure (Figure 4.1). One fragment contained only the N-terminal three α -helices, one contained the third α -helix and the coil-coil domain and a last construct contained only the coil-coil domain.

The mRFP-tagged constructs were transfected into standard calcium keratinocytes and localisation was assessed visually (Figure 4.3 A). Most overexpressed full-length and truncated LZTFL1 was found in the cytoplasm, but some cell-cell contacts showed partial accumulation of LZTFL1 (Figure 4.3 B). Truncation mutants seemed to localise to contacts slightly more often than full-length LZTFL1, but the difference was not significant.

Since LZTFL1 has the leucine zipper motif and this motif is common in transcription factors, I also checked whether LZTFL1 localises to the nucleus. Indeed, a small proportion of cells showed higher intensity LZTFL1 staining in the region of the nucleus, but it cannot be excluded that this is just background or random accumulation of the protein (Figure 4.3 C). However, strikingly the N-terminal fragment of LZTFL1 accumulated in the nucleus in nearly all overexpressing cells. It is unlikely that this accumulation is due to small size and unspecific entry of the protein to the nucleus, since the C-terminal fragment of LZTFL1 is nearly identical in size to the N-terminal fragment and does not accumulate significantly in the nucleus. Furthermore, similar results were obtained with LZTFL1 N-terminal fragments with a 6myc- instead of the mRFP-tag (data not shown).

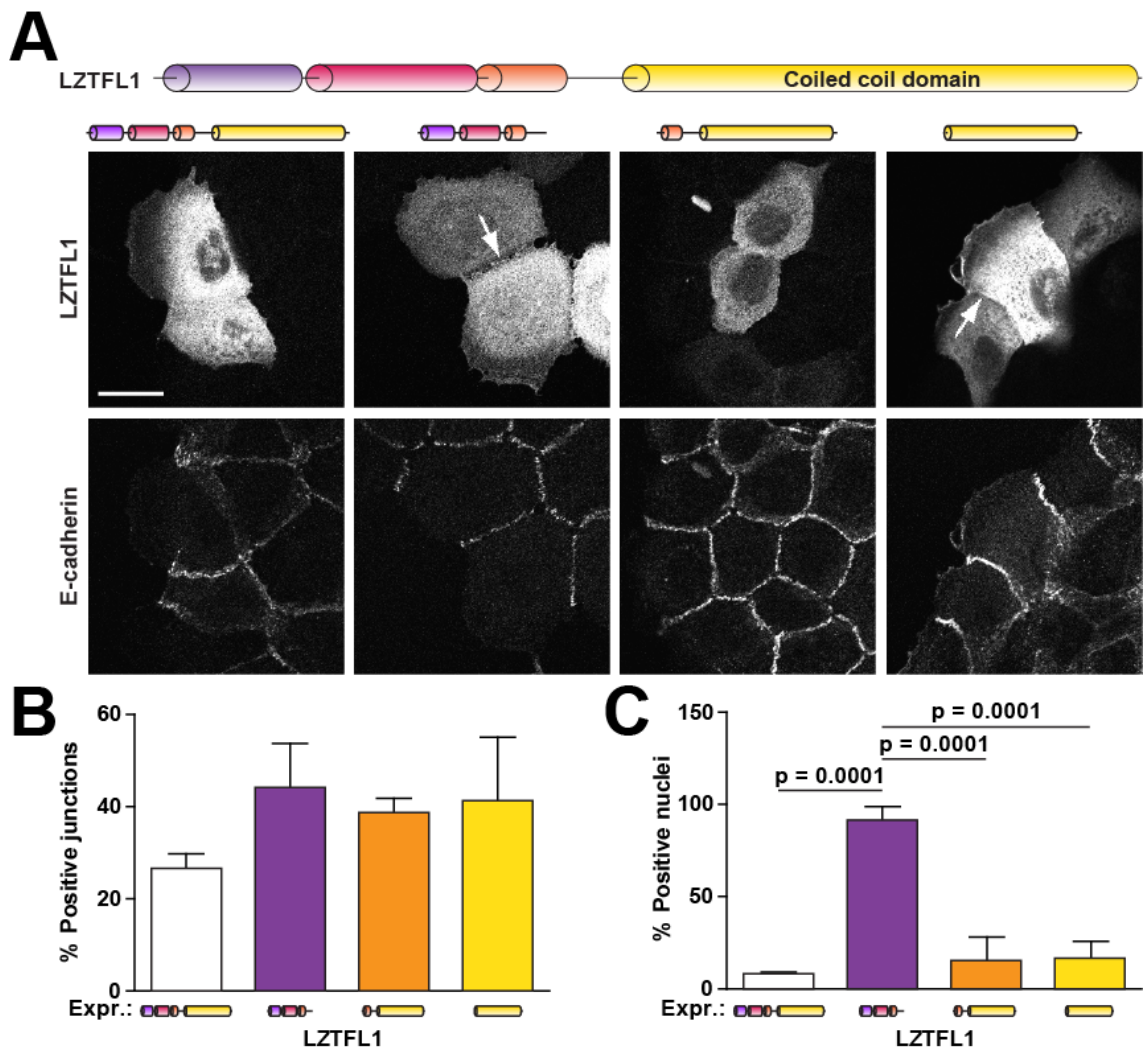


Figure 4.3: The N-terminal domain of LZTFL1 localises to the cell nucleus. LZTFL1 full-length and truncation proteins fused to mRFP were transfected into standard calcium keratinocytes before fixation and staining for E-cadherin and nuclei. A) Representative immunofluorescence images of keratinocytes transfected with the mRFP-LZTFL1 constructs depicted above. Arrows point at LZTFL1 at cell-cell contacts. Semi-quantitative quantification of LZTFL1 B) at cell-cell contacts or C) in nuclei. Graphs show mean $\pm$ SD. Data was analysed statistically by two-way ANOVA and Tukey's post-hoc test. N = 3. Scale bar = 20 μ m.

Subsequently, an antibody became available that appeared to work for endogenous staining of LZTFL1. Blocking the antibody either with GST or GST-LZTFL1 fusion protein was used to test the specificity of the antibody for use in Western blot and immunofluorescence staining. In Western blot, the antibody detected a band of the expected size (about 35 kDa) in both human keratinocyte and mouse heart lysates with the GST-blocked but not the GST-LZTFL1-blocked antibody, confirming that these bands represent LZTFL1 (Figure 4.4 A). Two additional higher molecular weight bands were detected in human lysates as well as a lower molecular weight band in mouse lysates. Interestingly, the higher molecular weight bands were not detected by LZTFL1-blocked antibody and the intensity of the lower molecular weight band was strongly reduced, suggesting that the bands must present some unidentified LZTFL1 products (Figure 4.4 A). It is unclear what the higher molecular weight bands could present, since no isoform with the molecular weight of about 45 kDa has been identified and stable posttranslational modifications are unlikely to cause such a large shift. Also, the bands are too low to represent LZTFL1 dimers and the Western blot conditions would not favour the detection of dimers as well. Therefore, it remains to be established what these bands represent. The low molecular weight band of about 27 kDa detected in mouse lysates could be *Lztfl1* isoform 3, which has a molecular weight of 29 kDa. RT-PCR could be used to test the presence of *Lztfl1* isoform 3 in mouse heart to support this hypothesis.

The antibody was further tested for immunofluorescence staining. Different fixation conditions were tested and good results were achieved with acetone or methanol fixation of human keratinocytes (Figure 4.4 B). With both fixations striking filamentous staining was obtained, but staining in methanol-fixed cells was stronger and was therefore chosen as the preferred fixation method. The specificity of the observed staining was confirmed by blocking the antibody with GST or GST-LZTFL1 (Figure 4.4 C).

LZTFL1 staining in human keratinocytes revealed that the LZTFL1 antibody labelled very straight and directional fibres (Figure 4.5 A). A subset of the fibres seemed to enclose the nucleus while another population of fibres run perpendicular to cell-cell contacts. Some fibres also appeared to pass through the nucleus and interestingly in many cells small bright dots were detected in the nucleus (Figure 4.5 A).

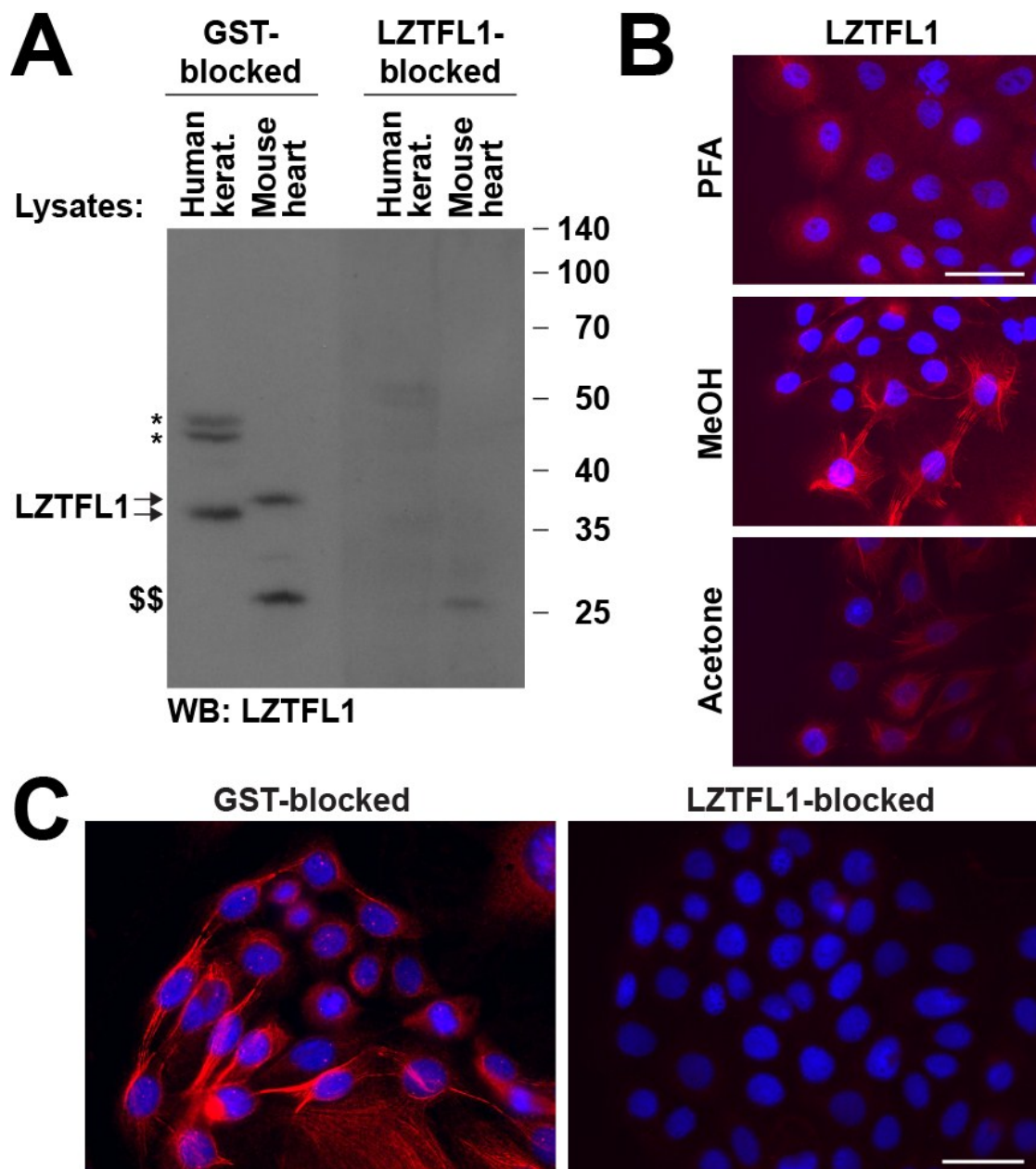


Figure 4.4: An antibody against LZTFL1 is able to specifically detect endogenous LZTFL1. An LZTFL1 affinity-purified antibody from ProteinAtlas was incubated with GST or GST-LZTFL1 bound to sepharose beads. The antibody dilutions were then used for A) Western blot or C) immunofluorescence staining of human keratinocytes. A) Western blot of human keratinocyte or mouse heart lysates using LZTFL1 antibody previously incubated with GST or GST-LZTFL1. Single stars mark higher molecular weight bands and two dollar symbols label lower molecular weight bands of unknown identity. Molecular weight markers are shown on the right. B) Immunofluorescence images of keratinocytes fixed with either paraformaldehyde (PFA), methanol (MeOH) or acetone and stained for LZTFL1. C) Immunofluorescence images of methanol-fixed keratinocytes stained for LZTFL1 with an antibody previously incubated with GST or GST-LZTFL1. Scale bar = 50 μ m.

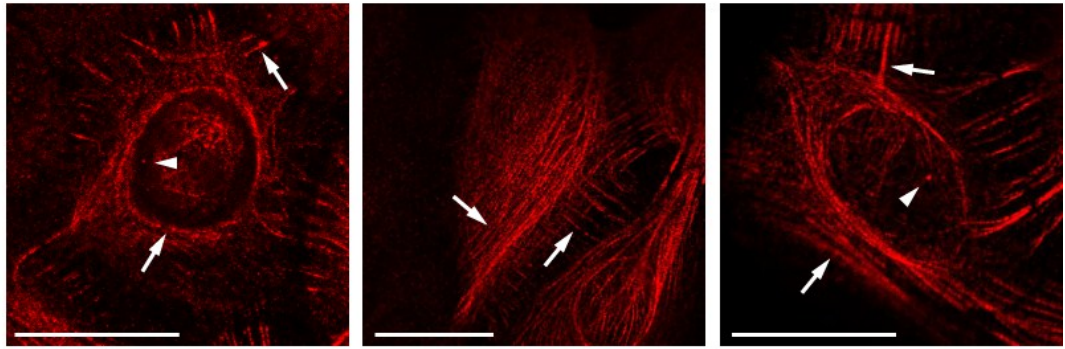
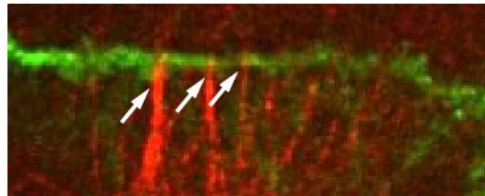
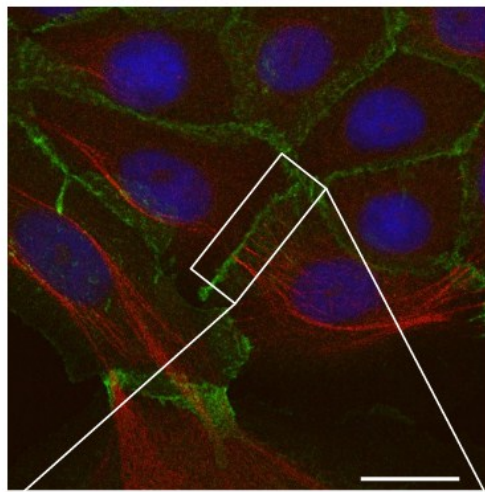
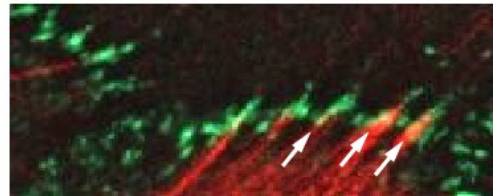
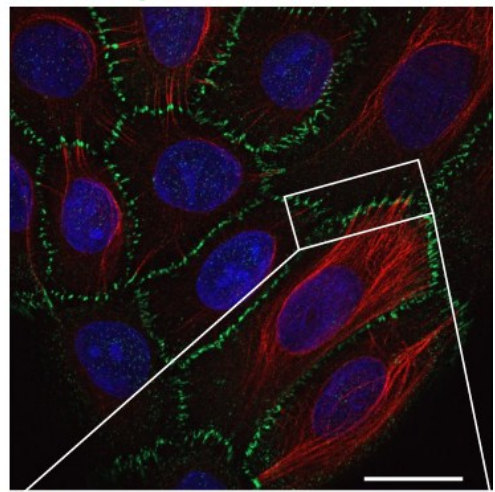
A**B****E-cadherin LZTFL1 DAPI****C****Desmoplakin LZTFL1 DAPI**

Figure 4.5: LZTFL1-labelled filaments co-localise both with E-cadherin and desmoplakin.
 A) Immunofluorescence images of keratinocytes stained for LZTFL1. Arrows point at filamentous structures and arrow heads at nuclear puncta. B-C) Keratinocytes were co-stained for LZTFL1 (red), nuclei (blue) and B) E-cadherin or C) desmoplakin (both green). Confocal immunofluorescence image of the Z-plane passing through B) adherens junctions or C) the desmosomes. Magnification of the boxed area is shown at the bottom of merged images. Arrows point at regions of partial co-localisation with LZTFL1. Scale bar = 20 μ m.

I then wanted to see how the fibres relate to AJ and desmosomes. Therefore, I co-stained LZTFL1 with E-cadherin or desmoplakin. Partial overlap at cell-cell contacts was detected between LZTFL1 and both E-cadherin and desmoplakin (Figure 4.5 B+D). As previously shown, E-cadherin staining was very continuous but desmoplakin staining appeared as short adjacent slashes. Interestingly, the LZTFL1-stained fibres seemed to directly insert into these slash-shaped patches of desmoplakin (Figure 4.5 C).

4.3.3 LZTFL1 is localising on keratin filaments

To identify which type of filament LZTFL1 co-localises with, I labelled keratinocytes for LZTFL1 and either actin, β -tubulin or keratin 14, as representatives of the three main cytoskeletal components (Figure 4.6). It was very clear that LZTFL1 co-localised with keratin 14, but interestingly, LZTFL1 only labelled a subset of keratin 14 fibres.

Although there might be an effect of LZTFL1 depletion on desmosomes in mature junctions, this effect was weaker than the effect of LZTFL1 depletion on AJ. As desmosomes form later than AJ during calcium switch (Gumbiner et al., 1988; Lewis et al., 1994), I asked whether the cellular localisation of LZTFL1 differs between nascent and mature cell-cell contacts.

Keratinocytes grown to confluence in low calcium medium were switched to standard calcium medium to induce cell-cell contact formation for one hour. Cells were co-stained for E-cadherin, keratin 14 and LZTFL1. As anticipated E-cadherin accumulated at cell-cell contacts as early as 15 minutes and cytoplasmic staining is reduced (Figure 4.7 A). In low calcium keratinocytes strongest keratin 14 staining was found in a perinuclear region and on short fibres at the cell periphery (Figure 4.7 A, 0 min). The short fibres were not unidirectional and were of different lengths. Fibres of adjacent cells were rarely aligned and did not seem to contact each other. Upon calcium switch the peripheral fibres became shorter, perpendicular to the cell surface and aligned with fibres of neighbouring cells (Figure 4.7 A, 15-60 min). This effect was observed as early as 15 minutes after switch, but the number of aligned fibres increased as contacts mature. By one hour after the switch, the aligned fibrils formed a tight border with very little spaces between laterally adjacent fibre pairs. A thin band between aligned fibrils of opposing cells was observed. LZTFL1 staining in this Z-plane is mainly cytoplasmic at all time points (data not shown).

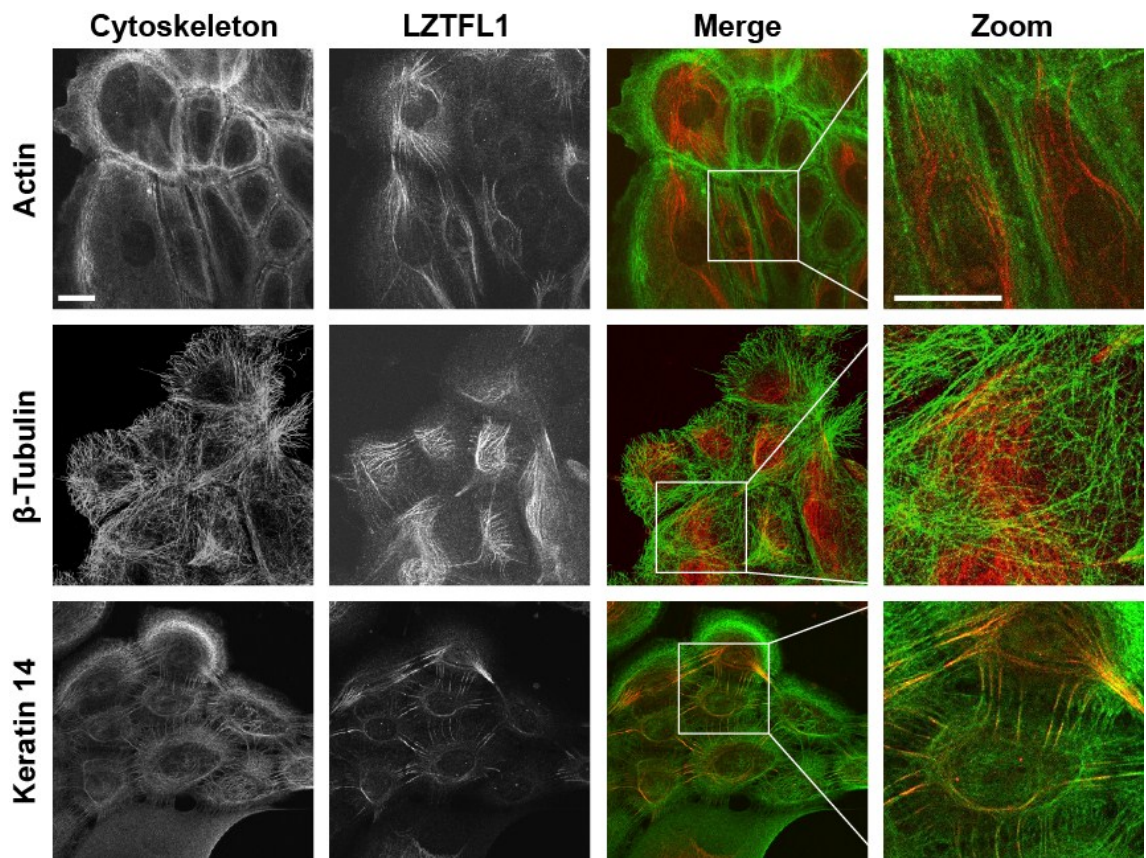


Figure 4.6: LZTFL1 localises to a subset of keratin fibres. Standard calcium keratinocytes were co-stained for LZTFL1 (red) and either actin, β -tubulin or keratin 14 (shown in green). Images were taken on a confocal microscope and up to three Z-planes covering the majority of the cytoskeleton were summed in ImageJ. Zoom is shown in the right panel and is a magnification of the boxed area. Scale bar = 20 μ m.

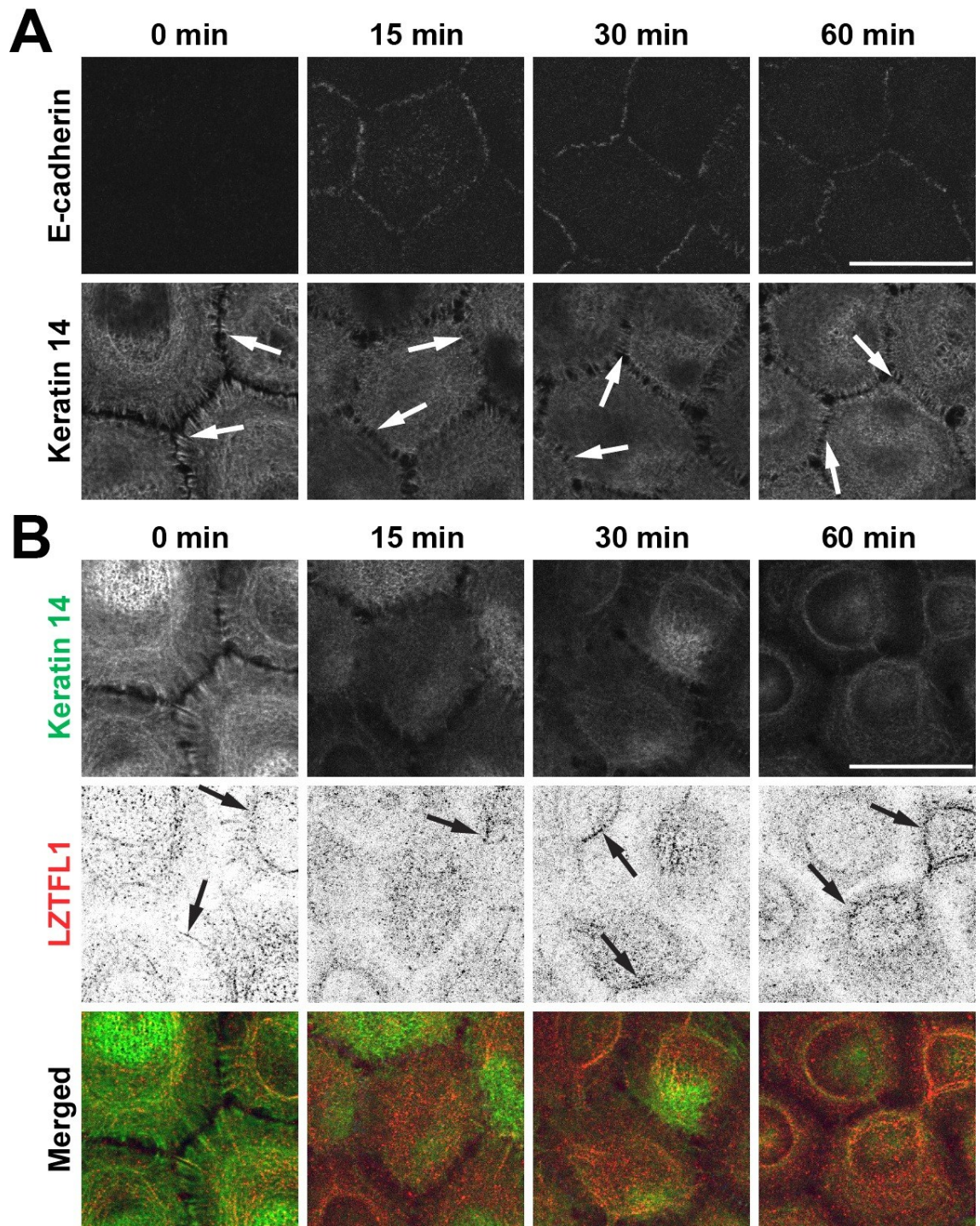


Figure 4.7: LZTFL1 and keratin 14 rearrange and compact during nascent cell contact formation. Cell-cell contact formation was induced in keratinocytes maintained in low calcium medium by the addition of 1.8 mM CaCl_2 to the medium. Cells were fixed after the times indicated and stained for E-cadherin, keratin 14 (green) and LZTFL1 (red). Images were taken on a confocal microscope. A) Immunofluorescence images of the Z-plane passing through the zonula adherens. B) Immunofluorescence images of the same cells as in (A) but showing the Z-plane with the most filamentous LZTFL1 staining. White arrows point at keratin filaments at cell-cell contacts and black arrows point at filamentous LZTFL1 structures. Scale bar = 20 μm .

In more basal regions of low calcium keratinocytes, a subset of keratin 14 fibres both in the perinuclear region and at the cell periphery co-stained with LZTFL1 (Figure 4.7 B, 0 min). At one hour after the switch some LZTFL1-positive filaments started to extend to contact sites and align with neighbouring cells (Figure 4.7 B, 60 min). However, the pattern was still very distinct to the LZTFL1 staining pattern observed in keratinocytes maintained in standard calcium (compare with Figure 4.4, Figure 4.5, Figure 4.6).

This experiment therefore suggested that some LZTFL1 is associated with a subset of keratin 14 filaments even in the absence of cell-cell contacts. Upon induction of contacts the association between LZTFL1 and keratin 14 seemed to increase. However, the formation of LZTFL1-positive filaments that extend across cells and align with adjacent cells at mature junctions appeared to be a late process that only starts by one hour after calcium switch.

4.3.4 LZTFL1 depletion reduces the association of keratin 14 fibres with cell-cell contacts

Considering the strong co-localisation of LZTFL1 with keratin 14 fibres associated with cell-cell contacts and the potential negative effect of LZTFL1 depletion on desmoplakin levels at mature desmosomes, I asked the question whether LZTFL1 depletion would affect keratin 14 fibres in standard calcium keratinocytes. Indeed, the keratin cytoskeleton looked very different in LZTFL1-depleted cells with fewer fibres contacting the membrane, although the alignment of fibres between adjacent cells seemed not affected (Figure 4.8 A). In fact, the keratin 14 filaments in LZTFL1-depleted cells seemed immature and resembled keratin 14 in keratinocytes 30 minutes after calcium switch (compare Figure 4.7A, 30 min) and did not seem to reach the density and compactness of mature cell-cell contact-associated filaments (compare to Figure 4.8A, control and Figure 4.7A, 60 min). The phenotype was quantified as mean grey values across lines drawn through the centre of cell-cell contacts as a relative readout for the number of fluorescent fibres attaching to cell-cell contacts. Mean grey values were significantly reduced in LZTFL1-depleted cells (Figure 4.8 C) suggesting that a reduced number of keratin 14 filaments attached to cell-cell contacts.

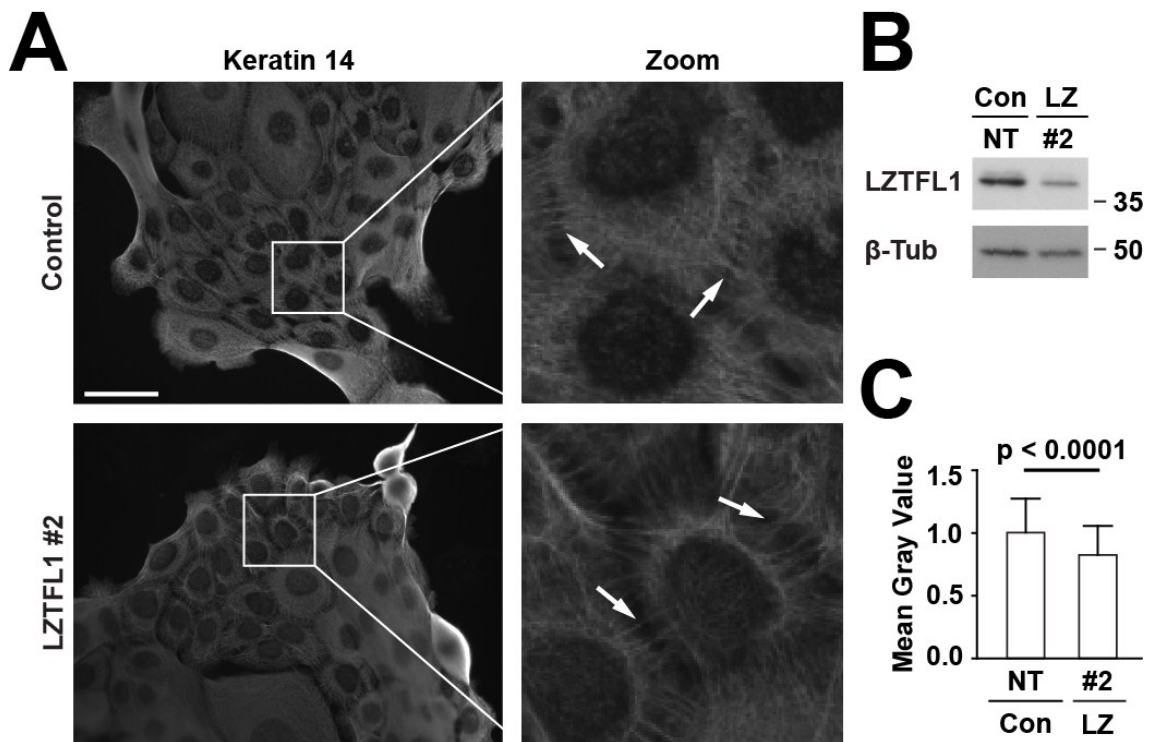


Figure 4.8: LZTFL1 depletion reduces compactness of keratin fibres at mature cell-cell contacts. Keratinocytes in standard calcium medium were treated with control or LZTFL1 (oligo #2) siRNA before fixation and staining for keratin 14. A) Representative immunofluorescence images of keratin 14-stained keratinocytes. The boxed area is magnified on the right. Arrows point at keratin fibres perpendicular to cell-cell contacts. B) Western blot of treated samples probed with antibodies against LZTFL1 (ProteinAtlas) and β -tubulin as loading control. Molecular weight markers are shown on the right. Average depletion of LZTFL1 with oligo #2 $70 \pm 13\%$. C) Keratin fibre density along cell-cell contacts was quantified as mean gray value of keratin staining across cell-cell contact sites. The graph shows mean $\pm$ SD. Data was analysed by 2-way ANOVA and Dunnett post-hoc test. N = 160 for NT, N = 180 for LZTFL1 oligo #2, from 3 experiments. Scale bar = 50 μ m.

4.3.5 LZTFL1 binds to keratin 14 through its N-terminus

In order to identify which part of LZTFL1 is responsible for localising to keratin 14, the different mRFP-tagged LZTFL1 constructs were transfected into keratinocytes and cells were fixed with methanol to mimic endogenous LZTFL1-staining conditions and remove free cytosolic proteins. The empty vector (mRFP) was found in small patches mainly in and around the nucleus of cells (Figure 4.9). Occasionally the staining seemed to exist along filaments. The C-terminus of LZTFL1 (142-299) did not localise to any filamentous structures, but was mainly found in the perinuclear region of the cell (Figure 4.9). Full-length LZTFL1 (1-299), N-terminal LZTFL1 (1-142) and LZTFL1 (95-299) all showed some filamentous localisation, with the strongest localisation to filaments exhibited by LZTFL1 (1-142) (Figure 4.9). These filaments co-localised with keratin 14 fibres. The same LZTFL1 mutants also accumulated at the free edge of overexpressing cells. In addition, LZTFL1 (1-142) notably accumulated at cell-cell contacts.

In order to confirm these results biochemically, I overexpressed the mRFP-LZTFL1 constructs in keratinocytes and used the cell lysates for pull-down assay experiments. RFP-tagged and associated proteins were extracted from lysates with RFP-trap beads and were tested for bound keratin 14 or GAPDH as control. All constructs expressed two proteins, a protein of the expected size and a second product about 10 kDa smaller in size (Figure 4.10, amido black, smaller product marked with asterisk). Since the second band was seen in all five constructs, it was concluded that it represents a N-terminally truncated mRFP-tag version, which is however still able to bind to the RFP-trap. Indeed, analysis of the mRFP sequence showed the presence of an alternative starting codon, which would produce a truncated version of mRFP about 10 kDa smaller in size. None of the constructs pulled-down GAPDH, but keratin 14 was found bound to full-length LZTFL1, LZTFL1 (1-142) and to a lesser extent to LZTFL1 (95-299) (Figure 4.10).

These experiments demonstrated that LZTFL1 can bind to keratin 14 via its N-terminus. The third α -helix of LZTFL1 has some binding affinity, but for efficient binding other regions of the LZTFL1 N-terminus are also required.

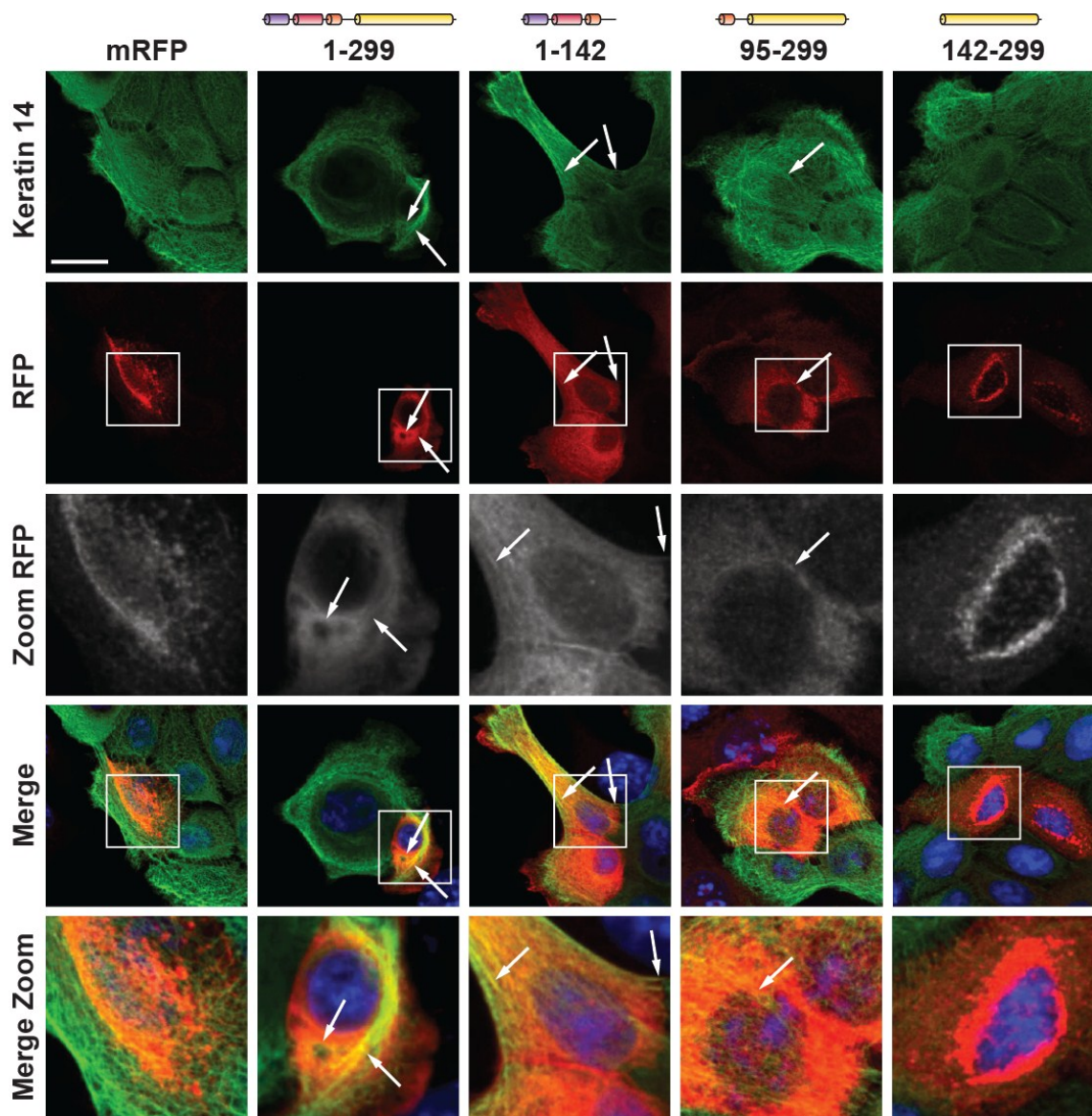


Figure 4.9: LZTFL1 localisation to keratin fibres does not require the C-terminal coil-coil domain of LZTFL1. Keratinocytes in standard calcium medium were transfected with cDNA constructs coding mRFP or mRFP-tagged LZTFL1 full-length (1-299) or truncation mutants as shown on top of the images. Cells were fixed with methanol and stained for keratin 14 (green) and RFP (red). Zoom shows the magnification of the boxed area. Arrows point at filaments where keratin 14 and LZTFL1 co-localise. Scale bar = 20 μm . N = 3.

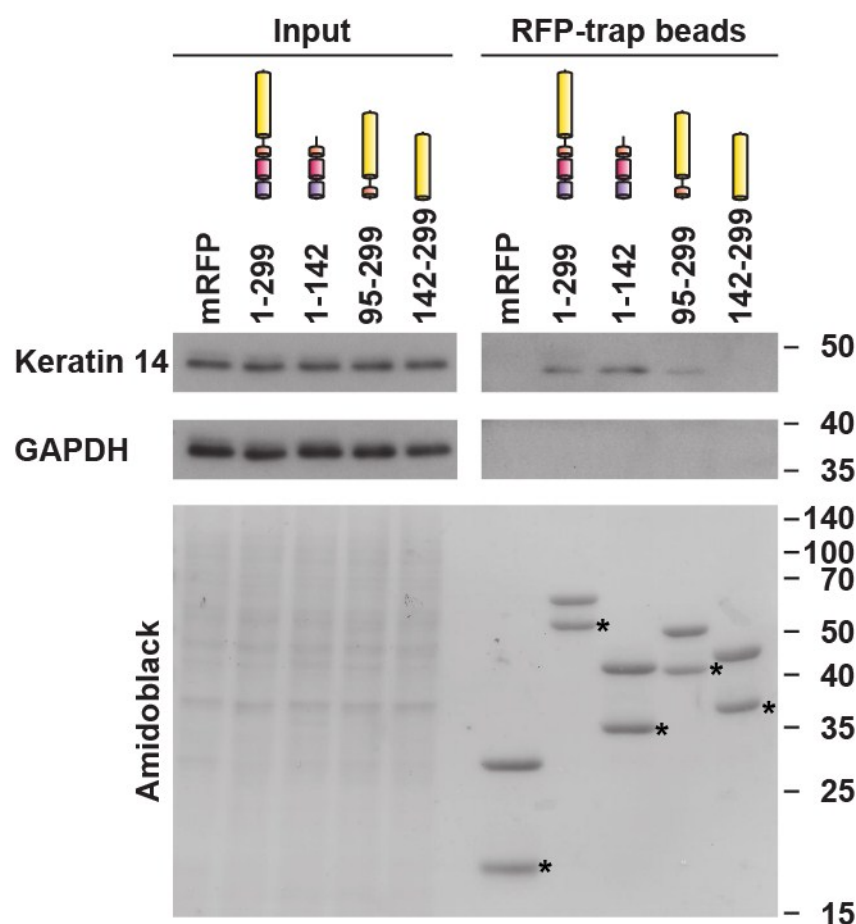


Figure 4.10: LZTFL1 interaction with keratin 14 requires the N-terminus of LZTFL1. Protein lysates from keratinocytes maintained in standard calcium medium and transfected with constructs coding mRFP or mRFP-tagged LZTFL1 full-length (1-299) or truncation mutants as shown on top of the blots. Lysates were tested for interaction between LZTFL1 and keratin 14 by pull down using RFP-trap beads. Samples were analysed by Western blot and probed with antibodies against keratin 14 and GAPDH as negative control. A sample of the lysates (0.5%) before pull-down assay was loaded as input control. Input was exposed for 10 seconds and RFP-trap bound samples were exposed for 2 hours. Amido black shows membrane bound proteins after transfer. The lower bands of the RFP-trapped proteins (marked with asterisks), represent fusion proteins tagged with a truncated version of mRFP, due to an alternative start codon in the mRFP sequence. Molecular weight markers are shown on the right. N = 5.

4.3.6 LZTFL1 binds to α -catenin

LZTFL1 depletion affects AJ and possibly desmosomes and I and others have shown that LZTFL1 partially localises to cell-cell contacts (Wei et al., 2010). Especially the junctional localisation of overexpressed LZTFL1 seemed quite distinct from the pool of cytoplasmic and filament-bound LZTFL1 (compare LZTFL1 (1-142) in Figure 4.2 A and Figure 4.9). Therefore, I wanted to test whether LZTFL1 could bind to core components of desmosomes or AJ.

For this purpose I optimised the bacterial production of LZTFL1 fusion protein. LZTFL1 was produced as fusion protein with a N-terminal His-GST-tag separated from LZTFL1 by a TEV-cleavage site. LZTFL1 was immobilised on glutathione beads and a batch of LZTFL1 was subsequently cleaved off the beads by TEV enzyme (Figure 4.11 A).

To test for binding of LZTFL1 to the desmosomal complex, LZTFL1 pull-down assays were tested for the presence of the desmosomal components desmoplakin and plakoglobin. Neither protein was detected (Figure 4.11 B). Although LZTFL1 pull-down assays have not been tested for other desmosomal proteins, the absence of desmoplakin and plakoglobin suggested that LZTFL1 does not bind to soluble desmosomal components.

In contrast, E-cadherin, α -catenin, β -catenin and p120 catenin were detected on the LZTFL1 beads, implying that LZTFL1 can bind to the AJ complex (Figure 4.12 A). In order to find out to which component of the complex LZTFL1 binds, reverse pull-down assay experiments were performed with the separate AJ components immobilised on beads and pull-down assays were probed for the presence of LZTFL1. Endogenous LZTFL1 strongly bound to α -catenin, but not to any other of the tested AJ components (Figure 4.12 B).

I aimed to establish whether LZTFL1 could directly bind to α -catenin. For this purpose, *in vitro* pull-down assays were performed with immobilised GST-LZTFL1 or GST- α -catenin and cleaved α -catenin or LZTFL1, respectively. Pull-down assays of α -catenin with β -catenin-beads was used as a positive control. LZTFL1 interacted with purified α -catenin and vice versa, which demonstrated that LZTFL1 is a direct binding partner of α -catenin (Figure 4.13 A).

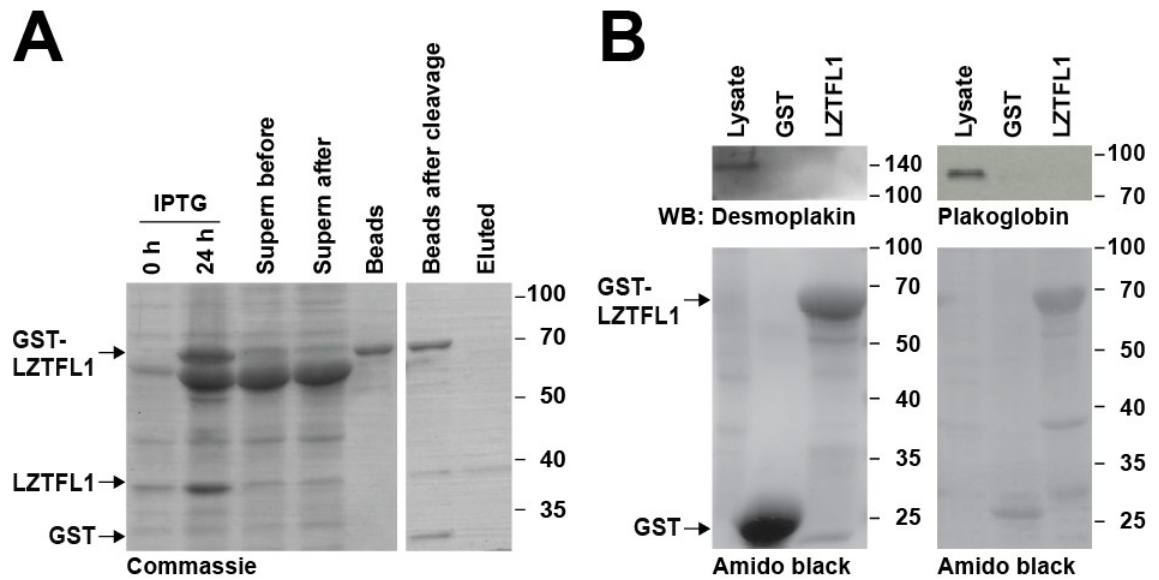


Figure 4.11: LZTFL1 does not bind the desmosomal components desmoplakin and plakoglobin. A) GST-LZTFL1 was produced in Arctic Express *E.coli* by induction with IPTG for 24 hours with lane 1 showing bacterial lysate before induction and lane 2 representing lysate after induction. Cells were lysed (Supern before) and GST-LZTFL1 was immobilised on glutathione sepharose beads (Beads) and bacterial lysate after incubation with beads is shown (Supern after). LZTFL1 was cleaved off the beads by incubation with TEV enzyme. Beads after TEV cleavage and cleaved and eluted LZTFL1 are shown on the left. B) Lysates of standard calcium keratinocytes were incubated with sepharose beads coated with either GST alone or GST-LZTFL1. Pulled down proteins were probed for the presence of desmoplakin or plakoglobin. Amido black represents the amount of fusion protein used for the pull-down assay. Molecular weight markers are shown on the right. N = 2.

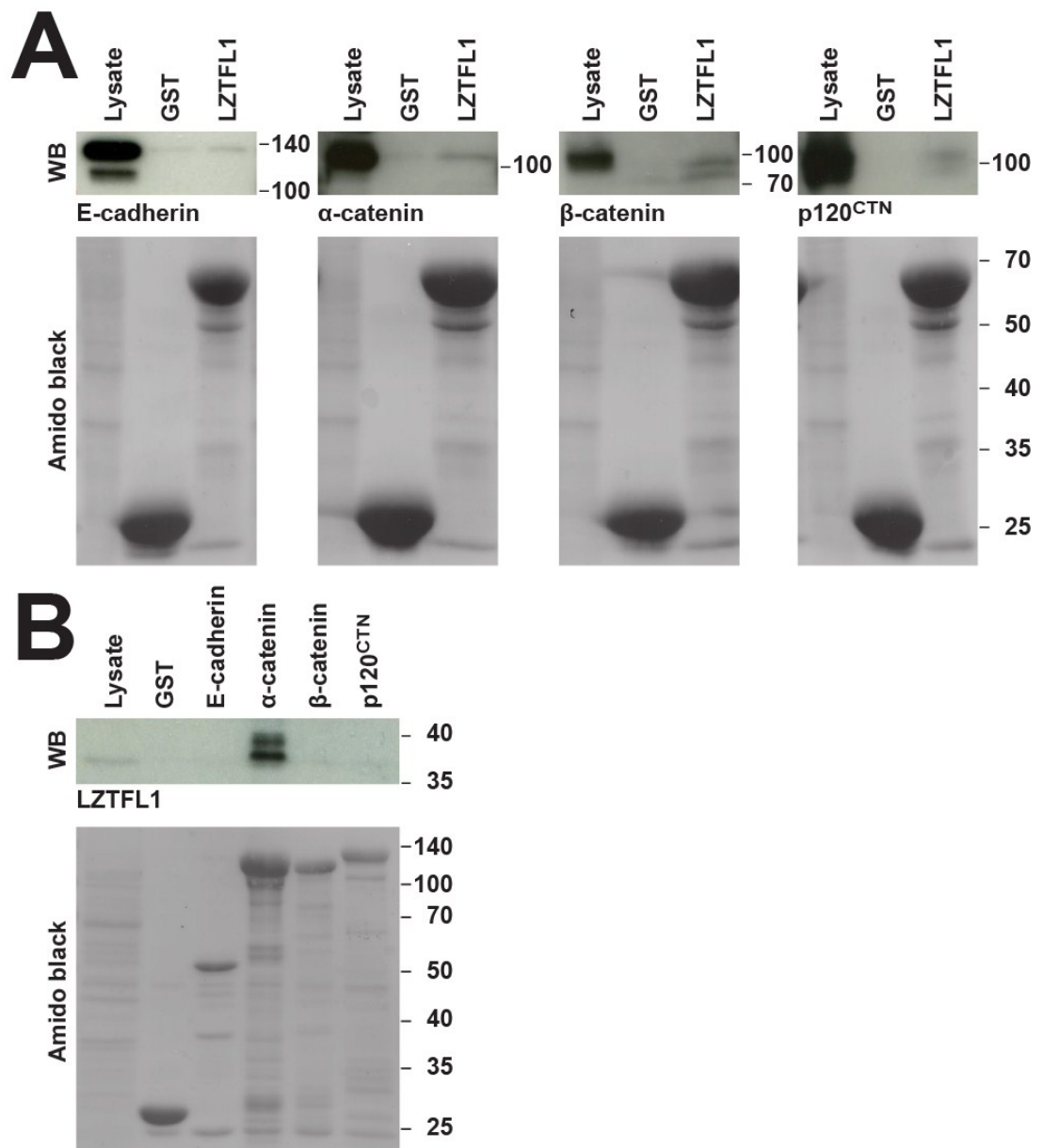


Figure 4.12: LZTFL1 binds adherens junctions via α -catenin. Lysates of standard calcium keratinocytes were incubated with sepharose beads coated with either GST alone or A) GST-LZTFL1 or B) components of the adherens junction complex (E-cadherin cytoplasmic tail, α -catenin, β -catenin or p120^{CTN}). Pulled down proteins were probed for A) the adherens junction components E-cadherin, α -catenin, β -catenin and p120^{CTN} or B) using an antibody against LZTFL1 (Proteinatlas). Amido black represents the amount of fusion protein used for the pull-down assay. Molecular weight markers are shown on the right. N = 5 (A); N = 3 (B).

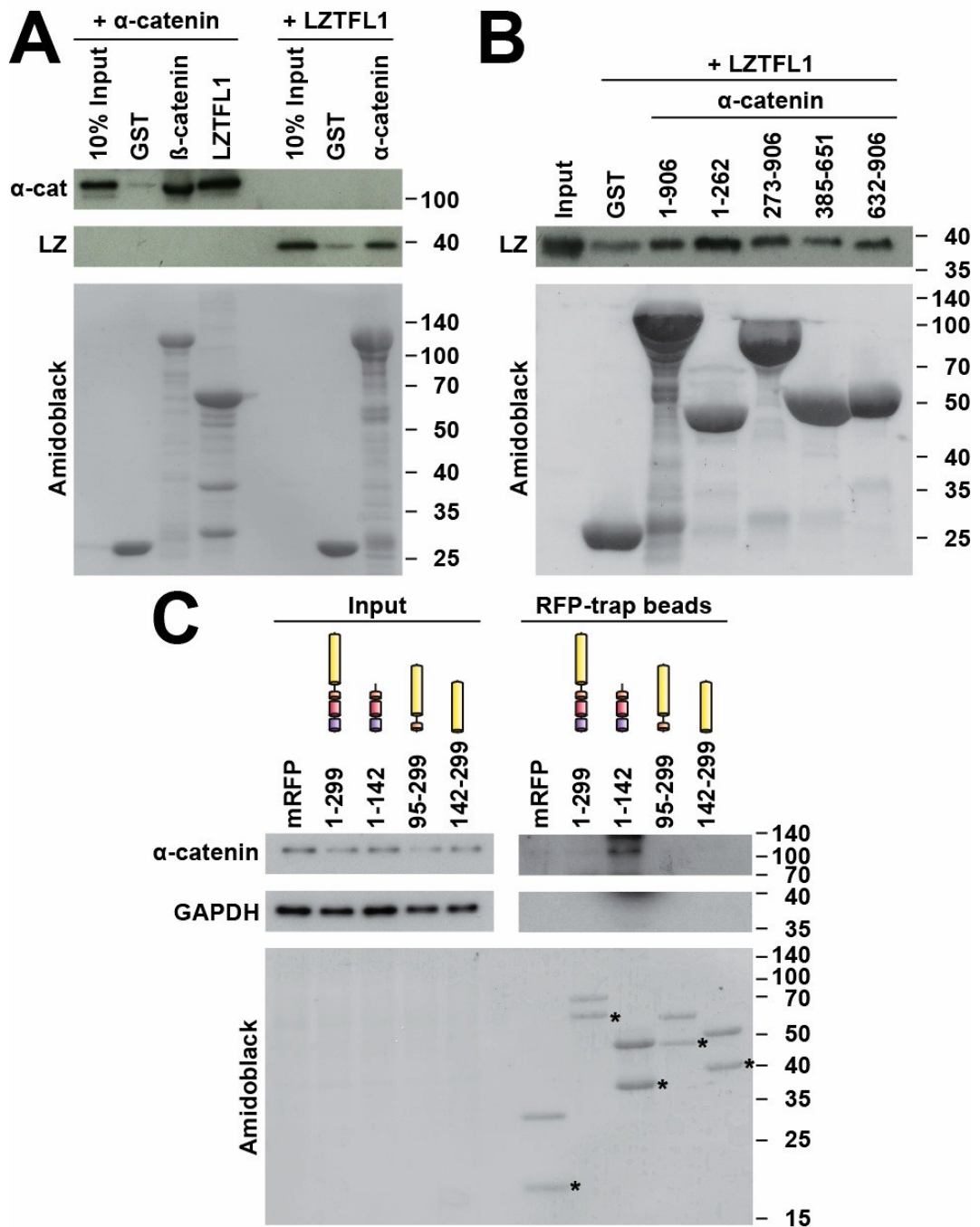


Figure 4.13: LZTFL1 and α -catenin directly interact via their respective N-termini. For *in vitro* pull downs A) GST-tagged full-length fusion proteins or B) α -catenin truncation mutants on beads were incubated with cleaved and eluted α -catenin or LZTFL1. The pulled down proteins were analysed by Western blot using an antibody against LZTFL1 (LZ) (ProteinAtlas) or α -catenin. C) Keratinocytes maintained in standard calcium medium and transfected with constructs coding mRFP or mRFP-tagged LZTFL1 full-length (1-299) or truncation mutants as shown on top of the blots were lysed for pull-down assays. Samples were tested for interaction between LZTFL1 and α -catenin by pull-down assays using RFP trap beads. Samples were analysed by Western blot and probing with antibodies against α -catenin and GAPDH as negative control. A sample of the lysates (0.5%) before pull-down assay was loaded as input control. Input was exposed for 1 minute and RFP-trap bound samples were exposed for 100 minutes. Amidoblack staining shows fusion proteins used (A, B) or lysates and bead-bound proteins (C). The lower bands of the RFP-trapped proteins, marks with asterisks, represent fusion proteins tagged with a truncated version of mRFP, due to an alternative start codon in the RFP sequence. Molecular weight markers are shown on the right. N = 3 (A, B) or 5 (C).

To further characterise the interaction between LZTFL1 and α -catenin, I used α -catenin truncation mutants bound to beads for *in vitro* pull-down assays of full-length LZTFL1. The strongest binding was observed with the N-terminal fragment of α -catenin (1-262) (Figure 4.13 B). The C-terminal part of α -catenin (273-906) also seemed to bind LZTFL1 above background binding, but the binding was less compared to the N-terminus (1-262).

To establish the α -catenin-binding region on LZTFL1, different truncation mutants of LZTFL1 were expressed and keratinocyte lysates subjected to RFP-trap pull-down assay to precipitate endogenous α -catenin. GAPDH was used as negative control (Figure 4.13 C). Weak binding to α -catenin was observed with full-length LZTFL1. However, LZTFL1 (1-142) strongly pulled down α -catenin. These experiments therefore showed that LZTFL1 can directly bind to α -catenin and that both proteins probably interact via their respective N-termini.

4.3.7 LZTFL1 increases internuclear distance and cell area of HaCaT in response to EGF

I have established that LZTFL1 is required to stabilise AJ and can bind to the AJ complex. LZTFL1 is a tumour suppressor and it appears likely that LZTFL1 could exert this function by stabilising AJ and thereby preventing EMT and metastasis. Although LZTFL1 levels are reduced in cancer tissue as compared to matched normal tissue (Wang et al., 2014; Wei et al., 2010), it has not been assessed whether there are differences in LZTFL1 levels between primary and secondary tumour of the same patient.

The head and neck squamous carcinoma cell lines HN4 and HN12 as well as HN30 and HN31 are pairs of cell lines derived from primary tumour and lymphnode metastasis from two different patients. HN4, HN30 and HN31 have a mainly epithelial phenotype, while HN12 is highly transformed and mesenchymal. By Western blot all HN cell lines had lower levels of E-cadherin as compared to keratinocytes (Figure 4.14 A). HN12 cells expressed the lowest levels of E-cadherin consistent with its mainly mesenchymal phenotype.

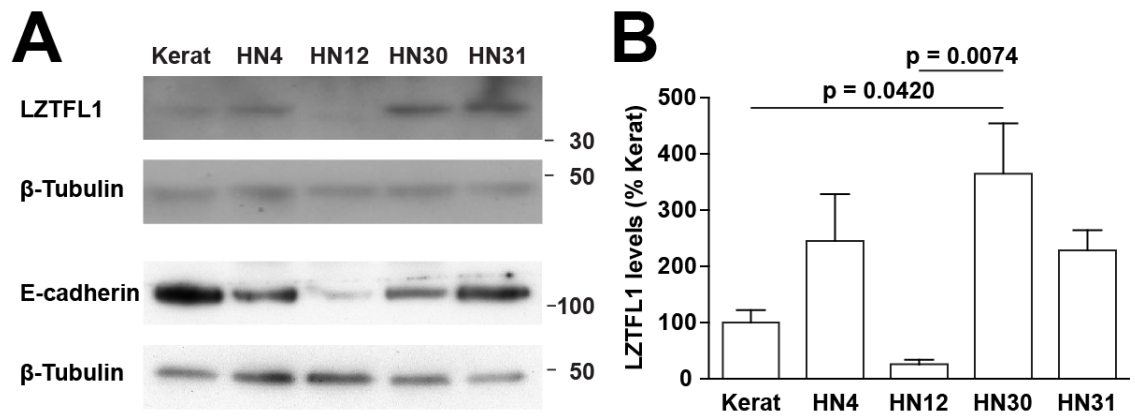


Figure 4.14: LZTFL1 is differentially expressed in head and neck squamous cancer cell lines. A) Protein lysates from keratinocytes and head and neck squamous carcinoma cell lines HN4, HN12, HN30 and HN31 were analysed by Western blot using antibodies against LZTFL1 (clone 7F6), E-cadherin and β -tubulin as loading control. Molecular weight markers are shown on the right. B) Quantification of LZTFL1 Western blots. LZTFL1 levels are represented relative to levels in keratinocytes. Graphs show mean + SD and data was analysed by 1-way ANOVA and Tukey's post-hoc test. N = 3.

LZTFL1 levels seem to be increased in HN4, HN30 and HN31 compared to keratinocytes, but the difference was only significant in HN30 (Figure 4.14 B). HN12 had highly reduced levels of LZTFL1, but again the difference was not significant. The lack of significance is likely due to small N numbers and the resulting lack of power. When comparing LZTFL1 levels between primary and secondary tumour, LZTFL1 was reduced in the metastatic tumour (HN12, HN31) of both patients, although the difference was not significant based on three Western blot repeats. This suggests that LZTFL1 levels inversely correlates with invasiveness and metastatic potential.

However, in contrast to colon cancer (Wang et al., 2014), LZTFL1 expression did not directly correlate with E-cadherin levels. HN12 cells had both strongly reduced levels of LZTFL1 and E-cadherin, but HN4, HN30 and HN31 had reduced E-cadherin but increased LZTFL1 levels compared to keratinocytes. Compared to HN30, HN31 had increased E-cadherin levels but decreased LZTFL1 levels.

Increased EGF signalling is a known inducer of EMT and leads to disassembling of AJ and cell scattering (Lu et al., 2003). I aimed to test whether LZTFL1 could stabilise AJ and prevent cell scattering after EGF treatment. For this experiment I used the immortalised keratinocyte cell line HaCaT, which is more prone to EGF-induced scattering than primary keratinocytes. Endogenous levels of LZTFL1 in HaCaT are higher as compared to primary keratinocytes (S. Bowling, data not shown).

HaCaT were transfected with vectors coding eGFP or eGFP-LZTFL1 and cell scattering was induced by treatment with 100 mM EGF (Figure 4.15 A). Internuclear distance between transfected cells and their direct neighbours was assessed for transfected cells at the colony border to quantify cell dispersion (Clark, 1994; Pope et al., 2008). LZTFL1 expression slightly, but significantly increased the internuclear distance after EGF scattering (Figure 4.15 B). This could suggest increased cell scattering in LZTFL1-transfected cells. However, LZTFL1-expressing cells appeared to be bigger than eGFP-transfected cells. Quantification confirmed that cell area of transfected cells was significantly increased in LZTFL1-expressing cells (Figure 4.15 C). This indicates that the enlarged cell area of LZTFL1-transfected cells probably caused the increase in internuclear distance. The increase in cell area seemed to be a specific response to EGF, since mRFP and mRFP-LZTFL1-transfected cells in the absence of EGF did not significantly differ in area (Figure 4.15 D). Due to time constraints, internuclear distance of empty vector- and LZTFL1-expressing cells in the absence of EGF was not determined, but should be assessed in the future.

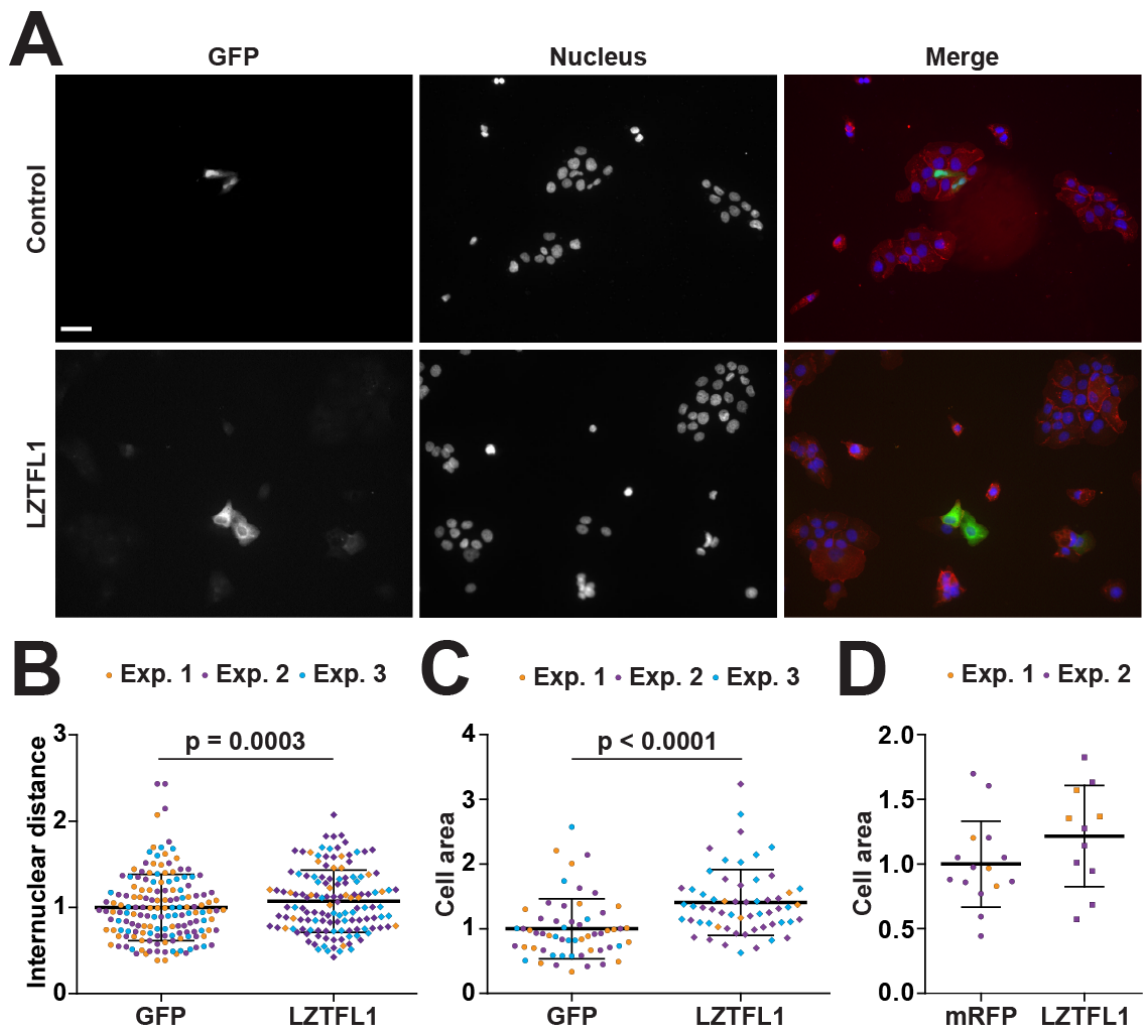


Figure 4.15: Overexpression of LZTFL1 increases internuclear distance and cell area upon EGF treatment. HaCaT cells were transfected with cDNA encoding eGFP or eGFP-tagged LZTFL1 before treatment with 100 mM EGF for 24 hours to induce cell scattering (A-C). Alternatively, cells were transfected with mRFP or mRFP-LZTFL1, but were not treated with EGF (D). Cells were fixed and stained for E-cadherin and nuclei. N = 3. A) Representative immunofluorescence images of HaCaT colonies with transfected cells (GFP – green, E-cadherin – red, nuclei – blue). Scale bar = 50 μ m. B) Quantification of internuclear distance of transfected cells. Internuclear distance was measured for up to a maximum of five directly neighbouring cells. C-D) Quantification of cell area of transfected cells. Only transfected cells at the colony border were analysed. Graphs show mean $\pm$ SD. Data was analysed by 2-way ANOVA and Tukey post-hoc test. N = 158 for GFP, N = 163 for LZTFL1, from 3 experiments (B); N = 57 for GFP, N = 59 for LZTFL1, from 3 experiments (C); N = 15 for mRFP, N = 11 for LZTFL1, from 2 experiments (D).

4.4 Conclusion

In this chapter, I demonstrate that LZTFL1 depletion might reduce F-actin compaction and decreases E-cadherin levels at mature AJ and the effect is stronger than in nascent cell-cell contacts. LZTFL1 can associate with AJ by direct binding to α -catenin and the interaction between LZTFL1 and α -catenin is likely mediated via their respective N-termini.

Furthermore, LZTFL1 depletion might reduce desmoplakin levels at mature cell-cell contacts. LZTFL1 does not seem to bind to soluble desmosomal core complex components, but it binds to keratin 14 filaments anchored to desmosomes, but also filaments in the cytoplasm and around the nucleus. This interaction also requires the LZTFL1 N-terminus. A pool of LZTFL1 seems to be associated with keratin 14 even in the absence of cell-cell contacts. The formation of the LZTFL1-labelled keratin 14 fibres anchored to desmosomes seems to be a late event during contact formation that only starts one hour after contact initiation. LZTFL1 appears to be required for the organisation of keratin 14 filaments at desmosomes, since LZTFL1 depletion reduces the number of fibres associated with cell-cell contact sites but does not seem to influence filament alignment.

LZTFL1 levels are reduced in head and neck squamous carcinoma cell lines derived from metastatic tumours when compared with primary tumours from the same patient. The cell line HN12 with the most transformed, mesenchymal phenotype had the lowest levels of LZTFL1. LZTFL1-overexpressing HaCaT cells increased in cell area in response to EGF as compared to cells expressing eGFP. This effect seems to be a specific response to EGF treatment.

4.5 Discussion

4.5.1 LZTFL1 as a stabiliser of adherens junctions

In this study I provide compelling evidence for a LZTFL1 function in the stabilisation of cell-cell contacts by influencing AJ and possibly desmosomes. I show that LZTFL1 depletion partially disturbs both types of junctions and that it can bind to the AJ core component α -catenin but also to keratin 14, the cytoskeletal filament underlying desmosomes in basal keratinocytes (Figure 4.16).

Given the partial junctional localisation of LZTFL1 and its ability to bind α -catenin, it is likely that binding to α -catenin recruits LZTFL1 to AJ where it then can exert its AJ stabilizing function. It remains to be answered how LZTFL1 supports AJ maintenance. LZTFL1 could sustain mature AJ by stabilizing α -catenin at AJ or by aiding the recruitment of actin-binding proteins required to link AJ to the actin cytoskeleton. However, a major structure-regulating role seems unlikely because the phenotype observed with LZTFL1 depletion is very mild compared to defects observed in α -catenin- or, for example, formin-1 or EPLIN-depleted cells (Abe and Takeichi, 2008; Kobiela et al., 2004; Vasioukhin et al., 2000).

Since actin remodelling did not seem strongly impaired with LZTFL1 depletion, a direct or indirect function in junctional actin formation downstream of AJ seems unlikely. However, circumferential thin actin bundles appeared less compact in cells depleted of LZTFL1, which coincided with an apparent overall reduction of compaction of neighbouring cells. Although these defects so far are merely observational and will require further experimental verification, they indicate a potential role for LZTFL1 in cell contraction or maturation of polarization. Compaction of circumferential thin actin bundles requires myosin-II activity and is involved in the increase of lateral height in epithelial cells during morphogenesis to achieve cuboidal shape (Zhang et al., 2005). Reduced cell compaction in LZTFL1-depleted cells could be one explanation for their lower resistance to mechanical stress. The question remains how LZTFL1 would affect thin bundle contraction. Preliminary experiments do not suggest an actin-binding capability of LZTFL1 (data not shown). Instead, LZTFL1 might regulate actin and cell compaction indirectly via Rho GTPases, activation of myosin II through recruitment of myosin II regulators or recruitment of actin-bundling proteins.

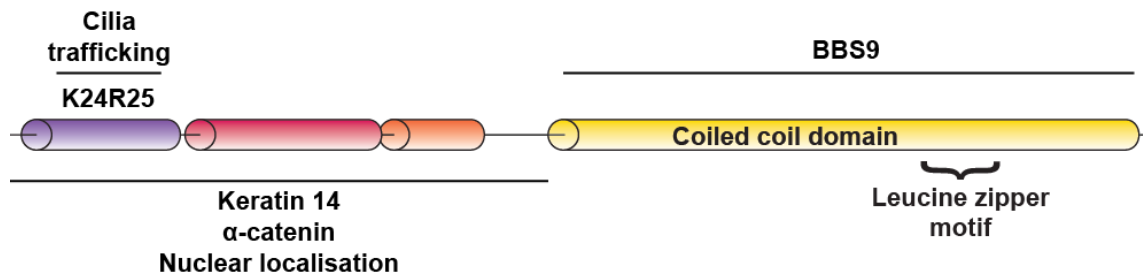


Figure 4.16: Summary of current knowledge of LZTFL1 structure and function. It has previously been shown that LZTFL1 binds to BBS9 and regulates the ciliary localisation of the BBSome complex for which lysine and arginine residues in position 24 and 25 (K24R25) are required (Seo et al., 2011). The current study has shown that LZTFL1 can bind to α -catenin and keratin 14 via its N-terminal domain and that nuclear localisation of LZTFL1 is also dependent on this part of the protein. LZTFL1 contains a leucine zipper motif, but so far no function involving this motif has been identified.

4.5.2 LZTFL1 as a regulator of keratin and desmosomes

LZTFL1 localises on keratin 14 filaments and LZTFL1 depletion seems to affect both desmosomes and keratin 14 filament density connected to cell-cell contacts. Keratin anchorage is required for desmosome stability (Kröger et al., 2013; Long et al., 2006; Wallace et al., 2012) and therefore it is likely that the reduction of desmoplakin at cell-cell contact sites observed with one LZTFL1 oligo is a consequence of decreased connectivity to keratin 14. An indirect effect on desmosomes is supported by LZTFL1 pull-down assay experiments indicating that LZTFL1 does not bind soluble components of the desmosomal core complex.

It is intriguing that LZTFL1 is only found on a subset of keratin 14 fibres. It will be interesting to investigate what distinguishes these fibres from others in the cells. Post-translational modifications are highly likely to directly or indirectly regulate LZTFL1 recruitment to keratin fibres. Keratin fibres represent scaffolds for a large number of keratin-binding proteins, which bind in response to certain keratin modifications (Green et al., 2005) and some such proteins might in turn recruit LZTFL1 to keratin fibres.

Interestingly, LZTFL1 is found specifically at keratin fibres that surround the nucleus and those that bind to desmosomes. These fibres have been shown to be the most stable and least dynamic keratin filaments in the cell (Strnad et al., 2002; Windoffer et al., 2011). It is believed that keratin maturation is anchorage-dependent and involves mechanosensing, which leads to alterations in the interaction with proteins that influence keratin dynamics. In the case of the perinuclear filaments, anchorage to the nuclear envelope is mediated via plectin-nesprin-3a (Wilhelmsen et al., 2005). The precise signal that alters interactions with keratin-binding proteins in response to attachment to desmosomes or the nuclear envelope is unknown, but keratin phosphorylation is thought to be a likely cue.

I therefore hypothesise that LZTFL1 specifically binds to anchored keratin filaments and aids their stabilisation, which in turn leads to reinforcement of cell-cell contacts. LZTFL1 recruitment might occur in response to mechanosensing of early desmosomes and correlate with desmosome maturation. This notion is supported by the staining pattern of LZTFL1 after calcium switch. LZTFL1 was found at perinuclear keratin fibres even in the absence of cell-cell contacts since the anchorage of these fibres to the nuclear envelope is independent of cell junctions. One hour after calcium switch LZTFL1-labelled fibres start appearing linked to cell membranes and this timing coincides with the maturation of keratin at cell-cell contacts. In the absence of LZTFL1,

keratin 14 maturation at contact sites is impaired as evident by the reduced number of keratin filaments found attached to cell-cell contacts. However, not all attachment of keratin 14 to desmosomes is prevented. These observations fit with the hypothesis of Windhoff *et al.* that cycling of keratins is a mechanism to probe the cell periphery for manifestation of new cell contacts and that established desmosomes are stabilised by turnover exit of attached keratin 14 filaments (Windoffer *et al.*, 2011).

A role in intermediate filament stabilisation by LZTFL1 might also explain LZTFL1 function in neurite outgrowth. LZTFL1-overexpressing Neuro 2a cells exhibited a larger number and increased length of neurites (Sakurai *et al.*, 2011). Phosphorylated neurofilament stabilises neurites, although it is not directly involved in neurite outgrowth (Shea and Beermann, 1994). In addition, neurofilaments are specifically involved in the elongation of long axons (Walker *et al.*, 2001). Although it needs to be tested whether LZTFL1 can bind to neurofilament, it is tempting to speculate that LZTFL1 is involved in the stabilisation of neurofilaments and thereby aids the stabilisation as well as elongation of neurites.

4.5.3 LZTFL1 as a cross bridge between AJ-mediated and desmosomal adhesion

LZTFL1 might stabilise cell-cell contacts by independently supporting the maintenance of AJ and desmosomes. However, given the proximity and functional interplay of both cell-cell contact complexes, I speculate that LZTFL1 may form a bridge between both types of adhesion complexes.

So far no such composite junction has been described in epithelial cells, possibly because of the presence of desmosomes as dedicated anchors of intermediate filaments. However, given the ability of LZTFL1 to bind to both α -catenin and keratin 14, I postulate that a pool of mature AJ might be coupled to keratin 14 via LZTFL1 and that such specialised mixed junctions might be specific mechanosensing complexes.

Several observations support this hypothesis. Firstly, LZTFL1 can bind to α -catenin and keratin 14. Secondly, LZTFL1-labelled keratin fibres attach to mature, but not early cell-cell contacts, which mimics the late association of plakoglobin, desmoplakin and supposedly vimentin with VE-cadherin in endothelial cells (Kowalczyk *et al.*, 1998; Lampugnani *et al.*, 1995; Valiron *et al.*, 1996). Thirdly, the LZTFL1-labelled fibres showed a degree of co-localisation with both E-cadherin and desmoplakin. Finally, LZTFL1 depletion weakens cell-cell contacts, but causes stronger defects on mature

AJ than nascent AJ and desmosomes. Thus, LZTFL1 has a stabilising effect on AJ and mechanosensing has recently emerged as a requirement for AJ reinforcement (Leckband et al., 2011).

Alpha-catenin is a key component of the tension-sensing machinery at AJ (Leckband et al., 2011), which is used to recruit more actin filaments to strengthen AJ under stress (Thomas et al., 2013; Yonemura et al., 2010). Keratin filaments also have mechanosensing abilities (Bordeleau et al., 2012; Gregor et al., 2014; Russell et al., 2004). Furthermore, they are linked to the nuclear envelope, which permits the translation of mechanical stress detected at the cell periphery into transcriptional changes (Bloom et al., 1996; Simpson et al., 2011). AJ that are linked to keratin filaments via α -catenin and LZTFL1 could therefore form a specialised mechanosensing apparatus that allows signals received at AJ to be transmitted to the nucleus and the metabolic machinery attached to desmosomes to provoke fast cellular responses.

4.5.4 LZTFL1 in the nucleus

Overexpression and endogenous staining show a pool of LZTFL1 in the nucleus and the N-terminus of LZTFL1 seems to be required for nuclear localisation (Figure 4.16). It is important to note, that computational prediction programs did not identify a non-canonical nuclear localisation signal (NLS) in the LZTFL1 amino acid sequence and that the N-terminus does not contain the leucine zipper motif. Therefore, it remains to be established how and why the LZTFL1 N-terminus enters the nucleus and why the full-length protein is found in the nucleus to a much lesser extent. It is likely that LZTFL1 might enter the nucleus bound to another protein. The identity of this binding partner is unclear as is the potential function of LZTFL1 in the nucleus. Recently, an interaction of LZTFL1 with β -catenin has been described (Wang et al., 2014). However, this interaction is unlikely to explain LZTFL1 localisation to the nucleus because LZTFL1 was shown to prevent β -catenin translocation to the nucleus (Wang et al., 2014). Furthermore, in our cell system, at least under the condition tested, β -catenin does not seem to bind significantly to LZTFL1.

Although further work is required to establish a role for LZTFL1 in the nucleus, it is interesting to note that LZTFL1 joins a group of other proteins that have been found both at cell-cell contacts and in the nucleus. Such protein as β -catenin (Valenta et al., 2012) or Ajuba (Kanungo et al., 2000; Marie et al., 2003; Nola et al., 2011) are likely to link adhesive and transcriptional events. Another interesting example is Pinin, that

binds to desmosomes and keratin but also localises to nuclear speckles and modulates transcription and splicing of the E-cadherin gene (Alpatov et al., 2004; Alpatov et al., 2008; Ouyang, 1999; Shi and Sugrue, 2000).

4.5.5 LZTFL1 in cancer and downstream of EGF

My results support a cell-cell contact-strengthening function of LZTFL1 in line with its described metastasis-inhibiting function (Wei et al., 2010). Although LZTFL1 levels are lower in metastatic tumour cell lines in head and neck squamous cancer, LZTFL1 expression does not directly correlate with E-cadherin levels as suggested in colon cancer (Wang et al., 2014). This indicates that LZTFL1 is not directly involved in the regulation of E-cadherin in keratinocyte-derived head and neck squamous carcinoma. This conclusion is supported by the lack of interaction detected between LZTFL1 and β -catenin in keratinocytes and unchanged E-cadherin protein levels in LZTFL1-depleted and -overexpressing cells (data not shown).

EGF signalling leads to cell spreading and contraction in HaCaT and these cell shape changes are required for migration (Kim and Kim, 2008). Cell scattering in response to EGF led to a small but significant increase in internuclear distances in LZTFL1-overexpressing compared to eGFP-expressing cells. This did not correlate with increased cell dispersion but rather increased cell area in LZTFL1-expressing cells. In HaCaT, cell spreading requires the phosphorylation of focal adhesion kinase (FAK), a regulator of cell-matrix interaction, while cell rounding is mediated by dephosphorylation of FAK (Kim and Kim, 2008). If dephosphorylation is inhibited, cell rounding is prevented and migration abrogated. Since LZTFL1-overexpressing cells are more spread in response to EGF, it could be speculated that LZTFL1 might enhance FAK phosphorylation after EGF treatment or prevent its dephosphorylation.

Furthermore, keratin filaments are also involved in cell spreading. In keratinocytes, cell spreading is inversely correlated with the extent of keratin bundling (Lee and Coulombe, 2009). In addition, keratin regulates actin organisation during cell spreading via the RhoA-ROCK signalling pathway (Bordeleau et al., 2012). Since LZTFL1 binds to keratin, LZTFL1 regulation of keratin remodelling might be a further explanation for increased cell spreading in overexpressing cells.

4.6 Future Work

4.6.1 LZTFL1 regulation of AJ

Direct evidence of LZTFL1 binding to α -catenin at AJ is still missing. Preliminary data from co-staining of α -catenin and LZTFL1 shows some co-localisation of LZTFL1-labelled filaments and α -catenin at AJ (data not shown) similar to the co-staining with E-cadherin. However, a significant pool of α -catenin and LZTFL1 is cytosolic and these pools are lost with methanol fixation. Therefore, no conclusions can be drawn about a potential interaction between both proteins in the cytoplasm. A good technique to assess protein co-localisation reliably would be the use of Duolink *in situ* PLA fluorescence staining.

It is possible that E-cadherin linkage to the actin cytoskeleton is disturbed when LZTFL1 is ablated. To test this hypothesis, experiments that assess E-cadherin mobility, such as E-cadherin FRAP or live-cell imaging with E-cadherin photoswitchable constructs, could be performed since actin-binding limits E-cadherin mobility. The TsMod-E-cadherin constructs could also be used to support this hypothesis, as they detect actin-pulling on E-cadherin by means of FRET read outs (Borghi et al., 2012). Finally, defects in actin recruitment could be detected using E-cadherin antibody-coated beads, which bind to and cluster E-cadherin thereby mimicking AJ formation (Braga et al., 1997).

LZTFL1-depleted cells seem to show defects in thin bundles and cell compaction but this phenotype needs further validation. Computational algorithms that should help quantify these defects are currently under development in our laboratory. Thin bundle and cell compaction lead to an increase in lateral height of cells (Zhang et al., 2005). Therefore, comparing the height between control and LZTFL1-depleted cells would provide further evidence for a potential role of LZTFL1 in cell compaction. If such a role can be confirmed, LZTFL1 function could be further investigated by studying potential defects in recruitment of myosin II regulators or actin bundling-proteins by immunofluorescence staining. Problems in myosin II activation could also be investigated by Western blot using phosphorylation-specific antibodies.

4.6.2 LZTFL1 regulation of keratin

It will be important to further characterise the interaction between LZTFL1 and keratin filaments. In vitro keratin binding assays could be used to test direct binding of LZTFL1 to keratin 14. In light of its proposed function in neurite outgrowth and considering that

epithelial cells change their keratin composition during differentiation, it would also be interesting to test binding of LZTFL1 to other intermediate filaments. For future functional studies, LZTFL1 mutants unable to bind to keratin could be generated after further characterisation of the keratin-binding site on LZTFL1. It would also be interesting to test whether LZTFL1 can bundle keratin filaments in *in vitro* bundling assays.

At the same time, identifying the specific properties of the keratin population that is labelled with LZTFL1 will be important to gain indications towards the function of LZTFL1. Modification-specific antibodies or modification inhibitors could be used to test for specific post-translational modifications of the keratin filaments in combination with LZTFL1 staining. Since my results suggest LZTFL1-binding to stabilised keratin 14 filaments and since phosphorylation is linked to intermediate filament stability, phosphorylation would be a good starting point for the investigation. Even if the keratin population is identified as being phosphorylated, the significance of this finding in primary keratinocytes will need to be assessed. Therefore, keratin filament stability should be evaluated. The tyrosine phosphatase inhibitor orthovanadate could be used as described before to break down keratin filaments (Strnad et al., 2002) and combined with LZTFL1 staining at different times after inhibitor treatment. If LZTFL1 indeed labels the most stable population of keratin 14, filamentous LZTFL1 staining should be observed even after other populations start breaking down. Similar experiments could also be performed in combination with siRNA-mediated LZTFL1 depletion to test whether LZTFL1 is required for the stability of cell-cell contact- and nucleus-linked filaments.

I hypothesised that LZTFL1 aids maturation of keratin filaments by supporting their stabilisation. Maturation is likely to be induced by detection of mature desmosomal contacts. This suggests that keratin filaments mature in response to a tension signal transmitted from mature desmosomes to the still immature keratin filaments. It would therefore be very interesting to test whether LZTFL1 is recruited to keratin filaments in response to mechanical stress. Cells could be grown on elastic membranes and changes in LZTFL1 localisation observed upon membrane stretch.

4.6.3 LZTFL1 as a bridge between AJ and keratin filaments

I proposed the idea that composite junctions might exist in keratinocytes as specialised mechanosensors linking AJ and the nucleus via keratin filaments. Evidence could come from co-localisation studies between α -catenin and keratin 14 using Duolink *in*

situ PLA fluorescence staining. Another important question to address in this context is whether LZTFL1 can bind to α -catenin and keratin 14 simultaneously. This could be confirmed by *in vitro* binding assays.

If the existence of these composite junctions in keratinocytes can be proven, the next step would be to establish their importance for mechanosensing. LZTFL1 mutants unable to bind to keratin could be used to disrupt these junctions. Although such constructs would also weaken desmosomes, the effect on AJ mechanotransduction could be specifically assessed with E-cadherin antibody-coated beads and optical or magnetic tweezers to only pull at AJ. It could be predicted that cells react to increased tension with upregulation of E-cadherin and an increase in E-cadherin mRNA transcript number could be assessed quantitatively at the single cell level by FISH (Raj et al., 2008).

4.6.4 LZTFL1 in the nucleus

Overexpressed and endogenous LZTFL1 is found in the nucleus and the nuclear localisation seems mediated by the LZTFL1 N-terminus. To supplement these findings cellular fractionation assays should be performed to help to quantitatively assess changes in LZTFL1 localisation in response to stimuli. For example, in low calcium keratinocytes only very little filamentous staining of LZTFL1 is observed and it would be interesting to test whether the nuclear pool of LZTFL1 is increased under these conditions as compared to standard calcium keratinocytes. This would implicate specific nuclear functions for LZTFL1 in sub-confluent cells, for example in regulation of proliferation, and that keratin binding might be a way of storing or sequestering LZTFL1 away from the nucleus.

So far, it is completely unknown what the nuclear function of LZTFL1 might be and high-through put techniques such as microarrays could help testing for changes in transcriptional profiles in the absence or presence of LZTFL1. In addition, the nuclear staining pattern of endogenous LZTFL1 in the form of small dotted structures is striking and might indicate a function in a specific nuclear compartment such as Promyelocytic leukaemia (PML) bodies (transcriptional regulation), Cajal bodies and Gemini of Cajal bodies (Gem) (regulators of small nuclear ribonucleoprotein biogenesis), Oct1/PTF/transcription (OPT) domains (transcriptionally active domains of unknown function) or Polycomb (PcG) bodies (unknown function, maybe protein storage or genetic silencing) (Spector, 2001). Co-staining could help identify to which sub-nuclear domain LZTFL1 localises.

4.6.5 LZTFL1 in EGF-induced cell spreading

LZTFL1-overexpressing HaCaT exhibit increased cell area in response to EGF treatment. It would be interesting to verify this phenotype using isolated cells and to test whether the effect is specific to EGF by testing cell scattering e.g. by hepatocyte growth factor (HGF).

Based on the available literature and the evidence gathered in this study, the increase in EGF-induced cell spreading could be induced by increased FAK phosphorylation or regulation of keratin signalling. Comparing FAK phosphorylation after EGF stimulation in eGFP- and LZTFL1-overexpressing cells could test the first idea. LZTFL1 mutants could check the latter theory. Keratin regulation would rely on LZTFL1 binding to keratin. Therefore, it can be expected that LZTFL1 mutants defective in keratin binding would prevent the observed increase in cell area after EGF treatment. Rho GTPases also regulate cell spreading and it would be interesting to test whether LZTFL1 associates and regulates any Rho GTPase.

Chapter 5 LZTFL1 expression during cardiovascular disease

5.1 Introduction

5.1.1 Intercalated discs

AJ and desmosomes form strong connections between neighbouring cells and thereby mediate tissue integrity. This is particularly important in cells that are under constant mechanical stress such as cardiac muscle cells. Neighbouring cardiomyocytes are connected via intercalated discs (Forbes and Sperelakis, 1985), which contain AJ complexes and desmosomal protein complexes side-by-side in *area composita* (Franke et al., 2006).

AJ and desmosomes mechanically couple the cells in muscle fibres so that they can act as a functional syncytium. For this purpose electrical and chemical coupling is also necessary and is mediated by gap junctions. The main gap junction component in the myocardium is connexin-43 (Jongsma and Wilders, 2000). Many studies of experimental models and cardiomyopathies have shown that the formation and stability of gap junctions is dependent on AJ and desmosomes (Li, 2014; Saffitz, 2005; Saffitz, 2011). Gap junction formation follows AJ formation temporally (Kostin et al., 1999) and disruption of AJ and/or desmosomes is also causing reduction of gap junctions (Hertig et al., 1996b; Kostetskii et al., 2005; Luo and Radice, 2003; Matsuda et al., 2006; Oxford et al., 2007). Overall, it has been established that the different components of the intercalated disc are highly interdependent.

5.1.2 The cytoskeleton in cardiomyocytes

The cytoskeleton in cardiomyocytes is highly specialised to fulfil its role in contraction. Its structure is very regular and formed of sarcomeres that are connected end to end to form fibrils (Sarantitis et al., 2012). At the transverse membranes of cardiomyocytes, they are inserted into the intercalated disc and exactly aligned with fibrils of neighbouring cells. Aside the sarcomere, cytoskeleton exists similar to other cells in the form of intermediate and actin filaments and microtubules (Sarantitis et al., 2012), with desmin being the intermediate filament found in cardiomyocytes.

5.1.3 Intercalated disc components and the cytoskeleton in cardiomyopathies

Cardiovascular disease is one of the leading causes of death especially in Europe (Nichols et al., 2014). Heart disease is frequently linked to ultrastructural changes of the intercalated disc and sarcomere arrangement and many disease-causing mutations in desmin, components of the intercalated disc or sarcomere have been identified to

date (see main introduction). On the other hand, changes in protein levels or localisation have also been associated with cardiomyopathies. In addition, the function of certain proteins is regulated by modifications in the expression ratios of different family members or isoforms of the same protein (see main introduction).

5.2 Hypothesis and aims

LZTFL1 is required for cell-cell contact stabilisation in epithelial cells. Skin and heart are both organs under constant physical stress. I therefore hypothesise, that changes in LZTFL1 levels or isoform expression may perturb intercalated disc function leading to or as a consequence of cardiovascular disease.

Using patient samples and animal models of physiological and pathological cardiac hypertrophy, heart failure and myocardial infarct I aim to correlate expression levels and isoform switches of LZTFL1 with cardiovascular disease. In addition, I will investigate whether changes in LZTFL1 levels relate to alterations in expression of AJ components of the intercalated disc.

5.3 Results

5.3.1 LZTFL1 is upregulated in DCM in human

LZTFL1 is likely involved in the regulation of both adhesive junctions and the linked filaments in keratinocytes. Therefore, it could be involved in the manifestation of any of the cardiovascular diseases that have been associated with changes in adhesive complexes or the cytoskeleton. To provide insights into which type of human cardiovascular diseases could be accompanied by changes in LZTFL1 levels, two microarrays of human cardiovascular disease were identified that showed changes in *LZTFL1* levels. Interestingly, both microarrays were performed on samples from patients with DCM (Figure 5.1).

The first microarray (GDS651) is from an unpublished study on normal donor organs or left ventricle biopsies taken from patients undergoing heart transplantation due to heart failure. The underlying cause for heart failure was either idiopathic DCM or ischemic cardiomyopathy. *LZTFL1* levels were probed with two different probe sets. One probe set showed a significant increase in expression signal for *LZTFL1* in DCM but not ischemic cardiomyopathy samples (Figure 5.1 A and data not shown). The second probe did not show a significant difference in *LZTFL1* expression for DCM or ischemic cardiomyopathy samples (Figure 5.1 A and data not shown). Similarly, septal myocardial samples from normal donor hearts or patients with DCM (microarray GDS2205) (Barth et al., 2006) showed significantly increased levels of *LZTFL1* in DCM (Figure 5.1 B).

In collaboration with Prof. Ralph Knöll (Imperial College), I analysed RNA from septal myocardial samples from normal donor hearts, patients with HCM undergoing myectomy and patients with heart failure undergoing heart transplantation. Two HCM patients were female and four male. None of them had arrhythmias and only two patients had a family history of cardiovascular disease. Of the heart failure patients four had been diagnosed with idiopathic DCM. Of these, one male patient had a history of alcohol abuse. A second male patient also had a history of alcohol abuse, as well as a virus infection and a family history of cardiovascular disease, all possible reasons for developing DCM. Another male patient likewise had a family history of cardiovascular disease. The fourth patient, patient FE, was the only patient classified as having familial DCM. Both his ventricles were dilated and he also presented with 50% blockage of his left anterior descending artery. The remaining two heart failure patients were not diagnosed with DCM. The only female patient had had atrial fibrillation since seven

years as well as rheumatic heart disease. Both, her left ventricle and atrium were severely dilated. The sixth patient was male and presented with atrial fibrillation and decompensated heart failure. My initial studies identified patient FE as particularly interesting. Therefore, six additional patients with familial DCM were included in the study. Unfortunately, no clinical data was available on these patients.

Qualitative real-time PCR (qPCR) was performed on the patient's RNA samples and results were normalised to the geometric mean of the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) (Figure 5.2 A+B). Expression levels of atrial natriuretic peptide (*NPPA*) and α -myosin heavy chain (*MYH6*) were determined as markers of hypertrophy (Chien et al., 1991). *NPPA* showed a trend to increased levels during hypertrophy although the difference was not significant (Figure 5.2 C). As expected, *MYH6* was significantly reduced in patients with HCM and heart failure (Figure 5.2 D). Levels were also reduced in familial DCM patients but the difference was not significant probably because of an outlier expressing very high levels of *MYH6*.

N-cadherin (*CDH2*) levels were increased in patients with familial DCM and decreased in heart failure. Patient FE expressed particularly low levels of *CDH2* (Figure 5.2 E). The difference between the DCM patient group and failing heart group was significant. I further tested expression levels of T-cadherin (*CDH13*), a member of the cadherin family that has been shown to exert cardioprotective functions as receptor of adiponectin (Denzel et al., 2010). *CDH13* levels were overall unchanged between all groups analysed, but it is interesting to note that two patients (D6 and FE) with familial DCM expressed very low levels of *CDH13* (Figure 5.2 F).

Next, I tested expression levels of distinct *LZTFL1* isoforms (all known isoforms, only isoform 1, 2 or a primer detecting isoforms 3 and X1). Total *LZTFL1* levels are increased in patients with familial DCM including patient FE from the heart failure group of patients (Figure 5.3 A). Patients D6 and FE expressed the highest levels of *LZTFL1*. Interestingly, *LZTFL1* isoform 1 did not show any changes in mRNA expression levels in any of the groups (Figure 5.3 B). However, patients D6 and especially FE seemed to express lower levels of *LZTFL1* isoform 1 in contrast to their overall high *LZTFL1* expression levels. The discrepancy between overall *LZTFL1* levels and isoform 1 levels indicate that another *LZTFL1* isoform must be upregulated in familial DCM patients and especially patients D6 and FE. Surprisingly, *LZTFL1* isoforms 3/X1 did not show any differences between normal patients and patients with HCM and DCM

(Figure 5.3 C). However, signal for these isoforms could not be detected in any of the patients with heart failure as well as patient D6 with familial DCM, one patient with HCM and one control. Considering that overall *LZTFL1* and isoform 1 levels in failing heart seem unchanged, this suggests that the lack of isoforms 3/X1 might be compensated by upregulation of another *LZTFL1* isoform. *LZTFL1* isoform 2 was below detection level in all patients, but one with HCM, and two each with familial DCM and heart failure (data not shown). Therefore, it is unlikely that changes in isoform 2 expression can explain the discrepancy between overall *LZTFL1* levels and isoform 1 levels. Primer analysis indicated good performance and no unspecific products, which suggests that technical errors are unlikely to explain the observed differences. It therefore stands to suspect that another unidentified *LZTFL1* isoform might get upregulated during DCM.

I finally tested the expression of α E- and α T-catenin (*CTNNA1* and *CTNNA3*) as further markers of intercalated disc structure and stability and as interaction partners for *LZTFL1*, at least in the case of α E-catenin. Levels of *CTNNA1* were increased in patients with familial DCM as compared to all other groups (Figure 5.3 D). The increase in *CTNNA1* correlated with the increase observed in *CDH2* expression in the same group of patients. However, expression of *CTNNA1* was strongly reduced in patient D6 compared to the other patients in the DCM group. Similar to his low expression of *CDH2*, patient FE also showed reduced levels of *CTNNA1*. The levels of *CTNNA3* were significantly increased in patients with familial DCM, but not in any other patient group (Figure 5.3 E). Patient FE again showed low expression levels, although one control also possessed only low levels of *CTNNA3*.

In conclusion, patients with familial DCM had increased levels of mRNA of AJ components and *LZTFL1*, suggesting increased intercalated disc stability supposedly to compensate increased pressure load. *LZTFL1* seemed to undergo an isoform switch in DCM patients, but the upregulated isoform could not be identified.

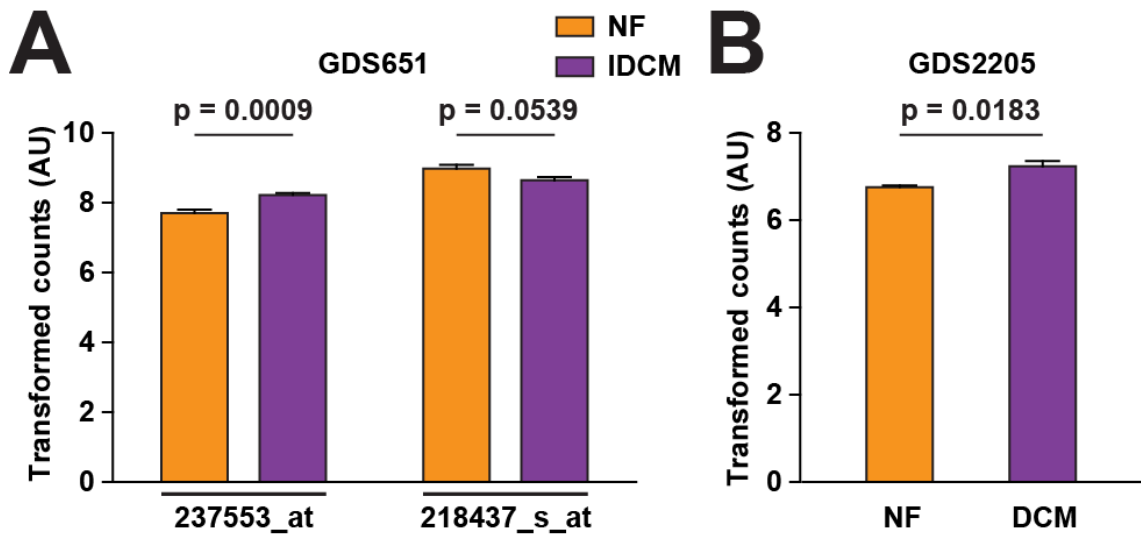


Figure 5.1: Microarray data suggests differential regulation of LZTFL1 levels in dilated cardiomyopathy. A) Microarray result for two probes (237553_at and 218437_s_at) targeting LZTFL1 from the data set GDS651. Samples were from the left ventricle of normal, non-failing human hearts (NF) (N = 11) versus those of patients with idiopathic dilated cardiomyopathy (IDCM) (N = 15). B) Microarray result for probe 218437_s_at targeting LZTFL1 from the data set GDS2205. Samples were from septal myocardial samples from normal, non-failing human hearts (NF) (N = 5) versus those of patients with dilated cardiomyopathy (DCM) (N = 7). Graphs represent mean + SD of transformed microarray counts (Arbitrary unit = AU). Data was analysed by t-test.

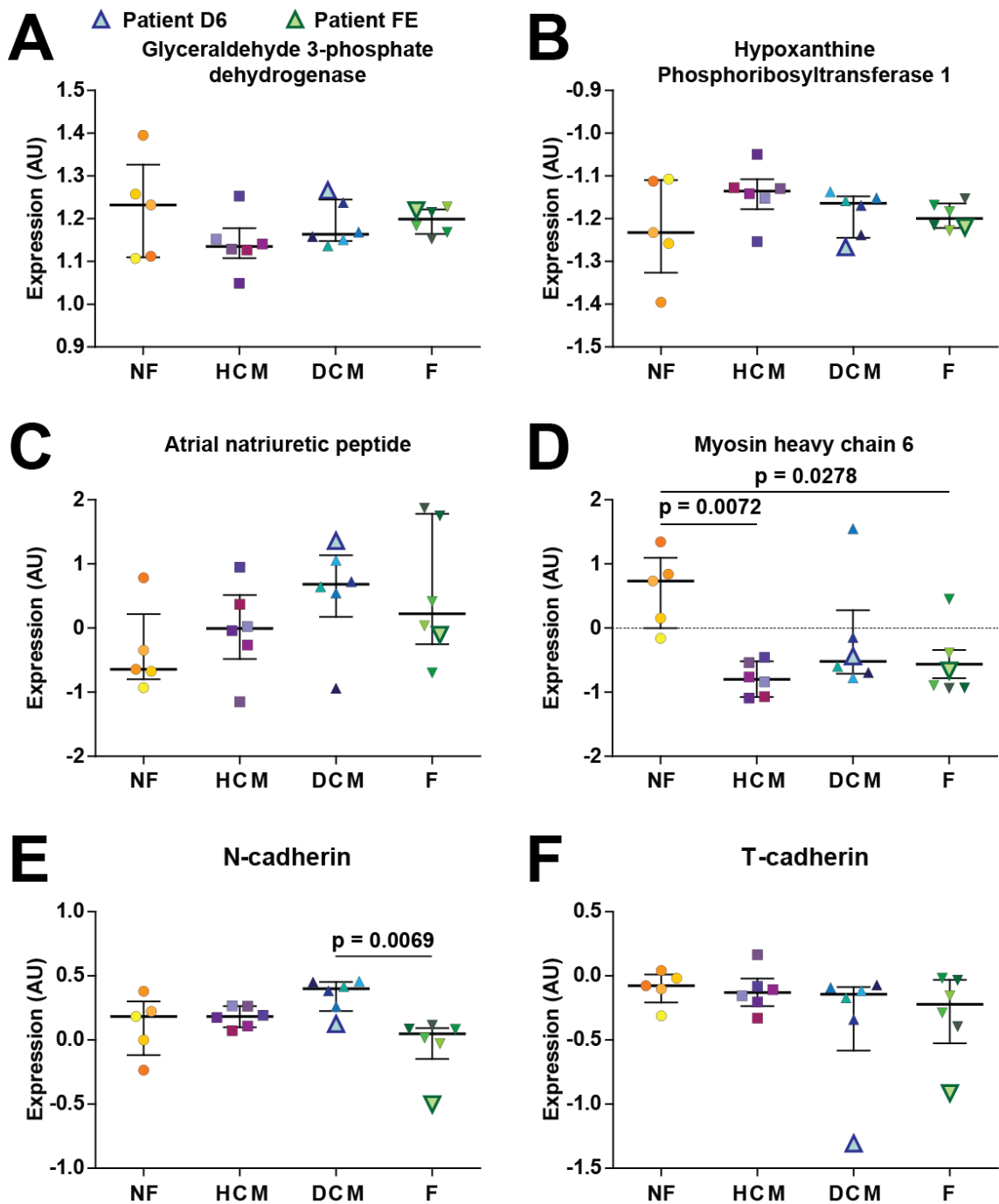


Figure 5.2: Patients with heart failure have reduced levels of N-cadherin as compared to patients with familial dilated cardiomyopathy. RNA from ventricular tissue of normal, non-failing hearts (NF) (N = 5) or from patients with hypertrophic cardiomyopathy (HCM) (N = 6), familial dilated cardiomyopathy (DCM) (N = 6) or failing hearts (F) (N = 6), was transcribed to cDNA and analysed by quantitative real-time PCR (qPCR) to test expression levels of A) glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and B) hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) as house keeping genes, C) atrial natriuretic peptide (*NPPA*), D) myosin heavy chain 6 (*MYH6*), E) N-cadherin (*CDH2*) and F) T-cadherin (*CDH13*). Values from each patient were normalised to the corresponding geometric mean of the house keeping genes *GAPDH* and *HPRT1*. Expression levels are represented as $\log_{10}(2^{-\Delta C_T})$. Graphs show mean and SD as well as individual values from each patient, with each patient represented by a specific colour across all graphs. Data points of patients D6 and FE are additionally enlarged and highlighted with coloured borders. Data was analysed by 1-way ANOVA and Tukey's post-hoc test. Expression in arbitrary units (AU).

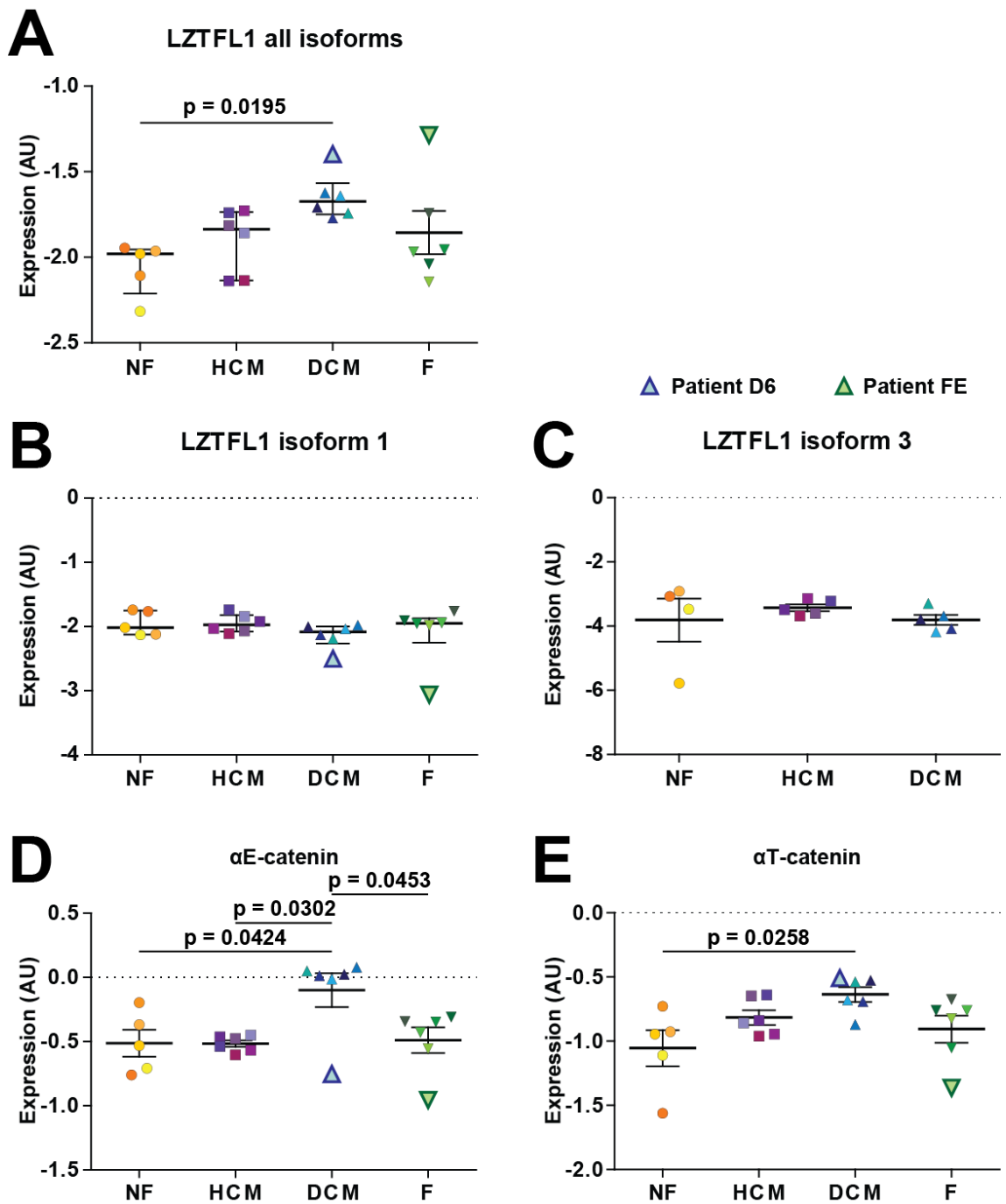


Figure 5.3: LZTFL1, αE- and αT-catenin levels are increased in patients with familial dilated cardiomyopathy. RNA from ventricular tissue of normal, non-failing hearts (NF) (N = 5) or from patients with hypertrophic cardiomyopathy (HCM) (N = 6), familial dilated cardiomyopathy (DCM) (N = 6) or failing hearts (F) (N = 6), was transcribed to cDNA and analysed by quantitative real-time PCR (qPCR) to test expression levels of A) *LZTFL1*, all isoforms, B) *LZTFL1*, isoform 1, C) *LZTFL1*, isoform 3 and X1, D) αE-catenin (*CTNNA1*) and E) αT-catenin (*CTNNA3*). Values from each patient were normalised to the corresponding geometric mean of the house keeping genes *GAPDH* and *HPRT1*. Expression levels are represented as $\log_{10}(2^{-\Delta C_T})$. Graphs show mean and SD as well as individual values from each patient, with each patient represented by a specific colour across all graphs. Data points of patients D6 and FE are additionally enlarged and highlighted with coloured borders. Data was analysed by 1-way ANOVA and Tukey's post-hoc test. Expression in arbitrary units (AU).

5.3.2 *Lztfl1* is upregulated during isoproterenol-induced hypertrophy in mice

When checking microarray datasets for changes in *LZTFL1* levels, I also identified a microarray on mouse models showing differences in *Lztfl1* expression. The study compared RNA expression profiles of male C57BL/6 mice, mice with isoproterenol-induced cardiac hypertrophy and mice with exercise-induced physiological hypertrophy (Galindo et al., 2009). Pathological hypertrophy was induced by subcutaneous infusion with 40 mg.kg⁻¹.d⁻¹ isoproterenol for 10 days. *Lztfl1* levels were tested with 5 different probes. All probes showed increased levels of *Lztfl1* in isoproterenol-induced cardiac hypertrophy compared to control mice and the difference was significant with 3 probes (Figure 5.4).

Based on these results, I collaborated with Dr Tommaso Poggioli (Imperial College). To induce hypertrophy, immunodeficient mice were injected with 1 mg.kg⁻¹.d⁻¹ isoproterenol for seven days (see methods for details). In addition, a group of mice had HEK293 cells, which transiently overexpressed IGF-1Ea, the propeptide of insulin-like growth factor 1 (IGF-1), injected in their hearts one day prior to isoproterenol treatment. Hearts of isoproterenol-treated animals exhibited increased values of heart weight/body weight ratio, left ventricular mass, stroke volume, end diastolic volume and cardiomyocyte cross-sectional area as markers of hypertrophy. All changes were avoided when mice were preconditioned with IGF-1Ea before isoproterenol treatment (PhD thesis Tommaso Poggioli).

RNA samples from untreated hearts or treated with isoproterenol with or without IGF-1Ea preconditioning were analysed by qPCR. Results were normalised to the house keeping genes *Hprt1* and Ribosomal protein, large, P0 (*Rplp0*) (Figure 5.5 A+B). Alpha skeletal muscle actin (*Acta1*) was tested as a marker of hypertrophy. Expression was increased with isoproterenol treatment (Figure 5.5 C). Although, IGF-1Ea preconditioning avoided hypertrophy onset, it did not inhibit the upregulation of *Acta1*.

Lztfl1 expression was decreased in hypertrophic hearts (Figure 5.5 D). The difference was not statistical significant when comparing all three conditions using post-hoc tests with correction for multiple comparison. It was significant when only comparing control animals and animals with hypertrophy using t-test ($p = 0.0377$). The lack of significance is therefore likely a lack of power. Preconditioning with IGF-1Ea increased *Lztfl1* levels significantly as compared to isoproterenol treatment alone. Although the mean of *Lztfl1* expression was also higher than in controls, with the current number of repeats the difference to control was not significant.

The hypertrophic marker *Nppa1*, as expected, was significantly upregulated in isoproterenol-induced hypertrophy and similarly to *Acta1* IGF-1EA preconditioning did not prevent this upregulation (Figure 5.6 A). *Cdh13* levels were increased during hypertrophy and even further if hearts had been pretreated with IGF-1Ea (Figure 5.6 B).

Since a cadherin switch had been observed in a rat model of HCM previously (Craig et al., 2010), I decided to determine RNA levels of *Cdh2* and E-cadherin (*Cdh1*). While *Cdh2* levels did not change between the three treatment groups, *Cdh1* was significantly upregulated in response to isoproterenol, if animals had been preconditioned with IGF-1Ea but not without this pre-treatment (Figure 5.6 C+D). Finally, I measure expression levels of the two α -catenin isoforms. Both isoforms were upregulated during hypertrophy, but levels were lower when hypertrophy was prevented by IGF-1Ea (Figure 5.6 E+F). While *Ctnna3* levels returned to control levels, *Ctnna1* levels were still significantly higher than in controls.

In conclusion, *Lztfl1* levels were reduced in isoproterenol-induced hypertrophy, while levels of *Cdh13*, *Ctnna1* and *Ctnna3* were increased. The cardioprotective effect of IGF-1Ea prevents or lessens changes of *Lztfl1*, *Ctnna1* or *Ctnna3* levels in response to isoproterenol and enhances expression of *Cdh1* and *Cdh13*.

Since isoproterenol treatment is a systemic approach to induce hypertrophy and the results might potentially be affected by the use of immunodeficient mice, I verified changes in *Lztfl1* levels during HCM using a different mouse model. I collaborated with Dr Lorna Fiedler (Imperial College), who uses different mouse models to study cardiovascular disease. She used transverse aortic constriction (TAC) to induce hypertrophy and provided me with protein samples of mice after two weeks of TAC, corresponding to the adaptive phase of hypertrophy, and after four weeks of TAC, at the transition to maladaptive hypertrophy and heart failure. Quantification of Western blots demonstrated a significant decrease of *Lztfl1* two weeks after TAC, but not four weeks after TAC (Figure 5.7), suggesting that the change is part of the adaptive response to increased pressure load, but is not sustained during progression to heart failure. Additional lower molecular weight bands of unknown identity are also observed in some samples and might correspond to different *Lztfl1* isoforms. However, no correlation between their expression profiles in relation to treatment could be recognised. This experiment verifies decreased *Lztfl1* levels during HCM in mouse models.

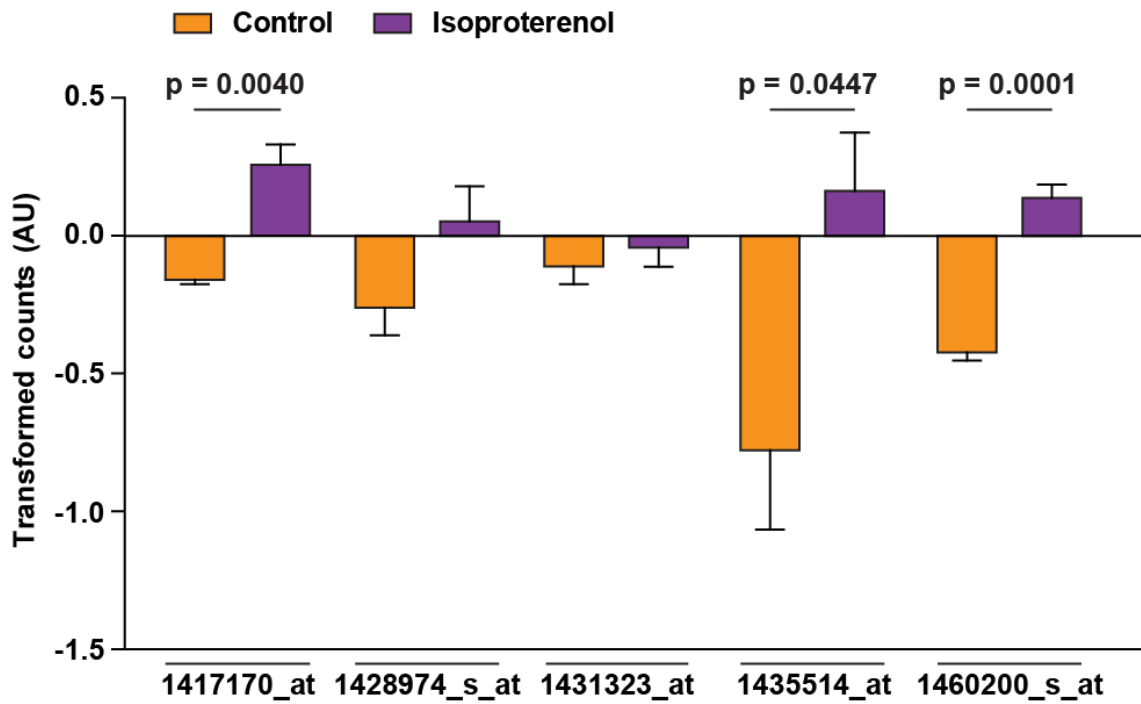


Figure 5.4: Microarray data suggests increased *Ltfl1* levels in mice with isoproterenol-induced hypertrophy. Microarray result for five probes (1417170_at, 142897_s_at, 1431323_at, 1435514_at, 1460200_s_at) targeting *Ltfl1* from the data set GDS3596. Samples were left ventricles from male mice of the strain C57BL/6 with (N = 3) or without (N = 3) isoproterenol-induced cardiomyopathy. To induce hypertrophy, mice were administrated with 40 mg.kg⁻¹.d⁻¹ isoproterenol for 10 days. Graphs represent mean + SD of transformed microarray counts (arbitrary units = AU). Data was analysed by 1-way ANOVA and Tukey's post-hoc test.

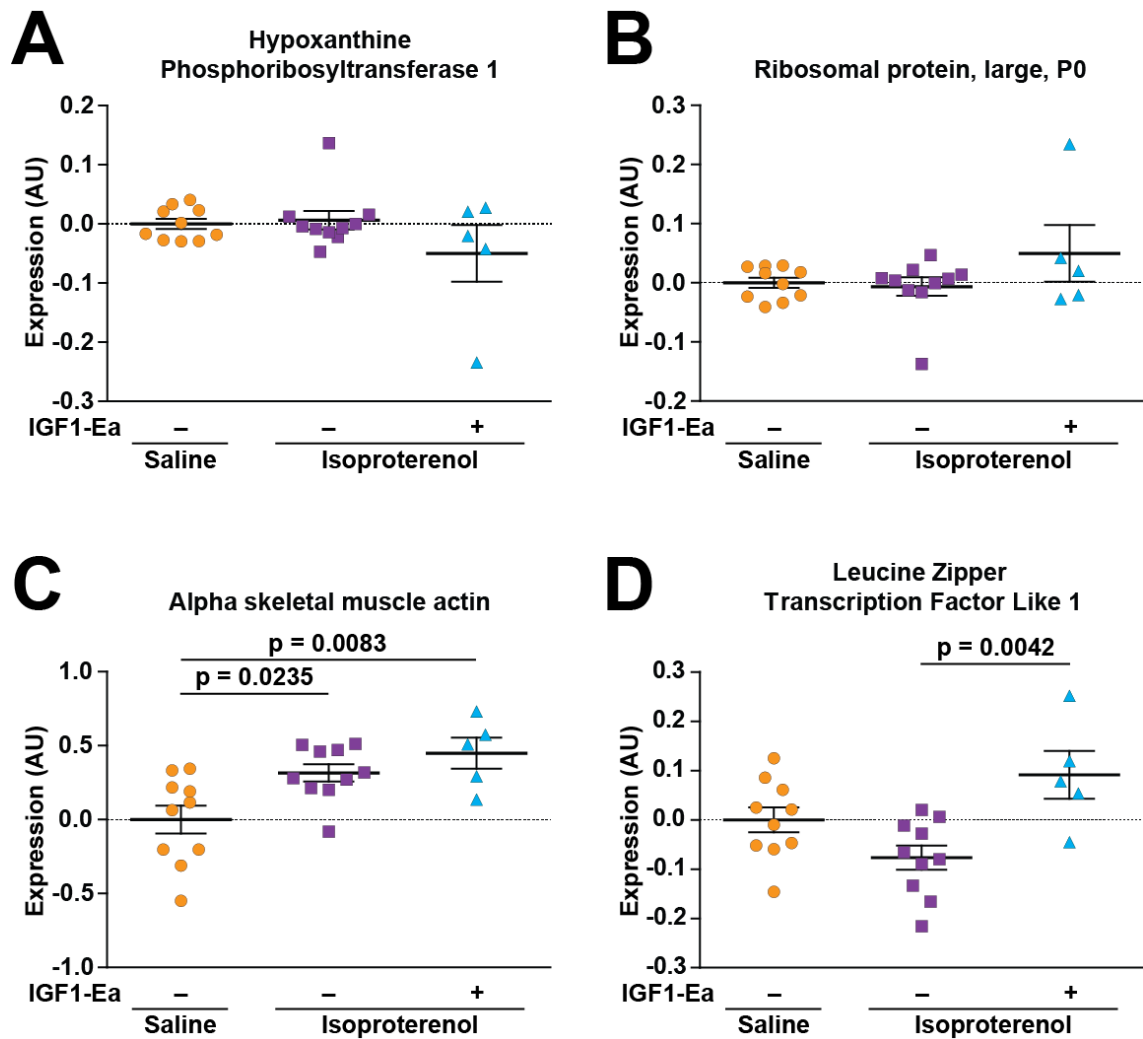


Figure 5.5: Mice with isoproterenol-induced cardiac hypertrophy have reduced cardiac *Lztf1* levels. Immunosuppressed, 8-9 weeks old mice of the strain NOD.CB17 Prkdcid/JHliHSD were injected daily with either saline (N = 10) or 1 mg.kg⁻¹.d⁻¹ isoproterenol (N = 10) for 7 days. A third group was injected with HEK293 cells transiently overexpressing IGF1-Ea 24 hours prior to the first injection with isoproterenol (N = 5). RNA from whole hearts was isolated, transcribed to cDNA and analysed by quantitative real-time PCR (qPCR) to test expression of A) hypoxanthine phosphoribosyltransferase 1 (*Hprt*) and B) ribosomal protein, large, P0 (*Rplp0*) as house keeping genes, as well as C) alpha skeletal muscle actin (*Acta1*) and D) leucine zipper transcription factor like 1 (*Lztf1*). Expression levels are represented as $2^{-\Delta C_T}$ in arbitrary units (AU). Graphs show mean and SD. Data was analysed by 1-way ANOVA and Tukey's post-hoc test.

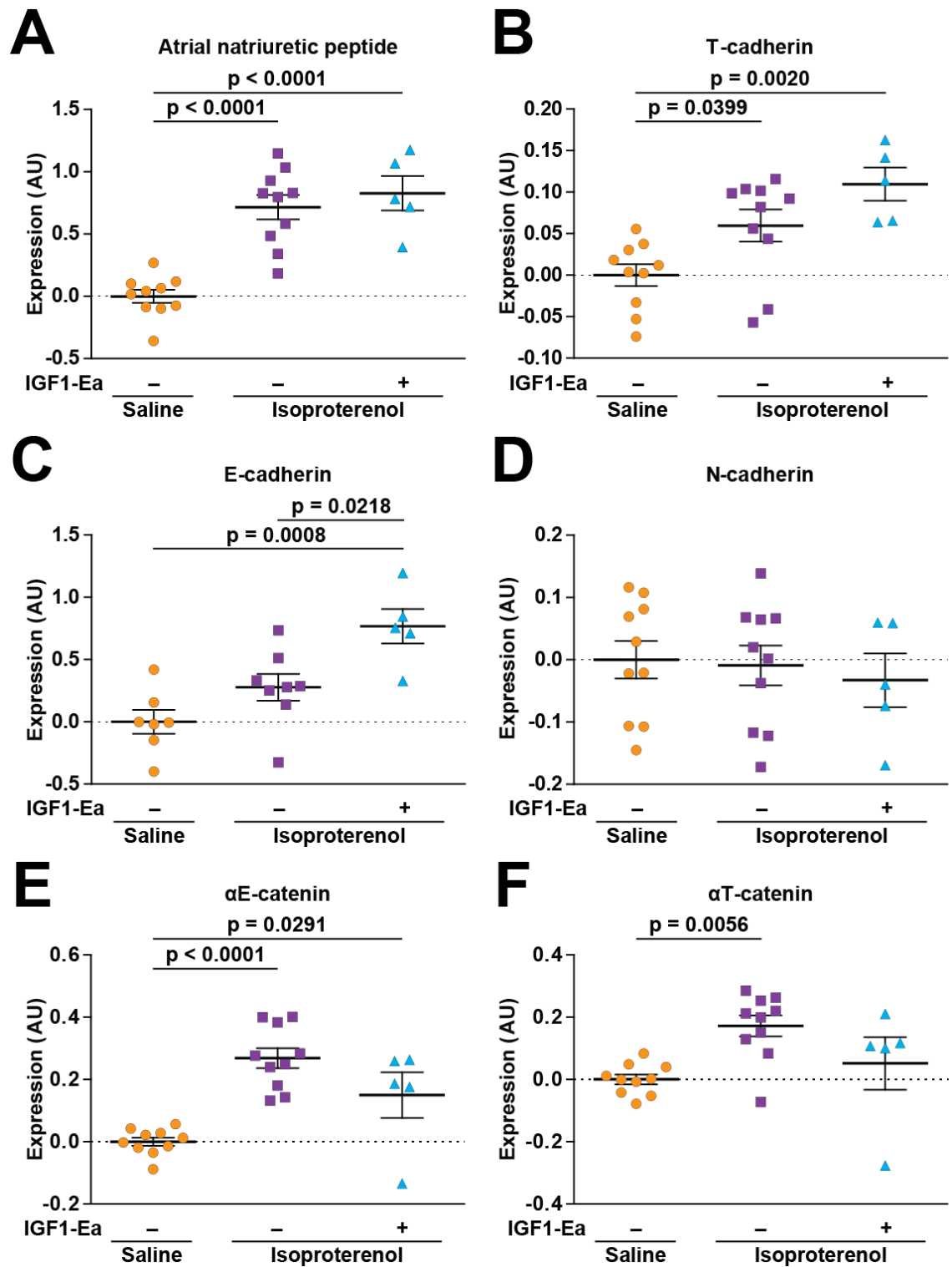


Figure 5.6: Isoproterenol-induced cardiac hypertrophy increases expression levels of intercalated disc-associated proteins. Immunosuppressed, 8-9 weeks old mice of the strain NOD.CB17 Prkdcid/JHliHSD were injected daily with either saline (N = 10) or 1 mg.kg⁻¹.d⁻¹ isoproterenol (N = 10) for 7 days. A third group was injected with HEK293 cells transiently overexpressing IGF1-Ea 24 hours prior to the first injection with isoproterenol (N = 5). RNA from whole hearts was isolated, transcribed to cDNA and analysed by quantitative real-time PCR (qPCR) to test expression levels of A) atrial natriuretic peptide (*Nppa*), B) T-cadherin (*Cdh13*), C) E-cadherin (*Cdh1*), D) N-cadherin (*Cdh2*), E) α E-catenin (*Ctnna1*) and F) α T-catenin (*Ctnna3*). Expression levels are represented as 2^{- Δ C_T} in arbitrary units (AU). Graphs show mean and SD. Data was analysed by 1-way ANOVA and Tukey's post-hoc test.

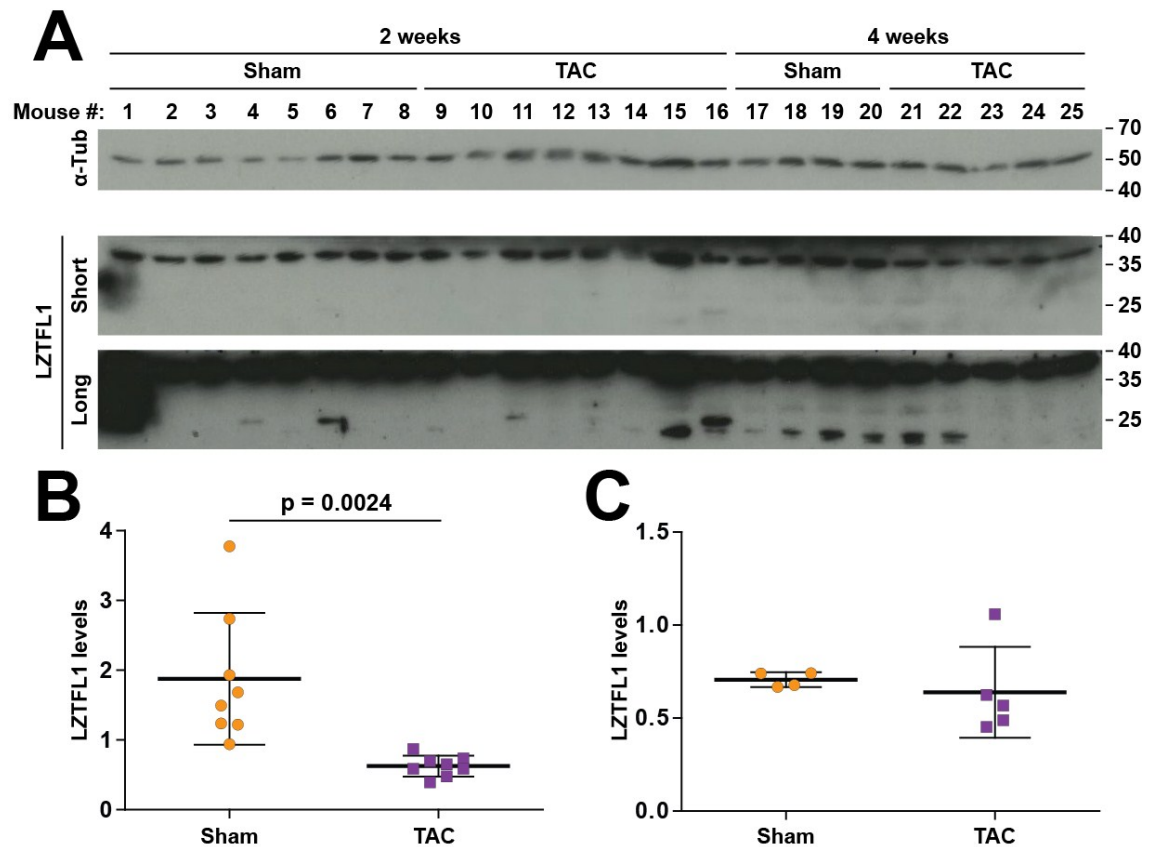


Figure 5.7: Lztfl1 levels are reduced during early but not late stage cardiac hypertrophy induced by transverse aortic constriction (TAC). Mice were sham-operated or cardiac hypertrophy was induced by transverse aortic constriction (TAC) for 2 or 4 weeks. A) Samples were analysed by Western blot and probed with antibodies against Lztfl1 and α -tubulin as loading control. A long and a short exposure are shown for Lztfl1. Molecular weight markers are shown on the right. B-C) Densitometric quantification of the Western blot showing mean $\pm$ SD of Lztfl1 levels (top band, short) corrected to α -tubulin after B) 2 weeks or C) 4 weeks of TAC. Data was analysed by t-test and was non-significant in samples from 4 weeks of TAC (C). N = 8 for sham, 2 weeks; N = 8 for TAC, 2 weeks; N = 4 for sham, 4 weeks; N = 5 for TAC, 4 weeks.

5.3.3 Lztfl1 is not upregulated during physiological hypertrophy

So far I have shown that *LZTFL1* levels are increased in humans with familial DCM and decreases in mouse models of pathological HCM. Hypertrophy can, however, also arise under physiological conditions such as exercise or pregnancy. I therefore wanted to test for changes in *LZTFL1* levels during physiological exercise-induced hypertrophy. Comparison by microarray of sedentary mice with mice exercised by swimming for 8 weeks (Galindo et al., 2009) only detected a significant increase in *Lztfl1* levels with one out of five probes (Figure 5.8 A).

Dr Lorna Fiedler provided me with protein samples of sedentary mice and mice exercised by running to induce hypertrophy. Samples were analysed by Western blot but no difference between both groups was detected (Figure 5.8 B+C). In combination with the microarray data, this suggests that *Lztfl1* levels are not changed during physiological exercise-induced cardiac hypertrophy.

5.3.4 Lztfl1 levels are decreased in the infarcted area after MI

Finally, I wanted to test whether changes in *LZTFL1* levels in cardiac pathologies are restricted to hypertrophic conditions. I studied samples from mice hearts one day after myocardial infarct (MI) or from sham-operated controls. Hearts were separated into samples from the left ventricle apex, in MI mice comprising the infarct site and border zone, and samples comprising the rest of the heart (right ventricle, left and right atrium and some left ventricle), referred to as distant sites. Although, only few sham-operated controls were available, the results suggest that *Lztfl1* levels are unchanged between both groups in distant sites, but are downregulated in the left ventricle apex in mice with MI compared to sham controls (Figure 5.9 A-C).

Interestingly, the results also showed large differences in *Lztfl1* levels between left ventricle apex and distant sites (Figure 5.9 A). However, these differences were observed both in sham-operated and MI mice. This suggests that this difference in *Lztfl1* levels might be caused by inflammation, since both groups had a needle passing through the left ventricle under the coronary artery (see methods), which causes some local inflammation. On the other hand, the different localisations, where samples were derived from, might also be able to account for distinct *Lztfl1* levels. The infarcted and corresponding control samples were only derived from the left ventricle, but distant samples contained the right ventricle and both atria. Therefore, there could be differences in *Lztfl1* expression between left and right chambers of the heart or between atria and ventricles. Indeed, microarray data from a study comparing gene

expression between atrial appendages and ventricular walls of the rat heart (GEO3625, (McGrath and de Bold, 2009), demonstrated significantly higher *Lztfl1* expression in atrial muscle compared to ventricular muscle (Figure 5.9 D). Although this result will need to be validated, it suggests that differences in *Lztfl1* expression between atrium and ventricle may also contribute to the lower experimental values obtained for *Lztfl1* in the left ventricle apex in both sham and MI samples.

5.3.5 *Lztfl1* levels might be regulated downstream of IGF-1

In the previous sections, I have established that LZTFL1 is differentially regulated in different forms of cardiovascular disease, especially during adaptive stages of diseases such as HCM and DCM but possibly not in later stages such as heart failure. I aimed to identify an upstream signal that might be involved in the regulation of LZTFL1 levels. IGF-1 is a key regulator of cellular processes in the heart including the control of hypertrophy and contraction (Troncoso et al., 2014). IGF-1 has well-established cardioprotective effects and can regulate gene expression leading to adaptive cardiac hypertrophy. It is therefore a good candidate for a regulator of LZTFL1 levels in heart disease.

To test this hypothesis, I analysed *Lztfl1* protein levels in wild type mice hearts or hearts with cardiac-specific overexpression of IGF-1. *Lztfl1* levels were reduced in IGF-1 overexpressing hearts (Figure 5.10 A+B). T-test did not show a significant difference between both groups ($p = 0.1189$), but Mann-Whitney test, which compares data ranking instead of means, did yield a significant p-value ($p = 0.0480$). Since the sample size is small any statistical test will only be able to be interpreted as an indication of significance. However, the data indicates that IGF-1 overexpression likely reduces *Lztfl1* expression, suggesting that *Lztfl1* levels might be regulated downstream of IGF-1 signalling in the heart. IGF-1 treatment of keratinocytes leads to significantly increased LZTFL1 levels after 24 hours (Figure 5.10 C), confirming the link between IGF-1 signalling and LZTFL1 levels. However, the effect of IGF-1 on LZTFL1 and therefore the regulatory mechanism seems to differ between cell types.

It is further interesting to note that in four out of five samples of IGF-1–overexpressing hearts, but none of the wild-type samples, a strong lower molecular weight band was detected for *Lztfl1* (Figure 5.10 A) that might correspond to a different *Lztfl1* isoform. Although further experiments will be needed to clarify the identity of this band, it might indicate an *Lztfl1* isoform switch induced by IGF-1.

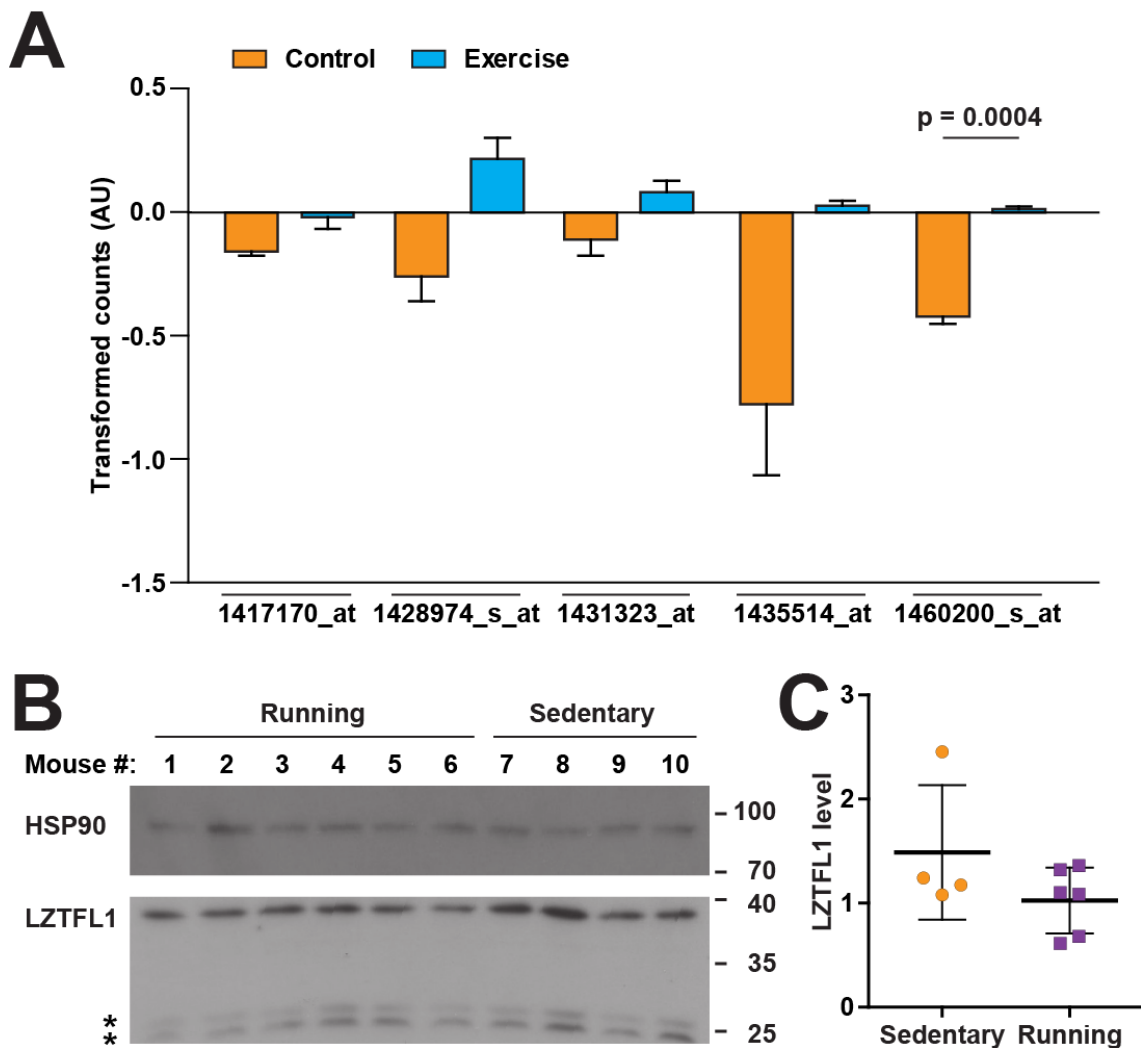


Figure 5.8: Physiological, exercise-induced hypertrophy is not associated with changes in *Ltzf1* levels. A) Microarray result for five probes (1417170_at, 142897_s_at, 1431323_at, 1435514_at, 1460200_s_at) targeting *Ltzf1* from the data set GDS3596. Left ventricles from male mice of the strain C57BL/6 with (N = 3) or without (N = 3) exercise-induced cardiac hypertrophy were analysed. Data from non-hypertrophic controls is identical to data shown in Figure 5.4. Expression is represented as transformed microarray counts (arbitrary units = AU). B) Eight weeks old mice were kept in cages with (N = 6) or without (N = 4) Fast-Trac Wheel for 4 weeks to induce physiological hypertrophy. Western blot of samples of left ventricle tissue from sedentary mice and mice with exercise-induced hypertrophy were probed with antibodies against *Ltzf1* and *Hsp90* as loading control. Molecular weight markers are shown on the right. C) Densitometric quantification of the Western blot showing *Ltzf1* levels corrected to *Hsp90*. Graphs represent mean $\pm$ SD. Data was analysed by A) 1-way ANOVA and Tukey's post-hoc test or C) t-test (non-significant).

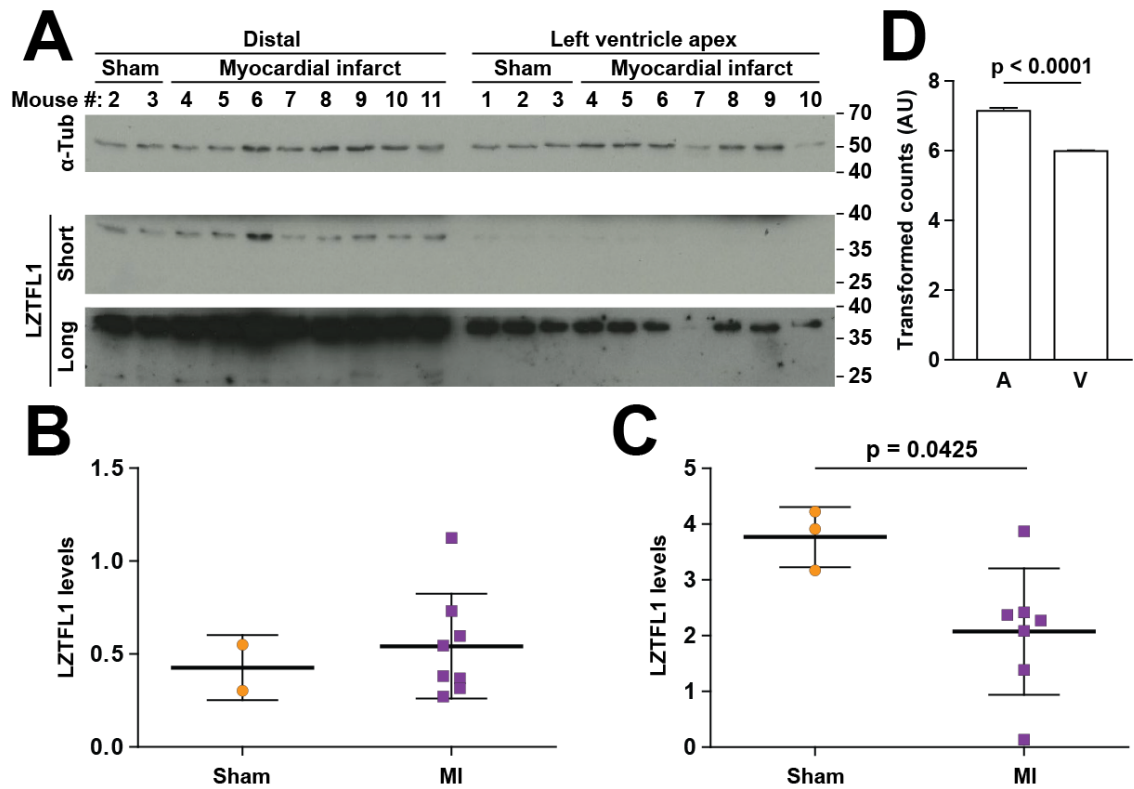


Figure 5.9: Lztf1 levels are reduced in the left ventricle apex after myocardial infarct. A) Myocardial infarct (MI) was induced by coronary artery constriction or as controls, mice were sham-operated. For analysis, the hearts from both animal groups were divided into two parts containing either the left ventricle apex (which in MI comprised the infarcted area and border zone) (N = 3 for sham, N = 7 for MI) or the remaining heart including atria, right ventricle and some left ventricle (referred to as distal) (N = 2 for sham, N = 8 for MI). Samples were analysed by Western blot and probed with antibodies against Lztf1 and α -tubulin as loading control. Molecular weight markers are shown on the right. B-C) Densitometric quantification of the Western blot showing mean $\pm$ SD of Lztf1 levels corrected to α -tubulin in B) distal area samples or C) left ventricle apex. Lztf1 levels were quantified from B) the short or C) the long exposure. Data was analysed by t-test and was non-significant in distal areas (B). D) Microarray result for probe 1375894_at targeting *Lztf1* from the data set GDS3625. Examined were samples from left atria appendage (A) (N = 4) and left ventricular free wall (V) (N = 4) from male Sprague Dawley rats. Graphs represent mean + SD of transformed microarray counts (arbitrary units = AU). Data was analysed by t-test.

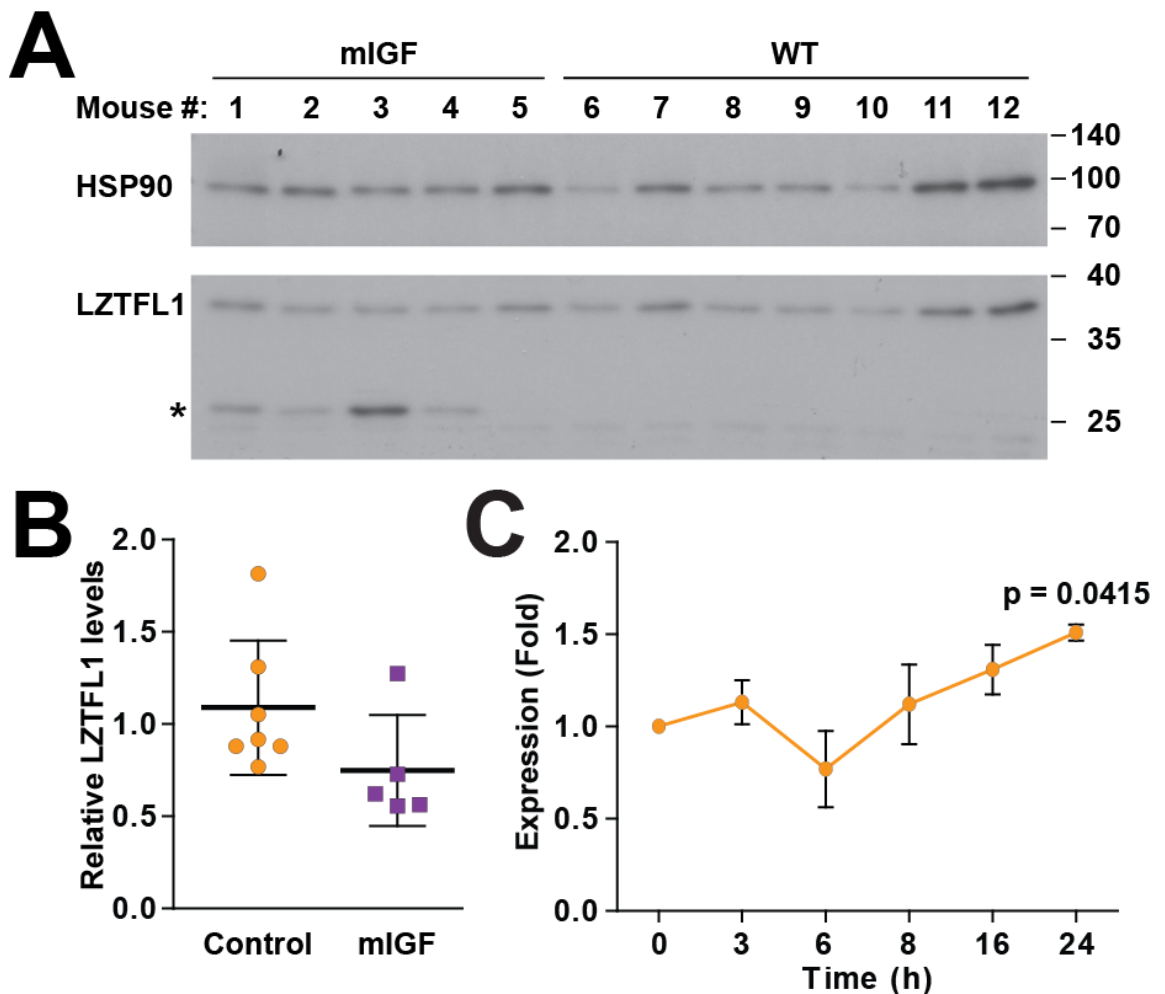


Figure 5.10: In mouse heart, mIGF1 seems to cause changes in *Lztf1* levels and isoform expression. A) Hearts of at least 7-months old mice of wild-type strain (N = 7) or those overexpressing mouse IGF1 specifically in cardiomyocytes (N = 5) were analysed by Western blot using antibodies against *Lztf1* or Hsp90 as loading control. Molecular weight markers are shown on the right. Asterisks labels lower molecular weight band of unknown identity. B) Densitometric quantification of the Western blot showing mean $\pm$ SD of *Lztf1* levels corrected to Hsp90. C) Standard calcium keratinocytes were treated over a time course of 0 to 24 hours with 200 ng/ml IGF1. RNA was isolated, translated to cDNA and analysed by qPCR for the expression of *LZTFL1* isoform 1. Expression levels are represented as $2^{-\Delta\Delta C_T}$ relative to 0 hours. N = 3. Data was analysed by B) t-test and was non-significant or C) 1-way ANOVA and Dunnett post-hoc test.

5.4 Conclusion

In conclusion, I demonstrate that LZTFL1 expression changes in cardiovascular pathologies both in human and mouse. The expression changes are specific for certain conditions, disease stage or location. In human, *LZTFL1* is specifically upregulated in patients with familial DCM, but not with HCM or heart failure deriving from conditions other than familial DCM.

Mouse models demonstrate decreased *Lztfl1* expression at the mRNA and protein level in pathological, but not physiological cardiac hypertrophy. TAC experiments suggest that downregulation occurs in the early, adaptive phase of the disease, but levels return to control levels at late stage hypertrophy. *Lztfl1* levels are also diminished in the area of MI, but not in distant sites.

Finally, IGF-1 overexpression in cardiomyocytes and IGF-1 treatment of keratinocytes suggest that IGF-1 signalling can regulate LZTFL1 levels. However, the experiments also suggest that the regulatory mechanism is cell type-specific.

5.5 Discussion

5.5.1 AJ and LZTFL1 in DCM

LZTFL1 levels were increased in patients with familial DCM but not in patients with heart failure derived from idiopathic DCM or other conditions. Unfortunately, no information were available on the familial DCM patients including information whether these patients were related, had developed heart failure and whether the samples were originating from endomyocardial biopsies during the stable phase of disease or were taken during heart transplantation in response to heart failure. This makes the interpretation of the data extremely difficult.

If the patients had heart failure and underwent heart transplantation, patient FE from the heart failure group, that also had a family history of DCM, belongs to the familial DCM-heart failure group and the data suggests a common and inherited underlying cause for the observed change in *LZTFL1* levels. In this case, the data would not provide information on whether *LZTFL1* levels are changed during earlier phases of disease or whether they are specifically linked to progression to heart failure.

On the other hand, if the samples of the familial DCM group were taken during the stable phase of disease, the data would suggest that *LZTFL1* is downregulated during the adaptive phase of disease. Since no data from patients without family history of DCM from the same phase of disease were available for comparison, it cannot be concluded whether this change would be a general feature of adaptive DCM or specific to this patient group. If it is a general feature of DMC, comparison with the heart failure group suggests that *LZTFL1* levels return to base levels during the maladaptive phase of DCM. In that case, patient FE would be an exception since his levels are still increased. On the other hand, if increased *LZTFL1* levels are specific to familial DCM patients, patient FE suggests that *LZTFL1* levels stay up during heart failure.

It is intriguing, that overall *LZTFL1* levels increased in familial DCM patients while isoform 1 and 3/X1 levels remained unchanged or undetectable and isoform 2 was undetectable in most samples. This suggests an isoform switch to an unidentified *LZTFL1* isoform. The existence of another unidentified isoform is supported by the detection of two unexplained higher molecular weight bands in Western blots with anti-*LZTFL1* antibody which are lost when blocking the antibody with *LZTFL1* fusion protein (Figure 4.4 A).

Overall, *LZTFL1* levels follow the same expression pattern as *CDH2*, *CTNNA1* and *CTNNA3*. Since familial DCM patients have increased levels of these AJ components and AJ-stabilising proteins, this might suggest that they have more stable intercalated discs. However, further data is needed to confirm upregulation on the protein level and to validate their localisation at intercalated discs. Augmented stability of cardiomyocyte connections might be beneficial to counteract higher myocardial strain caused by ventricular dilation. However, it has been suggested that larger amounts of intercalated disc components might enhance myocardial stiffness (Craig et al., 2010; Masuelli et al., 2003). Indeed, diastolic stiffness is increased during DCM (Vahl et al., 1997) and results in higher end-diastolic pressure and disease progression. Therefore, more stable intercalated discs might be beneficial at the onset of disease, but contribute to disease progression at later stages.

Increased levels of N-cadherin and other junctional components have also been observed in other studies of DCM. A histological study on post-mortem specimens from patients with end-stage dilated cardiomyopathy demonstrated that levels of N-cadherin are upregulated at intercalated discs and intracellularly compared to control specimens (Tsipis et al., 2010). In a mouse model of DCM, levels of AJ components are also elevated and this is linked to a reduction in cellular connexin 43 levels and gap junctions in the intercalated disc (Ehler et al., 2001). Both studies suggest that AJ components are upregulated in an attempt to counteract disturbances of the contractile cytoskeleton, but eventually lead to disorganised intercalated discs. In this context, it is interesting to note that sequencing data from patient D6 revealed a truncation mutation in titin that is likely to be disease causing (personal communication with Prof Ralph Knöll). Titin mutations have been associated with impaired sarcomere function (References in Table 1.2) and might induce compensatory upregulation of AJ components. Many studies have shown that connexin 43 is highly sensitive to stoichiometric changes in intercalated disc proteins (Ehler et al., 2001; Kostetskii et al., 2005; Li et al., 2005; Saffitz, 2011). The resulting reduction in connexin 43 leads to weakening of electrical coupling of cardiomyocytes and impaired contractility thereby promoting disease progression.

Patients D6 and FE differ from other samples in their respective groups, but exhibit a similar expression profile to one another. They possess the lowest levels of *CDH2*, *CTNNA1*, *CTNNA3* and *LZTFL1* isoform 1 in their particular group with levels comparable or below control group levels. The exception is *CTNNA3* level of patient D6, which is in fact the highest level observed in all samples. The missing upregulation

of AJ components correlates with strongly reduced levels of *CDH13*, a known cardioprotector. It is therefore possible that cardioprotective signalling through T-cadherin is required to induce the compensatory upregulation of AJ components. The lack of compensatory stabilisation of intercalated discs might also explain the more severe phenotype observed in patient FE, who presented with both ventricles severely dilated. Since DCM most commonly affects the LV, it could be speculated that reinforcement of intercalated discs is sufficient to prevent dilation of the RV where pressure is lower, but is insufficient to withstand the pressure in the LV. Similarly, an increase in wall stiffness would have a weaker detrimental effect in the RV than in the LV due to the differences in pressure.

5.5.2 AJ components in HCM

In isoproterenol-induced hypertrophy, *Cdh2* levels are unchanged, while *Ctnna1* and *Ctnna3* are increased. This result suggests that AJ components are not generally increased in this model of HCM. This is in agreement with the spontaneously hypertensive stroke-prone rat, in which only the epithelial cadherin E-cadherin and its localisation at intercalated discs are upregulated (Craig et al., 2010). Since none of the other AJ component levels are changed, the authors suggested that elevated E-cadherin might serve to sequester β -catenin at the membrane and thereby inhibit canonical Wnt signalling. Several studies have provided evidence that inhibition of β -catenin leads to attenuation of hypertrophy and improved cardiac performance as reviewed by (Bergmann, 2010).

Interestingly, my results also demonstrate *Cdh1* elevation in mice preconditioned with IGF-1Ea that did not develop HCM in response to isoproterenol treatment. So far, the mechanisms underlying the cardioprotective effect of IGF-1Ea are unknown. The observed upregulation of E-cadherin and proposed inhibition of Wnt signalling by sequestration of β -catenin provides an attractive hypothesis to explain the beneficial effect of IGF-1Ea.

Furthermore, I also observed upregulation of *Cdh13*. T-cadherin has cardioprotective properties by binding to adiponectin and promoting adiponectin signalling (Denzel et al., 2010). Although *Cdh13* levels are already elevated in isoproterenol-induced hypertrophy, IGF-1Ea preconditioning further enhances the levels and this mechanism might contribute to the cardioprotective effect of IGF-1Ea.

Finally, *Ctnna1* and *Ctnna3* levels are increased in isoproterenol-induced hypertrophy without detectable increase of other typical intercalated disc components. Alpha-E-catenin has other AJ-independent functions that might make upregulation of α E-catenin beneficial. Importantly, recent studies have shown that α E-catenin is an inhibitor of β -catenin function in Wnt signalling (Choi et al., 2013; Daugherty et al., 2014; Giannini et al., 2000) and therefore elevated levels of free α E-catenin would be expected to reduce hypertrophy by constraining Wnt signalling. Furthermore, α E-catenin is able to regulate actin and tubulin cytoskeleton independently of cell-cell adhesion (Benjamin et al., 2010; Shtutman et al., 2008). Thus α E-catenin may be upregulated to aid cytoskeletal remodelling in order to adapt cardiomyocytes to increased pressure load.

5.5.3 *Lztf1* in HCM

Lztf1 level is decreased in isoproterenol-induced hypertrophy in our study, in contrast to higher levels observed in a microarray study (Galindo et al., 2009). However, although both systems use isoproterenol to induce hypertrophy, the experimental set-up is very different in terms of isoproterenol dose, way of administration and duration of treatment. Low doses of isoproterenol, as used in this study, have been shown to induce hypertrophy, fibrosis and necrosis, while medium doses as used by (Galindo et al., 2009) also cause changes in the energy metabolism of cardiomyocytes and infarct-like myocardial damage (Nichtova et al., 2012). Furthermore, Tommaso Poggioli used immunodeficient mice for his study to allow for the engraftment of the HEK293 cells. Isoproterenol is known to regulate immune cells via β -adrenergic receptors (Busse and Sosman, 1984). Analysis of the microarray data identified immune system function and response as the top functions affected in isoproterenol-treated mice (Galindo et al., 2009). Therefore, the distinct experimental set-up is likely to explain the different *Lztf1* expression profiles obtained.

Lztf1 protein levels are reduced in early adaptive stages of TAC-induced hypertrophy. Interstitial fibrosis is a hallmark of HCM and appears early during disease development (Weber and Brilla, 1991). Increased fibrosis is associated with increased passive stiffness and impaired contractile function (Conrad et al., 1995). Since intermediate filaments are a determinant of cell stiffness in other cell types, it is likely that desmin content and organisation impacts on cardiomyocyte stiffness. Indeed, desmin levels negatively correlate with cardiac performance (Monreal et al., 2008; Seltsmann et al., 2013) and are upregulated in end-stage HCM, leading to increased cardiomyocyte stiffness (Helming et al., 2000). I have proposed that LZTFL1 is required for keratin stability in keratinocytes. Reduced *Lztf1* levels in early HCM might destabilise desmin

and decrease the desmin filament pool. This would lead to reduced cardiomyocyte stiffness to counterbalance the increased wall stiffness due to fibrosis. After four weeks of TAC, which corresponded to onset maladaptive HCM, *Lztfl1* levels are returned to normal and therefore might promote stabilisation and accumulation of desmin at this state of disease. Clearly, further studies will be needed to test whether LZTFL1 can bind to and stabilise desmin.

Another possible explanation for changes in LZTFL1 levels might be related to the control of gene expression. Endogenous LZTFL1 staining has been observed in the nucleus of keratinocytes and the atrial cardiomyocyte cell line HL-1 (data not shown). The nuclear localisation of LZTFL1 strongly suggests a function for LZTFL1 in transcriptional regulation. *Lztfl1* might therefore be downregulated in early HCM to change transcriptional programs.

5.5.4 *Lztfl1* in physiological hypertrophy and in response to IGF-1 signalling

IGF-1 signalling is thought to be highly important for physiological hypertrophy in response to exercise. Mice overexpressing IGF-1 receptor (IGF1R) in cardiomyocytes develop physiological hypertrophy with greater hypertrophic growth response to exercise (McMullen et al., 2004). On the contrary, mice carrying a cardiac deletion of the IGF1R are resistant to exercise-induced hypertrophy (Kim et al., 2008). Similarly, cardiac-specific overexpression of human IGF-1 causes the development of physiological hypertrophy in young mice and this correlated with increased cardiac output (Delaughter et al., 1999). However, older animals (from about 30 weeks) have poor cardiac performance in conjunction with increased hypertrophy and fibrosis. Extended IGF-1 exposure therefore leads to progression from physiological to pathological hypertrophy (Delaughter et al., 1999). Since *Lztfl1* levels are unchanged in exercise-induced hypertrophy and IGF-1 signalling induces physiological hypertrophy, it could be expected to observe no change of *Lztfl1* levels in IGF-1-overexpressing cardiomyocytes. Instead, *Lztfl1* levels seem reduced in overexpressing compared to wild-type cardiomyocytes. However, at the time of sampling, IGF-1-overexpressing mice were at least 7 month (= 30 weeks) old and therefore likely in the early phase of pathological hypertrophy. Accordingly, these results can be interpreted as a further verification of reduced *Lztfl1* levels during early phases of HCM in mice. *Lztfl1* level attenuation could be a direct result of extended IGF-1 signalling or of the onset of hypertrophy with changes in wall stress and contraction or altered intracellular signalling. In support of a direct effect of IGF-1 signalling is the fact that LZTFL1 levels are regulated by IGF-1 treatment in keratinocytes.

5.5.5 Lztfl1 levels in MI

Lztfl1 levels were reduced in the area of myocardial infarct but not in distant areas. This finding is consistent with an AJ-linked function of Lztfl1, since cell-cell contacts are lost in the area in and around the infarct probably caused by cell death (Matsushita et al., 1999). Compromised cell-cell contacts might lead to destabilisation and degradation of Lztfl1. However, other mechanisms could also explain the decreased levels of Lztfl1 such as changes in transcriptional regulation induced by post-infarct inflammatory response (Christia and Frangogiannis, 2013).

5.6 Future work

5.6.1 Towards defining the role of LZTFL1 in cardiomyocytes

A function for LZTFL1 in cardiomyocytes remains elusive. A first step in identifying a function for LZTFL1 would be the documentation of the cellular location. It will be particularly interesting to test localisation at intercalated discs, the nucleus and desmin cytoskeleton, which would suggest similar functions as in keratinocytes. In this context, testing binding to desmin would be desirable and could also be investigated by *in vitro* binding assays.

LZTFL1 is a binding partner of α E-catenin. Cardiomyocytes additionally express the related isoform α T-catenin, which can crosslink AJ and desmosomal protein complexes. I hypothesised that LZTFL1 might be able to bridge AJ and desmosomes in keratinocytes by binding to α E-catenin and keratin. It would therefore be interesting to test whether LZTFL1 can bind α T-catenin. This would support the linkage between different protein complexes in the intercalated disc by potentially binding α E- and α T-catenin and desmin.

To test that LZTFL1 helps stabilising intercalated discs, LZTFL1 depletion in cardiomyocyte cell culture models would help to verify this assumption. Since gap junctions rely on stable intercalated discs, the effect on connexin 43 should also be tested.

My results suggest that LZTFL1 isoform switches take place in several of the studied pathologies. However, the known human isoforms could not explain the *LZTFL1* increase in human DCM and, in mouse, only one *Lztfl1* isoform has been described to date. It would be interesting to identify other LZTFL1 isoforms, by scanning the NCBI database for expressed sequence tags (ESTs) with similarity to LZTFL1, and investigate differences in their domain structure and function.

My results suggest that LZTFL1 levels might be downregulated by IGF-1 or adiponectin/T-cadherin signalling in cardiomyocytes. Cell culture models could help to verify this connection. Since LZTFL1 is also reduced in MI, it might additionally be interesting to test the effect of proinflammatory factors such as tumour necrosis factor alpha (TNF α) and interleukin 1.

5.6.2 Towards a better understanding of AJ in cardiovascular disease

My results demonstrate increased mRNA levels of N-cadherin in human familial DCM and of α E-catenin and α T-catenin in human familial DCM and isoproterenol-induced hypertrophy. These results should be substantiated at the protein level and by investigating the cellular localisation of the increased pool of protein. Since the isoproterenol model results might be affected by the use of immunodeficient mice, the results should be confirmed in a different model of HCM, for example in the TAC model.

It is intriguing, that several patients with familial DCM have a very similar expression pattern of AJ components and *LZTFL1*, but that this pattern was different from patients with idiopathic DCM. Since the clinical data on the DCM patients was not available to exclude patient relation or disease state as explanation for this observation, analysis of additional patients will be required to confirm the result. If the distinction between familial and idiopathic DCM holds up it will be important to identify the underlying cause by sequencing patient DNA and search for mutations in known disease-related genes. Familial DCM is caused by mutations in a large number of genes. This fact makes the uniform expression pattern in the familial DCM group in this study surprising, especially because the pattern is distinct from idiopathic DCM. It has been suggested that titin mutations are very common and might be disease-supporting rather than disease-causing (Herman et al., 2012; Roncarati et al., 2013). It might therefore be useful to focus the scan on titin mutations and look for similarities in affected protein domains.

Alternatively, if further analysis of clinical data suggests that the alterations in AJ components and *LZTFL1* is connected to pre-heart failure disease state rather than common inherited causes, it would be useful to observe these changes in a timely manner in an animal model of DCM. A potential model system could be the MLP knock-out mouse since upregulation of AJ components in this animal model has been reported (Ehler et al., 2001).

In HCM, upregulation of α E-catenin was observed and in IGF-1Ea preconditioned mice also *de novo* expression of E-cadherin. Both changes might lead to anti-hypertrophic signalling by repression of the Wnt pathway and it would therefore be useful to investigate the activity of Wnt signalling under these conditions to confirm the functional connection.

Chapter 6 Final discussion

6.1 AJ formation and stabilisation is governed by complex pathways

Establishing novel AJ involves the activation and coordination of a number of different processes including trafficking events and cytoskeletal rearrangement. GTPases are key regulators of AJ formation. Mainly three GTPases, Rac1, RhoA and Cdc42 have been studied in the context of AJ formation and yet they have been found to stimulate a multitude of different pathways (Menke and Giehl, 2012). This is possible, because every GTPase is able to activate a number of different effector proteins dependent on the precise upstream signals. Different GEF proteins mediate GTPase activation in response of different signals and they are believed to exist in complex with a specific subset of GTPase effector proteins that will be induced upon GTPase activation (Cherfils and Zeghouf, 2013). Early events triggered include E-cadherin recruitment to sites of contacts and actin polymerization. Later, the contacts are expanded and matured by the formation and compaction of an underlying circumferential actin belt (Harris and Tepass, 2010).

E-cadherin recruitment can occur by two pathways, lateral diffusion or vesicular delivery (de Beco et al., 2012). Some E-cadherin is found at the cell surface even in the absence of cell-cell contacts and moves relatively freely by diffusion. Upon initial contact with E-cadherin molecules of neighbouring cells, diffusion is thought to be tuned down by the formation of actin patches that help cluster cadherin receptors and by the formation of a contractile actin network that limits the mobility of the clusters (Cavey et al., 2008). Further E-cadherin molecules are then incorporated that arrive by diffusion and as well as molecules that are actively delivered by vesicular transport.

In this study, I identified two proteins that regulate availability of E-cadherin at AJ. My data suggests that EF1 α is involved in restricting E-cadherin levels at junctions while CIP4 seems to be required to increase E-cadherin levels. EF1 α is able to bundle actin, but also binds to and targets β -actin mRNA to sites where actin polymerization is required (Demma et al., 1990; Liu et al., 2002). By doing so, one can speculate that EF1 α might help with the formation of junctional actin. The junctional actin network is then required to limit lateral diffusion and to stabilise E-cadherin at contact sites. It is therefore likely that, in concert with other proteins, EF1 α can regulate junctional actin formation via actin polymerization and bundling to stabilize E-cadherin at novel contacts and to restrict delivery by lateral diffusion.

During AJ formation, CIP4 might act to deliver E-cadherin to contact sites by vesicular transport. CIP4 is involved in DE-cadherin trafficking in *Drosophila*, although so far it has only been indicated in DE-cadherin endocytosis (Leibfried et al., 2008). My results support a role for CIP4 in E-cadherin endocytosis at least in the absence of cell-cell contacts. I propose that during junction formation and in response to altered GTPase signalling, CIP4 switches to an exocytosis promoting function. A similar switch in CIP4 function has been described for GLUT4 receptor transport in response to altered insulin levels (Chang et al., 2002; Hartig et al., 2009). Elevated insulin levels cause activation of the Rho GTPase TC10, which activates CIP4 and causes its translocation to the membrane (Chang et al., 2002). CIP4 is constitutively bound to GAPEX5, a GEF for Rab5 and Rab31 (Lodhi et al., 2007). Through translocation of CIP4, GAPEX5 becomes sequestered at the membrane away from its substrates. This inactivates Rab31 and diverts GLUT4 transport to the membrane. I show that CIP4 is recruited to the membrane early during AJ formation. I propose that in a mechanism analogous to GLUT4 transport, CIP4 might sequester a regulator of vesicle transport at the membrane thereby diverting E-cadherin trafficking to the membrane.

CIP4 localisation at junctions is dependent on the Rho GTPase GEF VAV2. VAV2 might recruit CIP4 directly or indirectly. As a GEF, VAV2 could promote CIP4 signalling through GTPase activation, which in turn might cause CIP4 translocation to cell-cell contacts. In addition to redirecting E-cadherin trafficking, CIP4 is retained in position ready to act in E-cadherin endocytosis when needed. This allows for fast contact remodelling during situations such as injury, cell division and tissue remodelling.

In its role as a Rho GTPase GEF, VAV2 is also thought to induce actin remodelling required for the stabilisation of AJ (Fukuyama et al., 2006; Kawakatsu et al., 2005). I therefore propose that EF1 α , CIP4 and VAV2 co-operate to maximise the availability and stabilisation of E-cadherin at cell-cell contacts via distinct processes: regulation of E-cadherin delivery through diffusion and exocytosis as well as actin remodelling to retain E-cadherin at junctions (Figure 6.1).

Early AJ are then further stabilised and matured through the formation and contraction of a circumferential actin ring, which is also required to complete cell polarization (Zhang et al., 2005). I identified two proteins that appear to be involved in this maturation and thereby help maintain cell-cell contacts. MTSS1 depletion led to the loss of thin bundles while bundle compaction seem to be impaired in LZTFL1 depleted

cells. The loss of both proteins weakened contacts as became apparent when exposing cells to mechanical stress.

MTSS1 has several actin- and GTPase-regulatory functions that make it a prime candidate of an AJ regulator. We and others showed that MTSS1 is required for actin assembly by activating Rac1 and promoting actin polymerisation through Arp2/3 (Dawson et al., 2012a; Saarikangas et al., 2011). Although so far untested I predict that MTSS1 further influences AJ stability through its abilities to regulate RhoA and bundle actin (Gonzalez-Quevedo et al., 2005; Lei et al., 2014; Yamagishi et al., 2004; Yu et al., 2011). Actin bundling might aid the compaction of thin bundles and RhoA regulation would support the fine balance in spatial and temporal GTPase activation that has been shown to be required for AJ formation (McCormack et al., 2013; Yamada and Nelson, 2007).

Our knowledge on LZTFL1 is very limited to date. I introduce LZTFL1 as a novel stabiliser of AJ and show that it can bind α -catenin and that LZTFL1 depletion likely leads to defective compaction of the circumferential actin belt. Binding to α -catenin might be required to recruit LZTFL1 to AJ, since the LZTFL1 N-terminus that binds to α -catenin was the fragment with the clearest junctional accumulation upon overexpression. It is unlikely that LZTFL1 is directly involved in thin bundle compaction, as preliminary results suggest that it does not bind to actin *in vitro*. Instead it might be involved in recruiting myosin-regulating or actin-bundling proteins such as MTSS1 or EF1 α to junctions and thereby promote actin compaction. In fact, EF1 α depletion produces a similar phenotype as LZTFL1 and MTSS1 in response to mechanical stress (J. Erasmus, unpublished) and therefore might cooperate with them in the regulation of thin bundles. At early stages of AJ formation, EF1 α might promote junctional actin formation and thereby limit diffusion by helping actin polymerisation through β -actin mRNA targeting and later further stabilise AJ by aiding thin bundle compaction.

In summary, I propose that MTSS1, LZTFL1 and possibly EF1 α cooperate in the maturation and maintenance of AJ and cell polarization by supporting actin polymerization and by compacting a belt of circumferential actin fibres underlying AJ (Figure 6.1). In this model MTSS1 promotes actin polymerisation through Rac1 and Arp2/3 and MTSS1 and EF1 α might aid circumferential actin compaction by actin bundling. LZTFL1 probably indirectly aids thin bundle compaction through recruitment of other factors.

Overall I confirmed the involvement of five proteins in AJ regulation in primary keratinocytes, that act side-by-side and through different pathways to enable the correct and timely formation and maintenance of cell-cell contacts. All proteins were identified as potential regulators through RNAi screens. These screens also identified a large number of additional proteins not yet studied in further detail, underscoring the complexity of the process. The cooperation among these pathways allows the precise regulation of formation and removal of AJ in space and time to appropriately respond to requirements during injury, development and differentiation.

6.2 Skin and heart have special requisites for contact stability

Heart muscle and skin are the organs under the most severe mechanical stress in the body. They therefore possess a second type of adhesive complex in their membranes, the desmosome, which anchors intermediate filaments and reinforces cell-cell contacts to ensure tissue integrity even under constant mechanical stress (Green and Simpson, 2007). In epithelial cells, desmosomes and AJ exist separately but adjacent at cell-cell contacts, with AJ being the more apical complex. In cardiomyocytes instead, AJ and desmosomal complexes exist side-by-side in the so-called *area composita* (Borrmann et al., 2006; Franke et al., 2006). However, it is believed that they not only coexist, but are actually crosslinked via α T-catenin, a protein that can bind to the AJ component β -catenin and to the desmosomal component plakophilin 2 (Goossens et al., 2007; Janssens et al., 2001). Similarly, composite junctions termed *complexus adhaerentes* have also been observed in vascular and lymphatic endothelial cells, in chicken lens and *Xenopus* mesendoderm, where classical cadherins are directly linked to intermediate filaments via plakoglobin and desmoplakin (Hämmerling et al., 2006; Kowalczyk et al., 1998; Leonard et al., 2008; Weber et al., 2012). This is possible, because plakoglobin is able to bind both to classical and desmosomal cadherins (Desai et al., 2009). These composite junctions and crosslinks between desmosomal and AJ complexes are believed to increase the strength of cell-cell contacts by tethering cadherins to actin and intermediate filaments simultaneously (Kowalczyk et al., 1998; Leonard et al., 2008).

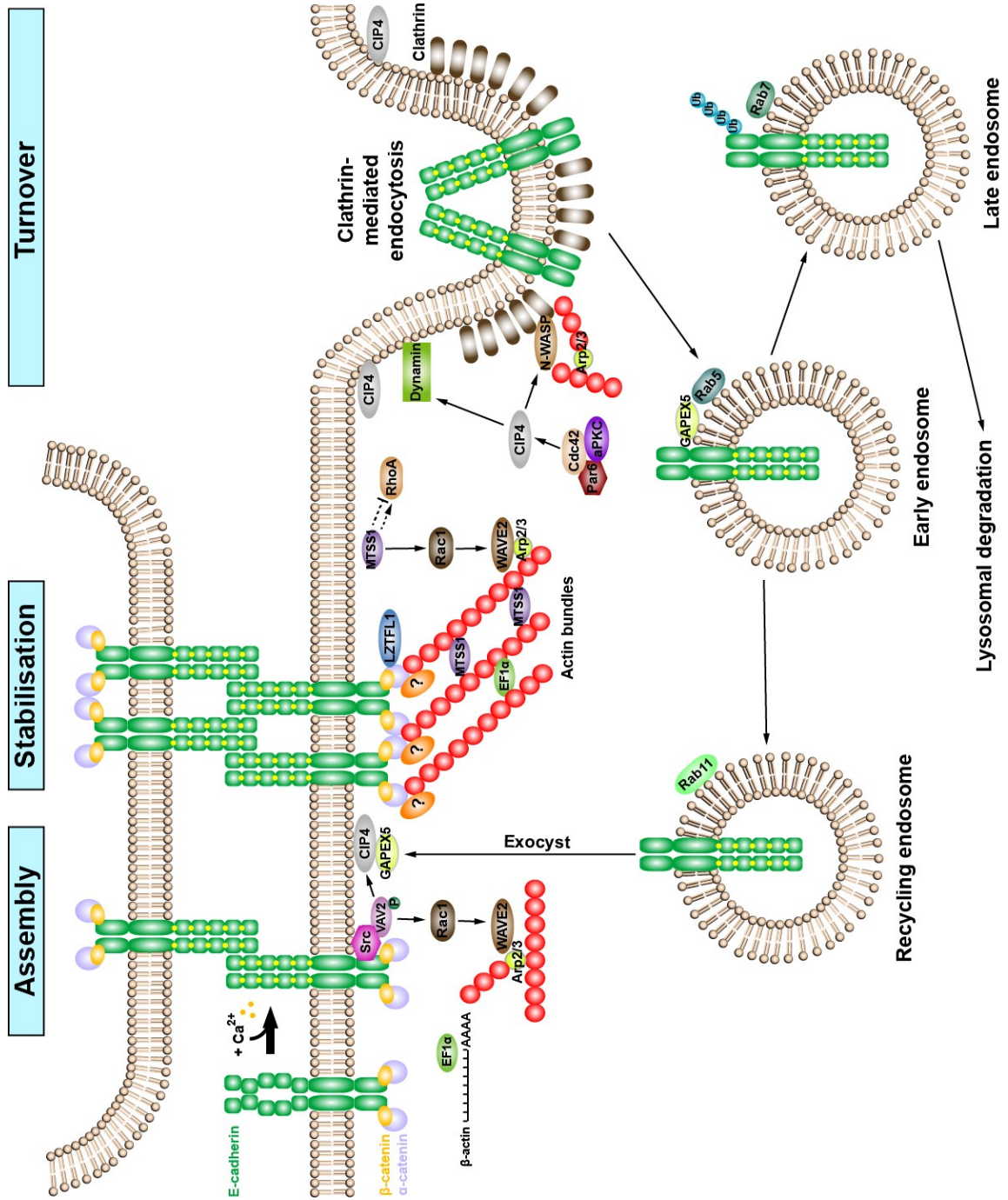


Figure 6.1: Model of proposed functions of CIP4, EF1α, LZTFL1, MTSS1 and VAV2 in AJ formation, stabilisation and turnover. In this model, EF1α regulates junctional actin formation in concert with other proteins by recruiting β-actin mRNA and by actin bundling to stabilize E-cadherin at novel contacts and to restrict delivery by lateral diffusion. VAV2 is activated by phosphorylation through Src and activates Rac1, which mediates actin polymerization through WAVE2-Arp2/3. VAV2 also recruits CIP4 to the membrane, from where it promotes E-cadherin delivery possibly by diverting vesicular transport to the membrane by sequestering Rab regulators such as GAPEX5. LZTFL1 is recruited to junctions by binding α-catenin and aids junction stabilisation. MTSS1 promotes actin formation by activating Rac1 and thereby WAVE2-Arp2/3 actin polymerization. MTSS1 might at the same time regulate RhoA and bundle and compact thin bundles. During clathrin-mediated E-cadherin endocytosis, CIP4 might be recruited to contacts through a Cdc42-Par6-aPKC complex. CIP4 aids membrane invagination through its F-BAR domain and recruitment of N-WASP to promote actin polymerization. CIP4 also recruits dynamin, which is needed for vesicle budding.

In addition, these specialised links have been shown to be required for mechanosensing. In *Xenopus* mesendoderm, plakoglobin is specifically recruited to C-cadherin upon stretch and this recruitment is required for polarized migration and keratin remodelling (Weber et al., 2012). AJ are also able to sense mechanical stress without the involvement of keratin and plakoglobin. It has been shown that α E-catenin changes conformation in response to tugging forces and exposes a binding site for vinculin (Yonemura et al., 2010). Bound vinculin is able to tether further actin filaments to the cadherin-catenin complex and therefore reinforce junction stability. However, additional recruitment of plakoglobin and keratin in response to stress might be a way to integrate and coordinate the remodelling of different cytoskeletal components.

Mechanosensing is not only required during collective cell migration, but also in situations such as growth control by contact inhibition. The formation of novel cell-cell contacts upon meeting adjacent cells is believed to signal information concerning cell density to the nucleus and thereby regulate proliferation (Kim et al., 2011a). Alpha-catenin has been shown to be required for this signalling and to control translocation of the transcriptional regulator YAP1 from the nucleus to the cytoplasm (Schlegelmilch et al., 2011; Silvis et al., 2011). How exactly the signal from AJ to the nucleus is transduced is however unknown.

Similarly, mechanosensing is required for cardiomyocytes to adapt to changes in pressure load. This involves changes in transcriptional programs, since isoform switches have been detected in different sarcomeric and other proteins during physiological hypertrophy and in pathological conditions (Table 1.3) (Gerilechaogetu et al., 2014; McCain and Parker, 2011). The intermediate filament desmin is thought to be involved in transcriptional regulation in response to mechanosensing because of its physical connection to the nucleus, sarcomeres and intercalated discs (Bloom et al., 1996). The crosslinking of AJ complexes to desmosomal complexes and desmin through α T-catenin might therefore be a way of integrating mechanosensing at AJ with nuclear gene expression.

No composite junction has to date been identified in epithelial cells. However, there are several considerations that suggest that such connections may exist similarly as they have been observed in other cell types. First, desmosomes and AJ are interdependent. Desmosomes are formed after AJ are established and if AJ formation is inhibited, desmosomes do not form (Gumbiner et al., 1988; Lewis et al., 1994). On the other hand, desmoplakin deletion impairs AJ maturation (Vasioukhin et al., 2001b), and

collapsing of keratin 8 leads to mistargeting of E-cadherin in simple epithelia (Hanada et al., 2005). The desmosome-binding protein pinin regulates E-cadherin transcription and splicing (Alpatov et al., 2004; Alpatov et al., 2008; Shi and Sugrue, 2000). A direct link to integrate signalling through both adhesive complexes would therefore make sense. Second, keratin, actin, AJ and desmosomes need to be regulated co-ordinately during morphogenic movement and wound healing (Ilna and Friedl, 2009; Roberts et al., 2011). Third, keratin links desmosomes to the nuclear envelope to allow mechanical signals to be converted into a transcriptional response. AJ also can convey signals to the nucleus to inhibit proliferation during contact inhibition (Kim et al., 2011a; Schlegelmilch et al., 2011; Silvis et al., 2011). However, how the signal is transmitted to the nucleus is unclear and could be explained with a connection between AJ and keratin analogous to desmosomes.

Plakoglobin is a protein that could form a link between AJ and desmosomes in epithelial cells in a similarly way as described in *Xenopus* mesendoderm (Weber et al., 2012). In this study I show that LZTFL1 also has properties that make it a good candidate to provide a physical link between AJ and the intermediate filament cytoskeleton. i) LZTFL1 can bind to α E-catenin and keratin 14. ii) LZTFL1 depletion negatively affects AJ, desmosomes, actin and keratin filaments, suggesting that it integrates the regulation of these different adhesive complexes and their associated cytoskeletal components. iii) Partial co-localisation was observed both with E-cadherin and desmoplakin. iv) LZTFL1-positive keratin filaments were strongest at colony borders and appeared weaker or were missing in cells at the centre of the colony. Since tensile stress increases towards the colony centre while traction is highest at the colony border (Treat et al., 2009), this observation suggests that LZTFL1 localisation at keratin filaments may be regulated by physical forces.

I therefore propose that LZTFL1 might be able to tether keratin filaments to AJ through binding to α -catenin (Figure 6.2). This connection would not only further stabilise AJ but also provide a mechanosensor complex to signal from AJ to the keratin cytoskeleton to regulate keratin remodelling during migration and cell polarisation. Since keratin is linked to the nuclear envelope, this connection would also provide a pathway to transform tension experienced at AJ into a transcriptional cue possibly by tugging on and distorting the nuclear envelop.

Although it will need to be tested whether LZTFL1 can bind to desmin, LZTFL1 could have a similar role in cardiomyocytes and support α T-catenin in bridging the different

junctional complexes. LZTFL1 expression is changed in a variety of cardiac pathologies that are linked to alterations in mechanical stress and tension properties of cardiomyocytes. Altered LZTFL1 levels might therefore represent an adaptation to different physical requirements of the cells or explain inappropriate responses to mechanical forces by interfering with mechanosensing.

6.3 LZTFL1 at the centre of transcriptional pathways

LZTFL1 mutations leading to production of truncated or no LZTFL1 have been identified in patients with Bardet-Biedl-Syndrome (Marion et al., 2012; Schaefer et al., 2014). Bardet-Biedl-Syndrome is genetic disorder linked to defects of the ciliary trafficking complex BBSome, a multi-protein complex. The BBSome and LZTFL1 are involved in the regulation of SHH signalling, a key developmental pathway, by controlling SMO transport to cilia (Seo et al., 2011). A clinical feature reported in the three cases of Bardet-Biedl-Syndrome patients with LZTFL1 mutations is mesoaxial polydactyly, a very rare form of polydactyly (Marion et al., 2012; Schaefer et al., 2014). It is therefore thought, that this symptom is specific to LZTFL1 mutations. Although the general symptoms of the patients are consistent with defective BBSome function and hedgehog signalling, this feature specific for LZTFL1 deficiency implies that LZTFL1 might be involved in developmental processes not solely through BBSome-SHH signalling.

In fact, I show in this study that a pool of LZTFL1 is found in the nucleus. It is thus likely that LZTFL1 has gene regulatory functions, although the nature of this function is entirely unclear to date. In addition, LZTFL1 is found on a subset of keratin filaments and particularly at the filaments surrounding the nucleus. These filaments are linked to the nuclear envelope and are thought to be involved in transcriptional regulation in response to mechanical cues (Bloom et al., 1996; Wilhelmsen et al., 2005). This places LZTFL1 in a position from where it could aid signal transduction, by stabilising the keratin filaments or the keratin-nuclear envelope interaction. At the same time it might be a mechanism to facilitate LZTFL1 nuclear translocation in response to signals such as stretch.

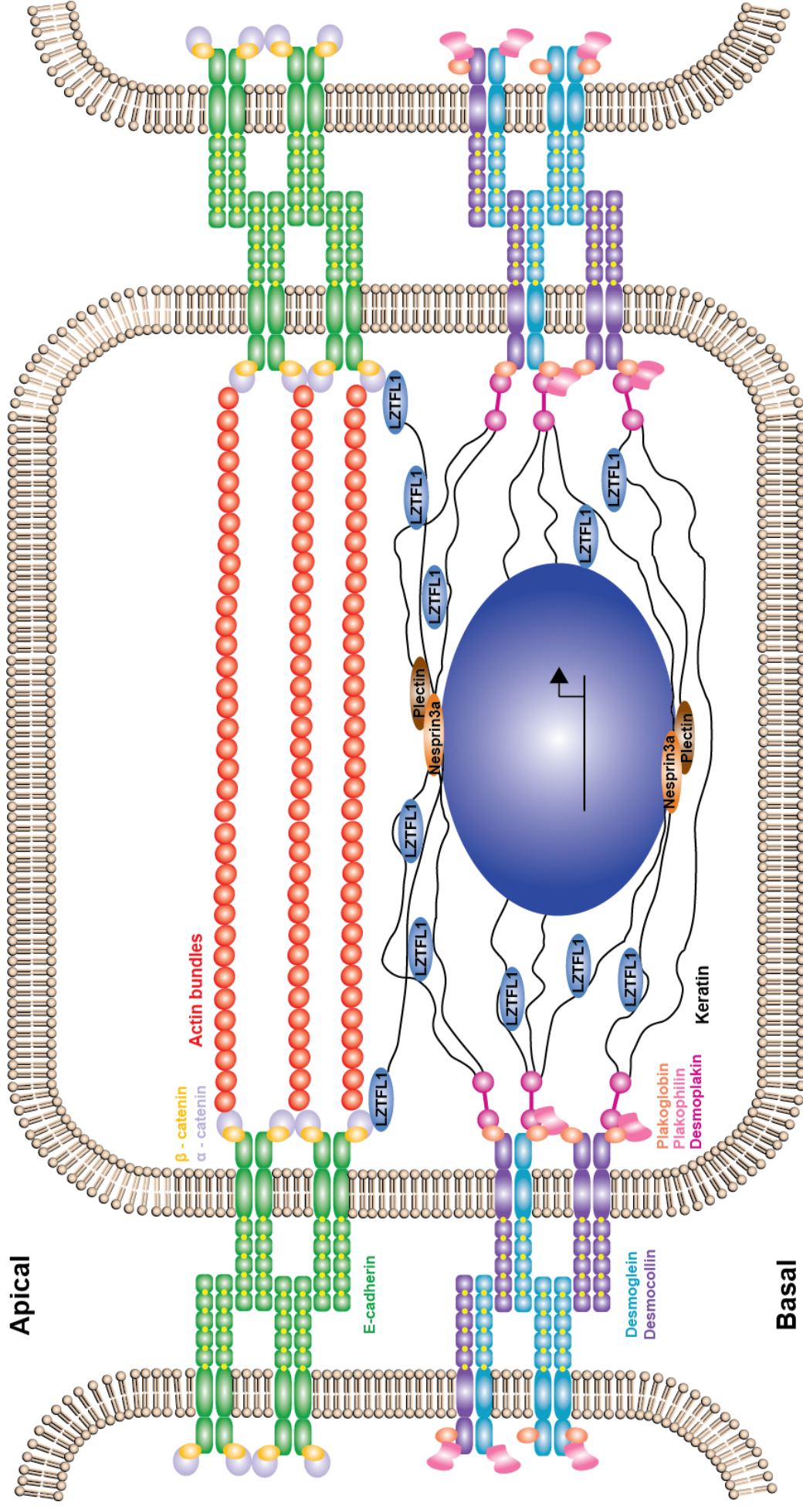


Figure 6.2: Proposed model of a composite AJ that is linked to keratin by LZTFL1. In addition to the existence of pure AJ linked to the actin cytoskeleton and desmosomes linked to keratin filaments, I propose the existence of AJ complexes that are linked to keratin filaments via LZTFL1 bound to α -catenin. Keratin filaments are linked to the nuclear envelop via Nesprin3a and plectin and this physical link enables mechanical stimuli received at AJ and desmosomes to be transformed into transcriptional changes.

What is more, in a recent study LZTFL1 has been shown to interact with β -catenin and to regulate the developmentally crucial Wnt signalling pathway by retaining β -catenin in the cytoplasm and preventing its translocation to the nucleus (Wang et al., 2014). Although I did not observe LZTFL1 interaction with β -catenin, I did show that LZTFL1 can directly bind to α -catenin. Alpha-catenin is a negative regulator of Wnt signalling: it promotes β -catenin ubiquitination and degradation, binds to β -catenin/TCF transcriptional complexes in the nucleus and attenuates their transcriptional activity (Choi et al., 2013; Daugherty et al., 2014; Giannini et al., 2000). LZTFL1 might therefore regulate Wnt/ β -catenin signalling through binding to α -catenin and possibly by sequestering α -catenin-bound β -catenin in the cytosol. In addition, α -catenin has been shown to regulate the proliferation-controlling Hippo/YAP1 pathway by localising YAP1 to the membrane and therefore away from the nucleus (Schlegelmilch et al., 2011; Silvis et al., 2011). As an α -catenin binding partner, it would therefore be possible that LZTFL1 could also play a role in the regulation of this pathway.

LZTFL1 so far has only been shown to be involved in the regulation of SHH and Wnt signalling. The negative regulatory role of LZTFL1 in Wnt and SHH signalling (Seo et al., 2011; Wang et al., 2014), could explain the downregulation of LZTFL1 observed during cancer and pathological cardiac hypertrophy, since both pathologies are associated with reinitiation of developmental gene programs (Ibsen and Fishman, 1979; Taegtmeyer et al., 2010; Taipale and Beachy, 2001). However, LZTFL1 nuclear localisation and interaction with keratin and α -catenin widens the spectrum of possible transcriptional functions of LZTFL1.

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