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Functional analysis of α-Parvin *in vivo*

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Erklärung

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Ehrenwörtliche Versicherung

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Meinen Eltern, meiner Frau Kerstin und meinen Kindern

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Contents

Abbreviations

aa	amino acid
ABI	Abelson-interacting protein
ABD	actin-binding domain
ABP	actin-binding protein
ADF	actin-depolymerizing factor
ADMIDAS	adjacent to metal-ion-dependent adhesion site
Ala	alanine
Alk	activin-receptor-like kinase
α-NAC	nascent-polypeptide-associated complex and co-activator-α
ANK	ankyrin
Arg	arginine
Arp2/3 complex	actin-related protein 2/3 complex
Asp	asparagine
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bFGF	basic firbroblast growth factor
BM	basement membrane
BMP	bone morphogenetic protein
Ca^{2+}	calcium-ion
Cdc42	cell division cycle 42
CH	calponin homology domain
CH-ILKBP	CH domain-containing ILK-binding protein
Col	collagen
CPI-17	protein-kinase-C-dependent phosphatase inhibitor of 17 kDa
CR16	corticosteroids and regional expression-16
Crk	v-crk sarcoma virus CT10 oncogene homolog
Dbl	diffuse B-cell-lymphoma
DEJ	dermal-epidermal junction
DLL4	Delta-like-4
DNA	deoxyribonucleic acid
Dock180	180-kDa protein downstream of CRK

DRF	diaphanous-related formin
E	embryonic day
EB	embryoid bodies
EC	endothelial cell
ECM	extracellular matrix
ELMO1	engulfment and motility 1
Ena/Vasp	enabled/vasodilator-stimulated phosphoprotein
Eng	endoglin
EPC	endothelial precursor cell
EPU	epidermal proliferative unit
EST	expressed sequence tag
FA	focal adhesion
F-actin	filamentous actin
FAK	focal adhesion kinase
FC	focal complexes
FERM	4.1, ezrin, radixin, moesin
FGF	fibroblast growth factor
FN	fibronectin
G-actin	globular actin
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GIT	G-protein-coupled receptor kinase interacting protein
GRAF	GTPase regulator associated with FAK
GSK3β	glycogen-synthase kinase-3β
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HeLa	Henrietta Lacks
HF	hair follicle
HM	hair matrix
HS	hair shaft
HSPC300	haematopoietic stem-cell progenitor
ICAM-1	intercellular adhesion molecule-1

I-EGF	integrin epidermal growth factor–like
IFE	interfollicular epidermis
Ig	immunoglobulin
IL	interleukin
ILK	integrin linked kinase
ILKAP	ILK-associated phosphatase
IMC	inner membrane clasp
IPP complex	ILK-PINCH-Parvin complex
IRS	inner root sheath
JNK	c-Jun N-terminal kinase
K	keratin
kAE1	kidney anion exchanger
kDa	kilodalton
LAD-III	leukocyte-adhesion deficiency type III
LAP	latency associated peptide
LDV	leucine-aspartic acid-valine
LLC	Lewis lung carcinoma
LIM	Lin11, Isl1, Mec3
LIMBS	ligand-induced metal ion binding site
LIMK	LIM kinase
Ln	laminin
LTBP	latent TGFβ-binding protein
MAdCAM-1	mucosal addressin cell adhesion molecule-1
Mg^{2+}	magnesium-ion
MHC	myosin heavy chain
MIDAS	metal-ion-dependent adhesion site
MLC	myosin regulatory light chain
MLCK	MLC kinase
MLCP	MLC phosphatase
MLP	muscle LIM protein
MM	metanephric mesenchyme
µm	micrometer
Mn^{2+}	manganese-ion
mRNA	messenger RNA

MSQ	main squeeze
MYPT1	myosin phosphatase-targeting subunit 1
Nap125	Nck-associated protein
NLS	nuclear localization signals
NPF	nucleation promoting factor
OMC	outer membrane clasp
ORS	outer root sheath
P	postnatal day
PAK	p21-activated kinase
PAT	paralyzed and arrested at the twofold stage
PDGF	platelet-derived growth factor
PDGFB	PDGF B
PDGFRβ	PDGF receptor β
PHI-1	phosphatase-holoenzyme inhibitor-1
PINCH	particularly interesting Cys-His-rich protein
PIR121	p53-inducible mRNA
PIX	PAK-interacting exchange factor
PKB	protein kinase B/Akt
PKL	paxillin-kinase linker
PLGF	placental growth factor
PSGAP	PH- and SH3-domain-containing RhoGAP
PSI	plexin-semaphorin-integrin
PTB	phospho-tyrosine binding
PtdIns(3,4,5)P3	phosphatidylinositol-3,4,5-trisphosphate
Rac1	Ras-related C3 botulinum toxin substrate 1
Ras	rat sarcoma
RGD	arginine-glycine-aspartic acid
RhoA	Ras homologous
RIAM	Rap1-GTP-interacting adaptor molecule
Rif	Rho in filopodia/RhoF
RNA	ribonucleic acid
RNAi	RNA interference
ROCK	Rho-associated kinase
s	second

S1P	sphingosine-1-phosphate
SC	sebaceous gland
Ser	serine
SFK	Src family of tyrosine kinases
SH2	Src-homology 2
SRF	serum response factor
TA	transit amplifying cells
TESK1	testicular protein kinase 1
TGFβ	transforming growth factor β
THD	talin head domain
Thr	Threonine
Tiam	T-cell lymphoma invasion and metastasis
TNFα	tumor necrosis factor α
TOCA-1	transducer of Cdc42-dependent actin assembly 1
UB	ureteric bud
UNC	uncoordinated
VCAM-1	vascular cell adhesion molecule 1
VN	vitronectin
vSMC	vascular smooth muscle cell
vWFA	von Willebrand factor A
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein
WICH	WIP- and CR16-homologous protein
WIP	WASP-interacting protein
Y2H	yeast two hybrid

Summary

The development and homeostasis of multicellular organisms critically depends on the ability of cells to migrate on and to adhere to glycoproteins of the extracellular matrix (ECM), which is secreted and organized by cells. Key receptors for components of the ECM are the members of the integrin protein family, which not only mediate adhesion to the ECM but also sense and transmit ECM-derived mechano-chemical cues to facilitate the appropriate cellular responses. This signaling function of integrins depends on the recruitment of various signaling and adaptor molecules to the cytoplasmic tails of integrins. Among the recruited adaptor molecules is the actin-binding protein α-Parvin. Although *in vitro* studies indicate that α-Parvin is essential for integrin signaling by providing a linkage to the actin cytoskeleton, its functions *in vivo* have not been analyzed.

In this study, we analyzed the *in vivo* functions of α-Parvin, which forms a ternary complex with the integrin linked kinase (ILK) and particularly interesting Cysteine-Histidin-rich protein (PINCH). Constitutive deletion of the α-Parvin-gene in mice resulted in embryonic lethality due to severe cardiovascular defects. The vascular defects were due to poor blood vessel-coverage by mural cells, compromised angiogenic remodeling, formation of aneurysms, blood vessel dilations and rupture of blood vessels leading to hemorrhages and edemas. Mechanistically, the vascular smooth muscle cell (vSMC) dysfunction resulted from increased contractility, which in turn was due to elevated RhoA-activity.

To investigate the *in vivo* functions of α-Parvin specifically in keratinocytes, we conditionally deleted α-Parvin-gene using the Cre/loxP system. The consequences ranged from severely compromised epidermal homeostasis to hair follicle morphogenesis *in vivo* and impaired adhesion and migration of α-Parvin -deficient keratinocytes *in vitro*. Impaired adhesion of α-Parvin-deficient keratinocytes resulted in locally confined detachments of the epidermis and displacement of integrin expressing cells into suprabasal layers of the epidermis and was accompanied by delayed differentiation and ectopic proliferation of suprabasal keratinocytes.

In conclusion, our data define a crucial function of α-Parvin in vascular development, epidermal homeostasis and hair follicle morphogenesis *in vivo*.

Introduction

1) The integrin family of adhesion receptors

Integrins are a family of glycosylated, heterodimeric, type I transmembrane adhesion receptors. Each integrin heterodimer is composed of one α- and one β-subunit that are non-covalently associated to form a shared ligand binding interface at their extracellular globular head domains (Arnaout et al., 2005; Luo et al., 2007). Therefore, both subunits contribute to the binding specificity of a given integrin heterodimer for its extracellular ligand(s). Their main ligands are components of the ECM but also a considerable number of soluble ligands and cell-surface molecules can be recognized by certain members of the integrin family (Humphries et al., 2006). The name "integrin" refers to their ability to integrate cues from the extracellular environment with the cells' interior organization (Tamkun et al., 1986). This property together with the ability to modulate growth factor receptor signaling makes integrins important regulators of a broad range of cellular processes including adhesion, migration, proliferation, survival and differentiation, which are crucial for the development and homeostasis of multicellular organisms (Humphries et al., 2006; Hynes, 2002; Legate et al., 2009; Sheppard, 2000). In line with their essential function in multicellular organisms, integrins are evolutionary conserved but restricted to metazoans (Whittaker and Hynes, 2002), where the number of subunits increases with the complexity of the organism. While the integrin repertoire of the nematode *Caenorhabditis elegans* comprises only two integrins, formed by two α- and one β-subunits, in the fruit fly *Drosophila melanogaster*, a set of five integrins is assembled by the combination of five α-subunits with one β-subunit. In mammals, 18 α- and 8 β-subunits are known to form 24 distinct integrin heterodimers that have overlapping substrate specificity and cell-type-specific expression patterns (Humphries et al., 2006; Hynes, 2002).

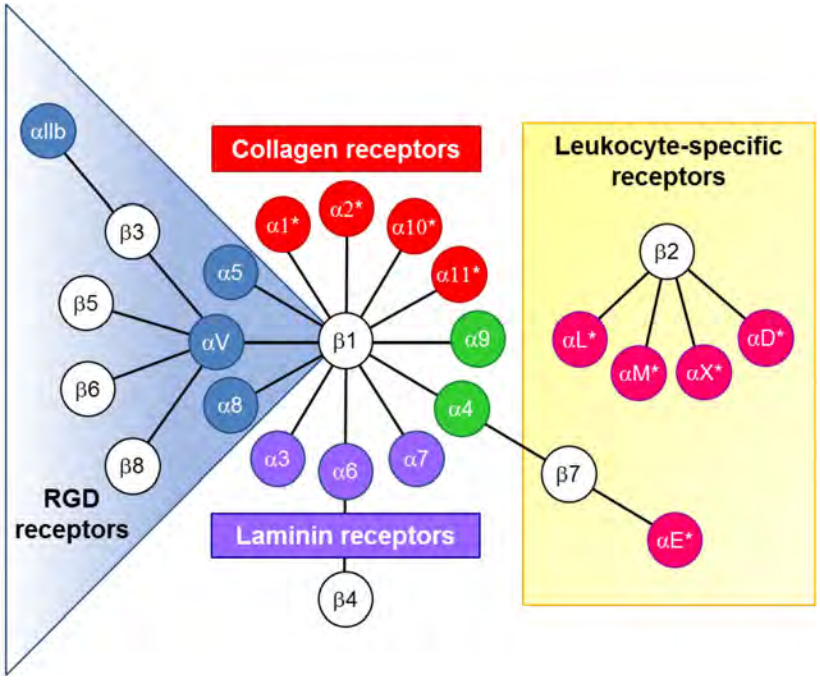

Figure 1: The integrin family of adhesion receptors

Depicted are the 24 mammalian integrin heterodimers arranged according to their main ligand binding specificity and leukocyte-specific expression. The nine α-subunits containing an αA/I-domain are indicated (*). See text for details. Figure modified from (Hynes, 2002).

According to their main ligand binding specificity and leukocyte-specific expression, integrins can be classified into four major groups (Figure 1) (Hynes, 2002):

1) Integrins that preferentially bind to ligands containing the tripeptide sequence RGD (arginine-glycine-aspartic acid); they consist of the αV heterodimers αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 as well as α5β1, α8β1, the platelet integrin αIIbβ3. Ligands for this group include fibronectin (FN), vitronectin (VN), thrombospondin, osteopontin and tenascin.

2) Collagen (Col) binding integrins are the β1 heterodimers α1β1, α2β1, α10β1 and α11β1.

3) The β1 heterodimers α3β1, α6β1, α7β1 and the α6β4 integrin are the main receptors for laminin (Ln). However, the collagen-receptors α1β1, α2β1 and α10β1 can also bind laminin (Humphries et al., 2006).

4) Leukocyte specific integrins are the α4β7 and αEβ7 heterodimers and the β2 heterodimers αLβ2, αMβ2, αXβ2, αDβ2. They recognize the tripeptide motif LDV (leucine-aspartic acid-valine) or structurally related motifs, and thereby mediate binding to ligands such as VCAM-1 (vascular cell adhesion molecule-1), mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and intercellular adhesion molecule-1 (ICAM-1).

The two related integrins α9β1 and α4β1 also bind to the LDV motif in FN and additionally recognize Ig-superfamily counter receptors such as VCAM-1.

2) Integrin structure and ligand binding

Both integrin subunits are type I transmembrane proteins, containing a short C-terminal cytoplasmic segment of about 20-50 amino acids (aa), a helical transmembrane segment of 25-29 aa and a long extracellular segment of up to 1104 aa for α- and 778 aa for β-subunits. The only exception is the β4 subunit, with a long cytoplasmic segment of about 1000 aa that links the α6β4 integrin to intermediate filaments.

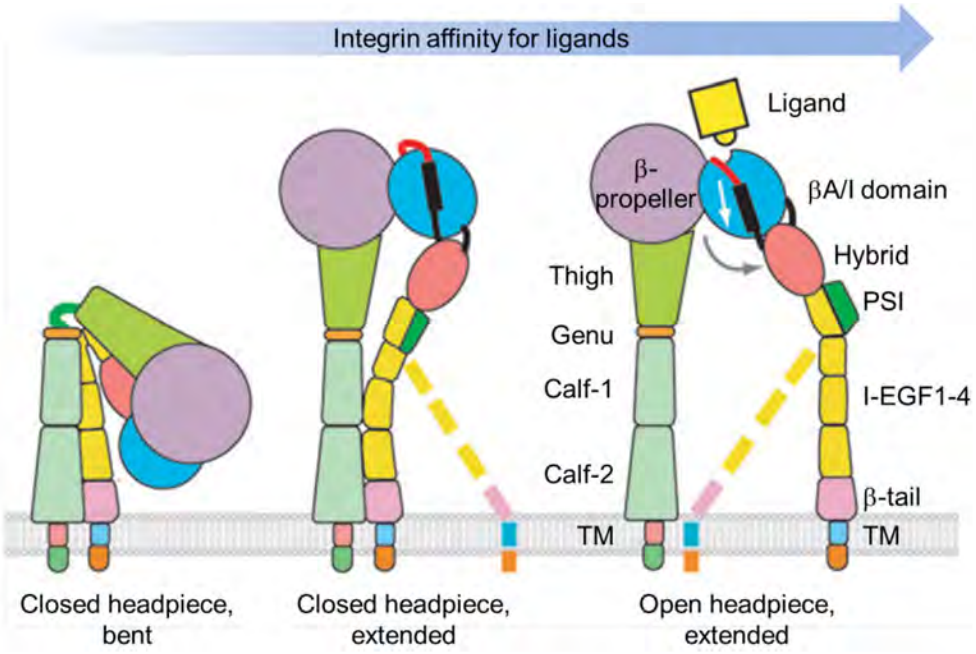

Figure 2: Integrin structure and conformational changes

Schematic representation of the integrin structure and conformational changes that switch integrins between states of low and high ligand-binding affinities. See text for details. Figure modified from (Luo et al., 2007)

At their extracellular N-termini, a seven-bladed β-propeller domain of the α-subunit non-covalently associates with the βA/I domain of the β-subunit to form the globular ligand-binding "head" of the integrin heterodimer. A long (~170 Å) stalk or leg region separates the globular head from the membrane. The β-subunits-stalk consists of a Hybrid, a PSI (plexin-semaphorin-integrin), four I-EGF (integrin epidermal growth factor–like) and a β-tail domain, whereas the α-subunit stalk is formed by Thigh (Immunoglobulin (Ig)-like), Genu, Calf-1 and Calf-2 (β-sandwich) segments. Importantly, the ectodomain of integrins can switch between an extended "active" and a bent "inactive" conformation, which is referred to as the "switchblade" model and involves separation of the cytoplasmic tails and the transmembrane

segments. Bending occurs at the Genu domain of the α-subunit and between I-EGF domains 1 and 2 of the β-subunit. Additionally, a "swing-out" of the Hybrid domain in the extended conformation results in a shift from a closed (low affinity) to an open (high affinity) conformation of the βA/I domain (Figure 2). The collagen-binding and the leukocyte specific α-subunits additionally contain an N-terminal von Willebrand factor A (vWFA) domain inserted into their β-propeller which is known as the αA/I domain. Structurally, this αA/I domain is highly similar to the βA/I domain, and likewise can adopt a closed (low affinity) or open (high affinity) conformation. Both domains contain a conserved metal-ion-dependent adhesion site (MIDAS), which physiologically is occupied by Mg^{2+} and is important for ligand binding. Substitution of Mg^{2+} by Mn^{2+} results in conformational alterations and induces the open (high affinity) conformation. Two additional metal-ion-binding sites, LIMBS (ligand-induced metal ion binding site) and ADMIDAS (adjacent to metal-ion-dependent adhesion site), that physiologically bind Ca^{2+}, are present in βA/I but not in αA/I and contribute to the regulation of the affinity state. Ligand binding is mainly mediated by the βA/I domain in integrins lacking an αA/I domain. However, in the nine integrins containing an αA/I domain, it is the αA/I domain that predominantly contributes to ligand binding (see Figure 1) (Arnaout et al., 2005; Hynes, 2002; Luo et al., 2007).

3) Bidirectional signaling of integrins

One important feature of integrins is their ability to transmit signals across the membrane in a bidirectional manner. Their ability to elicit intracellular responses upon ligand binding is referred to as "outside-in signaling", which in turn depends on the reversible activation of integrins by intracellular signals, referred to as "inside-out signaling".

3.1) Inside-out signaling

The affinity of integrins for their ligand(s) is tightly regulated. Without activating signals, integrins are believed to adopt a bent, inactive conformation. Upon the appropriate stimuli long range conformational changes take place in the transmembrane- and extracellular-domains, resulting in extended integrins with high affinity for their ligands (Figure 2). Physiologically, this is particularly important in platelets that must aggregate only upon

activation. While inappropriate activation of the platelet integrin αIIbβ3 and subsequent binding to its major ligand fibrinogen results in thrombosis, defective integrin αIIbβ3 signaling results in bleeding disorders such as Glanzmann thrombasthenia (Bennett, 2005; George et al., 1990; Lefkovits et al., 1995). Integrin activation is achieved by the binding of cytoplasmic proteins to the β integrin tails to enable separation of α- and β-transmembrane and cytoplasmic segments (Shattil et al., 2010; Wegener and Campbell, 2008; Wegener et al., 2007). An electrostatic salt bridge between Asp723 and Arg995 in the β3 and αIIb cytoplasmic tails, respectively, is implicated in preventing integrin activation by mediating a super weak interaction between integrin cytoplasmic tails (Hughes et al., 1996) and mutations of the corresponding residues in the conserved GFFKR motif in α4 and αL that disrupted this interaction resulted in integrin activation (Imai et al., 2008; Lu and Springer, 1997). However, no obvious phenotype was observed in mice upon replacement of the corresponding Asp by an Ala residue in the β1 integrin tail, questioning the importance of the putative salt bridge, at least for β1 integrins *in vivo* (Czuchra et al., 2006). Several studies also indicate an important regulatory function of transmembrane domain interactions for integrin activation, which are primarily mediated by an inner (IMC) and an outer (OMC) membrane clasp (Lau et al., 2009), although the αIIbβ3 salt bridge might contribute to the association of the transmembrane domains (Kim et al., 2009). While artificially preventing the separation of the transmembrane domains inhibited integrin activation (Lu et al., 2001; Luo et al., 2004; Zhu et al., 2008), mutations that interfere with the transmembrane association resulted in constitutive integrin activation (Gottschalk, 2005; Hughes et al., 1996; Li et al., 2005b; Luo et al., 2005; Luo et al., 2004; Partridge et al., 2005).

3.1.1) Cytoplasmic regulators of integrin inside-out signaling

In vitro and *in vivo* studies identified talin as key-regulator of integrin activation. Talin is a large (~270kDa) cytoplasmic protein composed of a globular head domain and a flexible rod domain. The 47-kDa talin head domain (THD) is comprised of a FERM (4.1, ezrin, radixin, moesin) domain, consisting of subdomains F1, F2 and F3, and a F0 subdomain (Calderwood et al., 2002; Garcia-Alvarez et al., 2003; Rees et al., 1990). Talin binds to lipids of the plasma membrane and to a conserved membrane proximal NPxY-motif and additional membrane proximal residues in the cytoplasmic tail of integrin β-subunits, leading to the separation of integrin cytoplasmic tails and transmembrane domains; this is thought to be the final common

step required for integrin activation (Lim et al., 2007; Nieswandt et al., 2007; Petrich et al., 2007; Simonson et al., 2006; Tadokoro et al., 2003). Binding to β-integrin tails mainly occurs via the phospho-tyrosine binding (PTB)-like F3 subdomain (Garcia-Alvarez et al., 2003), which is sufficient for β3 integrin activation (Calderwood et al., 2002). However, additional regions of the THD are required for β1 integrin activation (Bouaouina et al., 2008).

Other PTB-domain containing proteins can also bind to the NPxY-motif, but in contrast to talin they are not able to activate integrins (Calderwood et al., 2003), suggesting that pure binding to the NPxY is not sufficient for integrin activation. Indeed, additional interactions between talin and membrane proximal regions of the β3 integrin cytoplasmic tail and plasma membrane lipids are required for integrin activation, and mutations that disrupt these interactions prevent integrin activation (Knezevic et al., 1996; Ulmer et al., 2003; Vinogradova et al., 2002; Wegener et al., 2007). Although the THD is sufficient for integrin activation (in the presence of kindlin), formation of multimolecular adhesion structures, such as focal adhesions (FA), additionally requires the talin rod domain (Zhang et al., 2008a). The rod domain primarily mediates the linkage to vinculin and the actin cytoskeleton, and it consists of 62 amphipathic α-helices that are assembled into helical bundles. In addition, the rod domain contains a second integrin binding site and a homodimerization motif, which might facilitate integrin clustering and FA formation (Critchley and Gingras, 2008).

Regulation of talin function can take several forms. An autoinhibitory interaction between the talin rod and head domains represents an important regulatory mechanism in integrin inside-out signaling. Phosphatidylinositol-4,5-bisphosphate disrupts this interaction and thereby contributes to talin activation (Goksoy et al., 2008; Martel et al., 2001). In hematopoietic cells, talin recruitment to the plasma membrane is controlled by the small guanosine triphosphatase (GTPase) Rap1 and its effector Rap1-GTP-interacting adaptor molecule (RIAM) (Lee et al., 2009), representing another example of how integrin activation by talin can be regulated. Additionally, tyrosine phosphorylation at the membrane proximal NPxY motif of β-integrin tails is thought to serve as a regulatory switch that negatively influences talin binding while at the same time promoting binding of proteins such as Dok1, that compete with talin for the NPxY motif (Legate and Fassler, 2009; Oxley et al., 2008). However, *in vivo*, no obvious phenotype was observed upon the replacement of the corresponding tyrosine by non-phosphorylateable phenylalanine in the β1 integrin tail (Chen et al., 2006; Czuchra et al., 2006), whereas the respective mutation in β3 integrins resulted in a mild bleeding phenotype due to defects in outside-in signaling (Law et al., 1999), and impaired pathological angiogenesis (Mahabeleshwar et al., 2006).

Although talin is essential for integrin activation, recent studies revealed that integrin activation additionally requires the presence of kindlins (Ma et al., 2008; Montanez et al., 2008; Moser et al., 2008; Ussar et al., 2008). In mammals, kindlin-1, -2, and -3 comprise the kindlin family (Siegel et al., 2003). Kindlin-1 expression is mainly restricted to epithelial cells of tissues such as skin, intestine, and kidneys. While kindlin-2 is widely expressed, most prominently in skeletal and smooth muscle cells, expression of kindlin-3 is confined to the hematopoietic system (Jobard et al., 2003; Siegel et al., 2003; Ussar et al., 2006). Structurally, kindlins closely resemble the THD. However, a pleckstrin homology (PH) domain is inserted into the F2 subdomain of the kindlin FERM domain (Goult et al., 2009; Kloeker et al., 2004). Like in talin, kindlin binding to the β integrin tails is primarily mediated by the PTB-like F3 subdomain (Moser et al., 2008; Shi et al., 2007; Ussar et al., 2008). However, in contrast to talin, kindlin binding to the β integrin tails does not occur at the membrane proximal NPxY motif but at the membrane distal NxxY motif, and additionally depends on Thr/Thr or Ser/Thr residues, located between these two motifs (Ma et al., 2008; Montanez et al., 2008; Moser et al., 2009a; Moser et al., 2008; Shi et al., 2007; Ussar et al., 2008).

Although it is widely accepted that talin and kindlin synergistically activate integrins and thus are both required for efficient integrin activation, mechanistic details about how this synergistic effect is achieved remain largely elusive. The non-overlapping binding sites could allow simultaneous binding of talin and kindlin to one integrin tail. However, sequential binding or binding to different integrin-tails and transactivation are also possibilities (Moser et al., 2009b).

The importance of kindlins for integrin activation and outside-in signaling is demonstrated by the severe consequences of kindlin loss-of-function *in vivo*.

Loss-of-function mutations in human Kindlin-1 were identified as cause of a rare genodermatosis known as Kindler syndrome, characterized by defects in epithelial cell-adhesion subsequently resulting in poikiloderma, cutaneous atrophy and susceptibility to skin cancer (Jobard et al., 2003; Siegel et al., 2003). Deletion of kindlin-1 in mice resulted in a similar skin phenotype, although intestinal defects were more pronounced and finally led to perinatal lethality due to severe ulcerative colitis (Ussar et al., 2008). In the meantime gastrointestinal abnormalities have also been reported in man.

Kindlin-2 deletion in mice resulted in early embryonic lethality at the peri-implantation stage due to severe endoderm and epiblast detachment from the basement membrane (Montanez et al., 2008).

Finally, the deletion of kindlin-3 in mice resulted in defective platelet aggregation and severe bleedings due to impaired integrin activation, although talin expression was unaltered (Moser et al., 2008). Furthermore, kindlin-3 was found to be required for leukocyte adhesion and extravasation (Moser et al., 2009a). In humans, kindlin-3 mutation leads to a rare disease known as leukocyte-adhesion deficiency type III (LAD-III) characterized by severe bleedings and leukocyte adhesion and extravasation defects (Kuijpers et al., 2009; Malinin et al., 2009; Mory et al., 2008; Svensson et al., 2009).

3.2) Integrin avidity and clustering

Affinity modulation is essential to control the binding of an integrin to its ligand. As individual integrin-ligand interactions are relatively weak, firm adhesion of a cell to the ECM requires the collective binding of multiple integrins. The synergistic effect of multiple weak interactions is known as avidity. Integrin avidity occurs by clustering integrins into adhesive units. In cultured cells, several types of adhesive units can be distinguished based on size, morphology, localization and protein composition. These include nascent adhesions (Choi et al., 2008), which can subsequently mature into focal complexes (FCs), FAs and fibrillar adhesions (Geiger et al., 2001). Podosomes and invadopodia are related but structurally distinct adhesive structures characteristic for monocytic and tumor cells, respectively (Linder, 2009).

A vast number of cytoplasmic adaptor and signaling molecules are recruited to and organized within these adhesive units, which not only mediate the linkage to the F (filamentous)-actin cytoskeleton but also function as a signaling platform, orchestrating complex intracellular responses and signaling crosstalks upon integrin ligand engagement. Collectively, these processes are referred to as outside-in signaling.

3.3) Outside-in signaling

Integrins regulate a vast number of cellular processes such as adhesion, migration, proliferation, survival and differentiation. However, their short cytoplasmic tails lack enzymatic activity. Instead, integrin signaling relies on the recruitment of signaling and adaptor molecules.

To date, the “integrin adhesome” comprises far over 180 molecules, which are found to be associated with integrin adhesions (Schiller et al., 2011; Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007). Among them, more than 40 proteins can bind directly, although not simultaneously, to β integrin cytoplasmic tails, while so far, only a few are known to directly interact with the cytoplasmic tails of α subunits (Legate and Fassler, 2009).
Recruitment and direct binding of adaptor and signaling molecules to the cytoplasmic tails creates a platform for the assembly of additional adaptor and signaling molecules and finally results in the formation of a highly complex and dynamic multimolecular adhesion and signaling machinery. Spatiotemporal control of assembly/disassembly and molecular composition as well as cell-type and developmental specific expression of its constituents additionally contribute to the complexity of this machinery.

3.3.1) Linkage to the actin cytoskeleton

One key function of the integrin adhesome is the linkage to and regulation of the actin cytoskeleton. The physical linkage to the actin cytoskeleton depends on and is mediated by the actin-binding capability of several members of the adhesome. Talin, α-actinin, filamin and tensin can directly link integrins to the actin cytoskeleton by binding to both the β integrin cytoplasmic tail and F-actin. However, firm linkage to the actin cytoskeleton additionally depends on actin-binding proteins indirectly associated with integrins via adaptor molecules. For instance, although talin can provide the initial connection to the actin cytoskeleton, vinculin recruitment to talin reinforces the linkage (Humphries et al., 2007; Legate et al., 2009). Vinculin is additionally connected to integrins by its association with paxillin, which in turn binds to the ILK-PINCH-Parvin (IPP) complex through a direct interaction with ILK and Parvin. ILK can also directly bind to β integrin cytoplasmic tails and through Parvin provides another crucial link to the actin cytoskeleton. Furthermore, ILK can bind to kindlin, which, by its association with migfilin, indirectly connects ILK to filamin and thus to the actin cytoskeleton. Taken together this exemplifies, that multiple proteins synergize to firmly link the actin cytoskeleton to integrins and that the individual components of the adhesome are highly interconnected. The IPP complex and especially α-Parvin are the main focus of this thesis and therefore will be discussed in more detail in a separate chapter.

4) Rho GTPases regulate actin cytoskeleton dynamics

The adhesome facilitates not only the physical anchorage of the actin cytoskeleton, but also regulates its dynamics. Key regulators of the actin cytoskeleton and its dynamics are members of the Rho family of small (~21kDa) GTPases (Rho GTPases). The Rho family is a subfamily of the Ras (rat sarcoma) superfamily and comprises more than 22 members in humans. The most prominent and best studied representatives are RhoA (Ras homologous), Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42). Like other small GTPases, most Rho GTPases cycle between an inactive (GDP-bound) and an active (GTP-bound) state and thereby function as molecular switches (Figure 3) (Bustelo et al., 2007). In the GTP-bound state, Rho GTPases specifically interact with diverse effector proteins to control not only cytoskeletal dynamics, but also many other essential cellular processes such as gene expression, membrane trafficking, microtubule dynamics, proliferation and cytokinesis (Heasman and Ridley, 2008; Jaffe and Hall, 2005).

Tight regulation of these processes requires the spatiotemporal control of Rho GTPases, which is mainly mediated by three classes of regulatory proteins: GEFs (guanine nucleotide exchange factors), GAPs (GTPase activating proteins), and GDIs (guanine nucleotide dissociation inhibitors) (Figure 3).

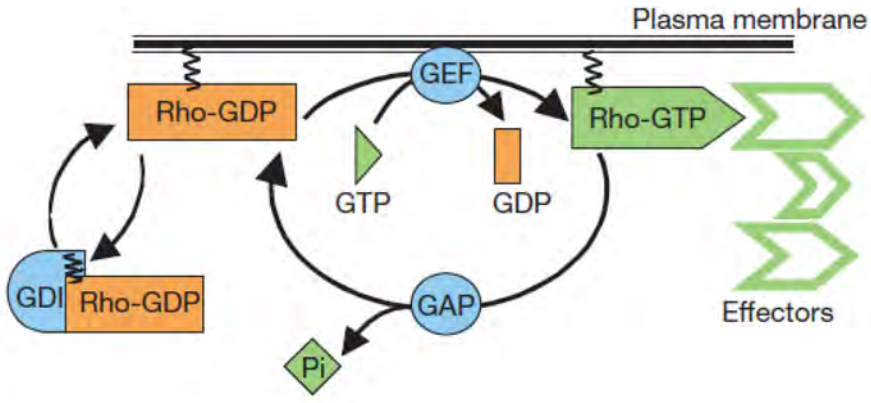

Figure 3: Cycling of Rho GTPases is controlled by GAPs, GEFs and GDIs

Cycling of Rho GTPases between an inactive GDP-bound and an active GTP-bound state is controlled by GEFs and GAPs. GDIs sequester Rho GTPase in an inactive state and negatively regulate their membrane association by masking a lipid moiety at the C-terminus of Rho GTPases. Figure taken from (Etienne-Manneville and Hall, 2002).

In humans, over 70 distinct GEFs are known, most of which belong to the Dbl (diffuse B-cell-lymphoma) family. GEFs facilitate the exchange of GDP for GTP and thus are required for the activation of Rho GTPases (Garcia-Mata and Burridge, 2007).

Similar to the large number of GEFs, around 80 GAPs are encoded in the human genome. GAPs inactivate Rho GTPases by enhancing their intrinsically inefficient GTPase activity (Garcia-Mata and Burridge, 2007; Moon and Zheng, 2003; Tcherkezian and Lamarche-Vane, 2007).

In contrast to GEFs and GAPs, only three GDIs have been identified in humans. GDIs inhibit the guanine nucleotide exchange and sequester Rho GTPases in an inactive state in the cytosol. Upon dissociation from the inhibitory GDIs, Rho GTPases can translocate to membranes and interact with GEFs, GAPs, and effector proteins. Membrane anchorage is facilitated by the post-translational isoprenylation at the C-terminus of most Rho GTPases. Binding of GDIs masks this hydrophobic lipid moiety and thus prevents membrane association of Rho GTPases (DerMardirossian and Bokoch, 2005; Wennerberg and Der, 2004).

Although Rho GTPases are primarily controlled by GEFs, GAPs and GDIs, accurate Rho signaling additionally requires tight regulation of the expression, stability, activity, localization and scaffolding of Rho GTPases, GEFs, GAPs, GDIs, effector proteins and upstream regulatory components in a spatiotemporal and cell context-dependent manner (Bustelo et al., 2007). Many GEFs and GAPs are controlled by a wide variety of regulatory mechanisms (Bos et al., 2007; Rossman et al., 2005; Schmidt and Hall, 2002; Tcherkezian and Lamarche-Vane, 2007). Both activity and subcellular localization of GEFs and GAPs are frequently determined by phosphorylation, binding of phospholipids, and/or interaction with regulatory and adaptor proteins. Additionally, GEFs and GAPs can function as scaffolding proteins and thereby couple the activity of Rho GTPases to specific effectors and downstream signaling pathways. Furthermore, a particular Rho GTPase often can be controlled by a number of different GEFs and GAPs, and although some GEFs and GAPs are specific for a certain Rho GTPase, others are more promiscuous. Cell type-specific expression of some GEFs and GAPs adds another layer of complexity to Rho GTPase signaling. The highly complex regulation of Rho GTPase signaling provides cells with the flexibility to coordinate and integrate actin cytoskeleton dynamics and other Rho-mediated processes with a variety of different stimuli and signaling pathways. Particularly for dynamic processes such as cell migration, Rho GTPase-mediated integration of actin cytoskeleton dynamics with integrin signaling and other signaling pathways is of utmost importance.

5) Actin cytoskeleton dynamics

The actin cytoskeleton is not a static entity, but is highly dynamic and flexible. Tight spatiotemporal control of its dynamics is essential for processes such as cell morphology, cell polarity, directional migration, cytokinesis, tissue homeostasis, wound healing, and embryonic development. Although Rho GTPases are key regulators of actin cytoskeleton dynamics, numerous additional proteins, collectively referred to as actin-binding proteins (ABPs), are required to facilitate the dynamic assembly and disassembly of actin fibers, their organization into higher order structures such as bundles or dendritic networks and the controlled rearrangement or breakdown of these structures.

Assembly of actin filaments requires the polymerization of globular actin monomers (G-actin) into F-actin, which generates the protrusive force required for cell migration. However, spontaneous de novo formation of actin filaments is kinetically hampered, due to the instability of short dimeric and trimeric actin intermediates. Instead, initiation of new actin filaments depends on and is regulated by ABPs, which can catalyze the rate limiting nucleation step and therefore are known as “nucleators”. The best characterized nucleators are the heptameric actin-related protein 2/3 (Arp2/3) complex, formins and spire (Firat-Karalar and Welch, 2011; Goley and Welch, 2006). Unlike formins and spire, which nucleate the linear actin filaments characteristic of protrusive finger-like structures known as filopodia, the Arp2/3 complex is thought to promote branched actin filaments by nucleating new daughter filaments at the side of existing mother filaments. Accordingly, the Arp2/3 complex is essential for the formation of lamellipodia, which are thin, sheet-like cellular protrusions at the leading edge of migrating cells, characterized by a branched network of actin-fibers. However, the existence of branched actin filaments in the lamellipodium was recently challenged and is debated (Insall, 2011; Koestler et al., 2008; Small, 2010; Urban et al., 2010). Alternatively, the lamellipodium might be comprised of cross-linked linear actin filaments. Once nucleated, the growth of actin filaments is kinetically favorable and eventually must be restricted. ABPs known as “capping proteins” can bind to the fast growing “barbed-ends” of actin filaments and thereby prevent further actin filament elongation. Conversely, proteins preventing the binding of capping proteins such as the diaphanous-related formin (DRF) mDia or Ena/Vasp (enabled/vasodilator-stimulated phosphoprotein), promote filament elongation (Ridley, 2006; Ridley et al., 2003). Other ABPs, including actin-depolymerizing factor (ADF)/cofilin, are critically involved in the disassembly and

reorganization of actin filaments. On the one hand, these proteins can sever and/or depolymerize actin fibers and thus facilitate actin fiber disassembly. On the other hand, they can also promote the assembly of new filaments by increasing the number of free barbed ends and the G-actin pool in the cell. Several ABPs also bind to G-actin and either inhibit actin polymerization by sequestering G-actin (e.g. thymosin β4), or promote F-actin formation by increasing the local availability of polymerization competent actin monomers (e.g. profilin). Finally, various ABPs, including α-actinin, filamin, fascin, and the molecular motor protein myosin II, can cross-link individual actin fibers and thereby facilitate the formation of more complex structures such as actin bundles and dendritic networks. Myosin II not only cross-links actin filaments, but also provides tension and contractility to the actin cytoskeleton. Myosin II is essential for the maturation of nascent adhesions and the formation of stress fibers, which are contractile bundles consisting of actin fibers cross-linked by bipolar myosin filaments and α-actinin (Vicente-Manzanares et al., 2009). Interestingly, although tension is known to promote the maturation of FA, the contractile properties of myosin II were found to be dispensable for initial adhesion maturation. Instead, the actin bundling activity of a motor-deficient myosin II mutant was sufficient to rescue the initial adhesion maturation in cells where myosin II or α-actinin had been depleted by RNA interference (RNAi) (Choi et al., 2008). Integrins play a major role in the complex organization and orchestration of actin cytoskeleton dynamics by establishing the physical linkage to the ECM and by controlling key signaling pathways, which frequently culminate in the activation or inhibition of Rho GTPases.

6) Regulation of actin cytoskeleton dynamics by Rho GTPases

Spatiotemporal control of actin cytoskeleton dynamics critically depends on and is mediated by Rho GTPases. Through their interaction with specific effector proteins, Rho GTPases not only control actin cytoskeleton dynamics and contractility but also regulate the formation and maturation of adhesion complexes. Although the constantly advancing knowledge about other Rho family members frequently reveals their important functions for actin cytoskeleton dynamics, RhoA, Rac1, and Cdc42 remain the best studied. Therefore, the discussion will be limited to these three prototypic members, which when activated, promote and regulate the

formation of prominent and morphologically distinct actin-based structures, namely lamellipodia (Rac1), filopodia (Cdc42) and stress fibers (RhoA).

6.1) RhoA promotes stress fiber formation and adhesion maturation

RhoA primarily promotes the maturation of nascent adhesions and the formation of stress fibers through its effectors Rho-associated serine/threonine kinase (ROCK) and the DRF mDia (Burridge and Wennerberg, 2004). ROCK is essential for the formation of stress fibers and FCs, and pharmacological inhibition of ROCK or expression of dominant-negative ROCK inhibits their formation (Riento and Ridley, 2003). Through the formin mDia, RhoA promotes polymerization, elongation, and bundling of linear actin filaments, whereas RhoA-mediated activation of ROCK results in increased phosphorylation of the regulatory light chain of myosin II (MLC) at Thr18 and Ser19, which activates the contractile and cross-linking functions of myosin II (Amano et al., 2010; Burridge and Wennerberg, 2004; Jaffe and Hall, 2005; Legate et al., 2009). ROCK primarily promotes MLC phosphorylation through the inhibition of MLC phosphatase (MLCP) by phosphorylating Thr696 and Thr853 of the regulatory myosin phosphatase-targeting subunit 1 (MYPT1). Although ROCK can directly phosphorylate MLC at Ser19 *in vitro*, the Ca^{2+}-dependent myosin light chain kinase (MLCK) might be the physiologically more relevant kinase *in vivo* (Amano et al., 2010; Jaffe and Hall, 2005). Additionally, the RhoA effector citron kinase also phosphorylates MLC at Thr18 and Ser19, although this may only play a role during cytokinesis (Burridge and Wennerberg, 2004).

ROCK not only controls MLC phosphorylation, but it also activates LIM (Lin11, Isl1, Mec3) kinase (LIMK). LIMK in turn phosphorylates cofilin at Ser3, resulting in the inactivation of cofilin and thus contributing to the stability of stress fibers (Jaffe and Hall, 2005).

However, RhoA controls stress fiber formation and adhesion maturation primarily through the regulation of myosin II activity. While assembly of nascent adhesions in the lamellipodium is myosin II-independent and instead depends on actin polymerization, adhesion maturation into FCs and FAs at the lamellipodium-lamellum interface is myosin II-dependent and depletion of myosin II by RNAi or pharmacological inhibition with blebbistatin prevents adhesion maturation and promotes the formation of nascent adhesions (Choi et al., 2008). Although the cross-linking activity of myosin II is sufficient for the initial maturation of nascent adhesions,

ATPase activity of myosin II contributes to adhesion maturation at later stages and is required for the formation of stress fibers and trailing edge retraction at the rear of migrating cells (Burridge and Wennerberg, 2004; Choi et al., 2008; Legate et al., 2009; Ridley et al., 2003; Riento and Ridley, 2003).

6.2) Rac1 and Cdc42 promote membrane protrusions at the leading edge of migrating cells

The formation of sheet-like membrane protrusions at the leading edge of migrating cells, known as lamellipodia, largely depends on, and is driven by, Arp2/3 complex-mediated actin polymerization. The Arp2/3 complex needs to be activated by nucleation promoting factors (NPFs) to enable efficient actin polymerization (Goley and Welch, 2006). Both Rac1 and Cdc42 can activate the Arp2/3 complex through members of the WASP/WAVE (Wiskott-Aldrich syndrome protein)/(WASP-family verprolin-homologous protein) protein families, which are class I NPFs (Goley and Welch, 2006). WAVE proteins together with ABI (Abelson-interacting protein), HSPC300 (haematopoietic stem-cell progenitor), Nap125 (Nck-associated protein), and PIR121 (p53-inducible mRNA) form a pentameric heterocomplex, referred to as the WAVE complex (Goley and Welch, 2006; Takenawa and Suetsugu, 2007). In the active state, Rac1 interacts with Nap125 and PIR121 and thereby activates the WAVE complex to stimulate Arp2/3 complex-mediated actin polymerization. Activation of the WAVE complex also involves IRSp53 (insulin-receptor substrate), which binds to both WAVE and Rac1, and additionally promotes the activity of the WAVE complex. Interestingly, IRSp53 binding to Cdc42 reduces the affinity of IRSp53 for WAVE (Goley and Welch, 2006; Jaffe and Hall, 2005; Takenawa and Suetsugu, 2007).

While Rac1 promotes Arp2/3 activity through the WAVE complex, WASP family members mediate the Cdc42-induced activation of the Arp2/3 complex. WASP proteins adopt an auto-inhibited conformation and direct binding of active Cdc42 is required for the activation of WASPs. Additionally, WASP proteins are thought to be inhibited by members of the WIP (WASP-interacting protein) family, including WIP, CR16 (corticosteroids and regional expression-16) and WICH (WIP- and CR16-homologous protein) (Takenawa and Suetsugu, 2007). Activation of the WASP-WIP complex is facilitated by the Cdc42 effector TOCA-1 (transducer of Cdc42-dependent actin assembly 1) and can be enhanced by phosphorylation of WASP through members of the Src family of tyrosine kinases.

Both Rac1 and Cdc42 also activate Ser/Thr kinases of the PAK (p21-activated kinase) family. PAKs phosphorylate and thereby activate LIMKs (Edwards et al., 1999), which results in the inhibition of cofilin. Additionally, PAKs are implicated in regulating myosin II activity. On the one hand, PAK-mediated phosphorylation of both MLCK and myosin heavy chain (MHC) decreases myosin II activity (Sanders et al., 1999; van Leeuwen et al., 1999). On the other hand, PAK can directly phosphorylate MLC, which increases myosin II activity (Chew et al., 1998).

Although Rac1 and Cdc42 use similar downstream signaling pathways to regulate the actin cytoskeleton, they promote morphologically distinct actin-based protrusive structures. While Rac1 promotes dendritic actin organization in lamellipodia, Cdc42 is thought to be the main mediator of the parallel linear actin filaments constituting filopodia. However, filopodia can also form in the absence of Cdc42, indicating that other Rho GTPases such as Rif (Rho in filopodia)/RhoF can compensate for Cdc42 function (Czuchra et al., 2005; Ridley, 2006). Furthermore, filopodia also form in the absence of either WASP (Snapper et al., 2001), the Arp2/3 complex or WAVE (Steffen et al., 2006), indicating that actin polymerization in filopodia neither depends on the formation of lamellipodia nor is mediated by the Arp2/3 complex but instead might be facilitated by other actin nucleators, such as formins, which in contrast to the Arp2/3 complex promote linear actin filaments (Schirenbeck et al., 2005). Indeed, Cdc42 and Rif bind to and activate the DRF mDia2, which also localizes to filopodia (Pellegrin and Mellor, 2005; Peng et al., 2003). RhoA also promotes actin polymerization through the DRF mDia1. Interestingly, although RhoA activity was thought to be restricted mainly to the cell body and the retracting rear, recent studies revealed that RhoA is also active at the leading edge and that its activity directly coincides with leading edge protrusion, whereas Cdc42 and Rac1 are activated 2µm behind the leading edge with a delay of approximately 40s (Machacek et al., 2009). Thus RhoA-mediated actin polymerization might initiate leading edge protrusion, whereas Rac1 and Cdc42 are required to sustain the protrusion (Spiering and Hodgson, 2011). This exemplifies, that tight spatiotemporal control of Rho GTPases and their effectors is essential for the complex orchestration of actin dynamics.

7) Integrin signaling contributes to the spatiotemporal control of Rho GTPases

Spatiotemporal control of Rho GTPases largely depends on the activity and localization of GEFs and GAPs. By controlling both the recruitment and activity of multiple GEFs and GAPs, integrins are critically involved in the regulation of Rho GTPases. Upon ligand engagement, integrins recruit non-receptor tyrosine kinases such as FAK (focal adhesion kinase) and members of the Src family of tyrosine kinases (SFK), which elicit multiple signaling pathways and critically contribute to the regulation of GEFs and GAPs (Huveneers and Danen, 2009). FAK recruitment to integrins and autophosphorylation at tyrosine 397 creates a high affinity binding site for the SH2 (Src-homology 2) domain of Src. Upon binding to FAK, Src subsequently trans-phosphorylates FAK on additional tyrosine residues, which fully activates the kinase activity of FAK and creates new binding sites for additional proteins (Huveneers and Danen, 2009; Mitra and Schlaepfer, 2006). This active FAK-Src complex facilitates the recruitment and phosphorylation of p130Cas, which in turn binds to Crk (v-crk sarcoma virus CT10 oncogene homolog) and thereby recruits a complex of Dock180 (180-kDa protein downstream of CRK) and ELMO1 (engulfment and motility 1), which serves as a GEF for Rac1. Additionally, the active FAK-Src complex promotes the phosphorylation of the adaptor protein paxillin, leading to the recruitment of a complex consisting of PAK, the ArfGAP PKL(paxillin-kinase linker)/GIT (G-protein-coupled receptor kinase interacting protein) and β-PIX (PAK-interacting exchange factor-beta), which is a GEF for Rac1 and Cdc42 (Huveneers and Danen, 2009). Interestingly, several studies also demonstrate an important function for β-Parvin in the regulation of α- and β-PIX (Filipenko et al., 2005; Matsuda et al., 2008; Mishima et al., 2004; Rosenberger et al., 2003). Finally, other Rac GEFs such as Vav and Tiam (T-cell lymphoma invasion and metastasis) are also regulated by SFK (Huveneers and Danen, 2009). Thus, integrin-mediated activation of the FAK-Src complex and recruitment of adaptor proteins such as β-Parvin, promote membrane protrusion by activating Rac1 and Cdc42 at sites of adhesion.

However, for efficient cell spreading and migration, protrusive and contractile activities need to be tightly balanced. This balance in part is facilitated by an extensive cross-talk between Rho GTPases. For instance, Rac1-mediated production of reactive oxygen species leads to the inactivation of phosphatases which otherwise inhibit p190RhoGAP-mediated RhoA inactivation. Conversely, through its effector ROCK, which phosphorylates and activates the

Rac1 GAP FilGAP, RhoA negatively influences Rac1 activity (Huveneers and Danen, 2009). The balance between contractility and protrusion also depends on FAK, which recruits and regulates not only multiple GAPs for RhoA, including p190RhoGAP, GRAF (GTPase regulator associated with FAK) and PSGAP (PH- and SH3-domain-containing RhoGAP) but also several Rho GEFs, such as PDZRhoGEF and p190RhoGEF (Schaller, 2010). Thus FAK essentially contributes to the balance between RhoA-mediated contractility and Rac1 facilitated protrusion by controlling both Rac1/Cdc42 and RhoA activities. In summary, integrin signaling profoundly affects the spatiotemporal coordination of actin cytoskeleton dynamics through Rho GTPases and thus integrates ECM derived mechano-chemical cues with the dynamic organization of the actin cytoskeleton. Although many more details than presented here already have been unraveled, precise understanding of how these complex processes are regulated in space and time, and how they are integrated with growth factor signaling and other signaling pathways still requires further extensive research.

8) The IPP complex

8.1) Formation of the IPP complex: identification, structure and expression of ILK, PINCH and Parvin

Integrin-mediated outside-in signaling depends on the recruitment of cytoplasmic adaptor and signaling molecules and is essential for the dynamic organization of the actin cytoskeleton and its linkage to integrin adhesions. Key components of the cytoplasmic integrin machinery are ILK, PINCH and Parvin, which together form the ternary IPP complex (Tu et al., 2001; Wu, 2001), whose assembly precedes its recruitment to integrin adhesions in mammalian cells (Zhang et al., 2002b) (Figure 4).

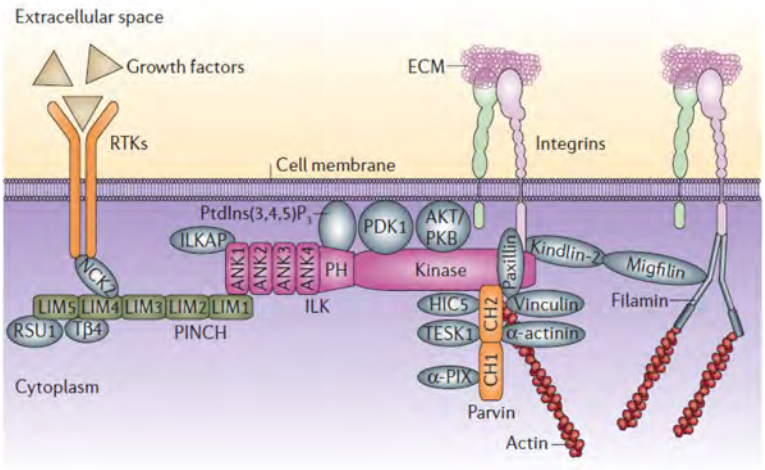

Figure 4: The IPP complex and its binding partners

Schematic representation of the IPP complex and some of its binding partners. Through Parvins, the IPP complex links integrins to the actin cytoskeleton. See text for details. Figure taken from (Legate et al., 2006)

ILK was identified in 1996 in a yeast two hybrid (Y2H) screen for β1 integrin-binding proteins (Hannigan et al., 1996), and was found to be ubiquitously expressed throughout development and adulthood (Li et al., 1997; Nikolopoulos and Turner, 2001; Sakai et al., 2003). ILK, which is conserved throughout the metazoan lineage (Bendig et al., 2006; Mackinnon et al., 2002; Yasunaga et al., 2005; Zervas et al., 2001), is composed of five ankyrin (ANK) repeats at its N-terminus (Chiswell et al., 2008) and a C-terminal Ser/Thr kinase-like domain (Hannigan et al., 1996). Additionally, a PH domain, which is believed to bind phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3), is interspersed between the N-terminal ANK repeats and the C-terminal kinase-like domain (Delcommenne et al., 1998; Pasquali et al., 2007).

In 1999, PINCH was identified as direct binding partner of ILK in an Y2H screen for proteins that bind to the ANK repeats of ILK (Tu et al., 1999; Wu, 1999). PINCH is a LIM-only protein, composed of five LIM domains (Rearden, 1994). PINCH is conserved among metazoans (Hobert et al., 1999) and in mammals, two highly homologous isoforms (82% identical at the amino acid sequence level in humans), have been identified (Rearden, 1994; Zhang et al., 2002a), referred to as PINCH-1 (also known as LIMS1) and PINCH-2 (LIMS2). In contrast to PINCH-1, which is ubiquitously expressed at embryonic and adult stages, PINCH-2 expression is slightly more restricted and absent in early embryonic development (Braun et al., 2003; Wickstrom et al., 2010b). Direct binding to ILK is facilitated by the first LIM domain of both PINCH-1 and PINCH-2 and the second ANK repeat of ILK (Li et al., 1999; Tu et al., 1999; Velyvis et al., 2001). However, recent structural data revealed that the

first LIM domain of both PINCH-1 and PINCH-2 binds in a highly similar and competitive manner not only to ANK 2 of ILK but also to ANK 3-5 and that ANK 4 provides the strongest contribution to the binding interface (Chiswell et al., 2010; Chiswell et al., 2008).

Parvin, the third member of the IPP complex, was identified in 2000 in a screen for paxillin LD1 (leucine-rich sequence) domain binding proteins and at that time was termed actopaxin due to its ability to bind to both paxillin and actin (Nikolopoulos and Turner, 2000). Subsequently, Y2H screening for novel ILK-binding partners and expressed sequence tag (EST)-database mining for proteins with homology to the actin-binding domain of α-actinin, revealed that Parvins form an evolutionary conserved family of ILK-binding proteins, with three members in mammals and a single isoform in the invertebrates *Caenorhabditis* and *Drosophila*, whereas the unicellular organisms *Dictyostelium* and *Saccharomyces* lack recognizable Parvin isoforms (Olski et al., 2001; Tu et al., 2001; Yamaji et al., 2001). The three mammalian isoforms are referred to as α-Parvin (also known as actopaxin or CH-ILKBP (CH domain-containing ILK-binding protein)), β-Parvin (also known as affixin) and γ-Parvin. While α-Parvin and β-Parvin are closely related (74% identity and 85% similarity), γ-Parvin is more divergent and only shares 42% identity and 67% similarity with α-Parvin. Structurally, Parvins are characterized by the presence of two calponin homology domains (CH1 and CH2) in the C-terminal region, which are separated by a linker-sequence of about 60 aa. Particularly the CH2 domain, which facilitates the direct binding of all three isoforms to the kinase-like domain of ILK (Fukuda et al., 2009; Tu et al., 2001; Yamaji et al., 2001; Yoshimi et al., 2006), is highly homologous, with 84% identity and 94% similarity between α-Parvin and β-Parvin, and 50% identity and 71% similarity between α-Parvin and γ-Parvin. The CH1-domain is preceded by an N-terminal region with little homology between the three isoforms. Two putative nuclear localization signals (NLS) and three potential SH3 (Src homology 3)-binding sites are situated in this region in both α-Parvin and β-Parvin but not in γ-Parvin (Olski et al., 2001). While α-Parvin is ubiquitously expressed, β-Parvin expression is enriched in heart and skeletal muscle, and γ-Parvin expression is restricted to the haematopoietic system (Chu et al., 2006; Olski et al., 2001; Tu et al., 2001; Yamaji et al., 2001).

8.1.1) Distinct IPP complexes assemble in mammalian cells

The binding of the different Parvin and PINCH isoforms to ILK is mutually exclusive and their partially overlapping expression patterns potentially permits the assembly of up to six molecularly and functionally distinct IPP complexes (Fukuda et al., 2003a; Montanez et al., 2009; Zhang et al., 2002a). Thus the ternary IPP complex in mammalian cells can consist of ILK, connected via its N-terminal ANK-repeats to the first LIM-domain of either PINCH-1 or PINCH-2, and via its C-terminal kinase-like domain to the second CH-domain either of α-, β-, or γ-Parvin. However, it is not clear if all combinations are physiologically relevant, or if there is a preference for certain IPP complex combinations.

It has been shown that the stability of IPP constituents critically depends on the assembly of a complete complex; depletion of either ILK, PINCH or Parvin results in proteasome-dependent degradation of the two remaining constituents, which complicates functional analyses of individual IPP members (Fukuda et al., 2003a; Li et al., 2005a). However, proteasomal degradation is not complete and the presence of residual amounts of IPP constituents suggests potential IPP-independent functions for the individual IPP members.

Although overexpression or up-regulation of the alternative PINCH or Parvin isoforms generally restores the protein levels of the IPP complex and is sufficient for its recruitment into adhesion complexes, this does not always functionally compensate for the loss of a particular Parvin or PINCH isoform (Wickstrom et al., 2010b).

For instance, whereas depletion of β-Parvin in vSMCs can be fully compensated by α-Parvin, β-Parvin is unable to functionally compensate for the loss of α-Parvin in these cells, even though expression of β-Parvin is elevated upon α-Parvin deletion and is sufficient to rescue protein-levels and recruitment of ILK and PINCH to FAs. However, β-Parvin is sufficient to functionally compensate for the loss of α-Parvin in fibroblasts (Montanez et al., 2009). Similarly, whereas depletion of PINCH-2 can be fully compensated by PINCH-1, overexpression of PINCH-2 in HeLa (Henrietta Lacks) cells is unable to functionally compensate for the loss of PINCH-1, despite stabilizing the protein levels of ILK and Parvin (Fukuda et al., 2003a). In contrast, PINCH-2 compensates for the loss of PINCH-1 in cardiomyocytes (Liang et al., 2009; Liang et al., 2005), and artificial expression of PINCH-2 functionally compensates for the depletion of PINCH-1 in mouse embryonic fibroblasts (MEFs) (Stanchi et al., 2005). Taken together, this indicates overlapping as well as cell-type- and isoform-specific functions of distinct IPP complexes, which likely depend on the recruitment and interaction with shared and isoform and/or cell-type specific binding partners.

8.2) IPP interaction partners

8.2.1) ILK-associated proteins

Apart from binding to Parvins and PINCHs, ILK either directly or indirectly associates with a variety of additional proteins. Since there are no alternative isoforms of ILK, direct ILK-binding partners may be shared by all the distinct IPP complexes. However, direct interaction with ILK was not confirmed for all ILK-associated molecules and thus might depend on the presence of specific PINCH and/or Parvin isoforms. Additionally, direct interactions might be stabilized by the simultaneous binding to both ILK and specific PINCH or Parvin isoforms, and thus might be favored by certain IPP complexes. Paxillin, for instance, has been shown to bind to ILK as well as to α- and γ-Parvin, but not to β-Parvin (Nikolopoulos and Turner, 2000; Nikolopoulos and Turner, 2001; Yoshimi et al., 2006). Similarly, thymosin-β4 binds to both ILK and PINCH-1, whereas its interaction with PINCH-2 has not been reported (Bock-Marquette et al., 2004; Fan et al., 2009).

Direct ILK binding partners, either shown by Y2H experiments, interaction of recombinant proteins or co-crystallization studies, include β1 and β3 integrins (Hannigan et al., 1996; Pasquet et al., 2002; Yamaji et al., 2002), paxillin (Nikolopoulos and Turner, 2001; Nikolopoulos and Turner, 2002), thymosin β4 (Bock-Marquette et al., 2004; Fan et al., 2009), ELMO-2 (Ho et al., 2009), EphA1 (Yamazaki et al., 2009), kAE1 (kidney anion exchanger) (Keskanokwong et al., 2007), the serine/threonine phosphatase ILKAP (ILK-associated phosphatase) (Leung-Hagesteijn et al., 2001), PKB (protein kinase B)/Akt (McDonald et al., 2008; Persad et al., 2001), Rictor (McDonald et al., 2008), Src (Kim et al., 2008) and the muscle LIM protein (MLP/CRP3) (Postel et al., 2008).

Additionally, *Caenorhabditis* UNC-112/kindlin-2 directly binds to ILK, suggesting that the ILK-kindlin-2 interaction in mammalian cells, observed by co-immunoprecipition of kindlin-2 and ILK, also might be direct (Mackinnon et al., 2002; Montanez et al., 2008).

α- and β-tubulin as well as the tubulin binding proteins ch-TOG, RUVBL1, (Dobreva et al., 2008; Fielding et al., 2008) and IQGAP (Wickstrom et al., 2010a) also have been shown to be associated with ILK. However, evidence for their direct interaction with ILK has not been reported.

Thus, the IPP complex, through the plethora of ILK interactions, can connect integrins not only to regulators of the actin and microtubule cytoskeleton but also to essential signaling pathways downstream of Src, PKB/Akt and Ephrins.

8.2.2) PINCH-associated proteins

PINCH-1 or PINCH-2 specific interactions could provide signaling specificity to distinct IPP complexes. Although several binding partners of PINCH-1 have been described, no PINCH-2 interactors have been reported so far. Among the identified PINCH-1 binding partners is the SH2- and SH3-containing adaptor protein Nck-2, which directly binds via its third SH3-domain to the LIM4 domain of PINCH-1. Mutations that interfere with the PINCH-1-Nck-2 interaction negatively affect both the recruitment of PINCH-1 into FAs and the organization of the actin cytoskeleton (Vaynberg et al., 2005; Velyvis et al., 2003). Through its SH3 domains, Nck-2 also associates with IRS-1, whereas its SH2 domain facilitates the interaction with growth factor receptors such as PDGFRβ (platelet derived growth factor receptor β). Interestingly, Nck-2 also associates with PAK, WASP and DOCK180. Thus, Nck-2 connects the IPP complex to both growth factor receptor signaling and mediators of actin cytoskeleton dynamics. Another PINCH-1-specific interaction partner, which binds to the LIM5 domain of PINCH-1, is the Ras-suppressor protein RSU-1, which negatively regulates Rac1 and JNK (c-Jun N-terminal kinase) signaling (Dougherty et al., 2005; Kadrmas et al., 2004; Legate et al., 2006).

Additionally, the G-actin binding and sequestering protein thymosin-β4 binds to LIM4 and LIM5 of PINCH-1, promoting cardiomyocyte migration and survival (Bock-Marquette et al., 2004). PINCH-2 can functionally compensate for the loss of PINCH-1 in ventricular cardiomyocytes (Liang et al., 2005), indicating that PINCH-2 might also be able to bind to thymosin-β4, although this needs to be experimentally verified.

Finally, PINCH-1 associates and negatively regulates the phosphatase PP1α, and thus indirectly promotes phosphorylation and activation of PKB/Akt. Association of PP1α and PINCH-1 depends on a KFVEF-motif in the LIM5-domain of PINCH-1 (Eke et al., 2010), which is conserved in PINCH-2, raising the possibility that PINCH-2 might also be able to associate with PP1α (Braun et al., 2003).

Since most studies focused on PINCH-1 and did not explicitly exclude a possible binding of the identified PINCH-1 interactors to PINCH-2, a direct comparison of the PINCH-1 and

PINCH-2 interactomes is required to determine whether binding partners are specific or shared between PINCH isoforms.

8.2.3) Parvin-associated proteins

The most prominent structural feature of Parvins is the presence of two tandemly arranged CH domains in the C-terminal region. Through the second CH domain, Parvins bind to the kinase-like domain of ILK, which is the only binding partner known to be shared by all three mammalian isoforms.

The tandem arrangement of two CH domains serves as the actin-binding domain (ABD) in a variety of actin binding proteins, including α-actinin, spectrin, filamin, plectin, dystonin, dystrophin, and utrophin (Gimona et al., 2002). Although Parvin CH domains diverge considerably from the typical type-1 and type-2 CH domains and are therefore classified as type-4 and type-5 CH domains (Gimona et al., 2002), they also can facilitate actin binding (Olski et al., 2001). However, direct binding to actin was verified only for α-Parvin (Olski et al., 2001) and could not be confirmed for β-Parvin and γ-Parvin (Yamaji et al., 2004; Yamaji et al., 2001; Yoshimi et al., 2006). Instead, β- and γ-Parvin might indirectly link the IPP complex to the actin cytoskeleton by binding directly to α-actinin (Yamaji et al., 2004; Yoshimi et al., 2006), which α-Parvin does not bind (Nikolopoulos and Turner, 2002). Binding of β-Parvin to α-actinin depends on the CH2 domain and the linker sequence between the two CH-domains (Yamaji et al., 2004). In contrast to β-Parvin, both α-Parvin and γ-Parvin, via their CH2-domains also bind to paxillin, which through its interaction with vinculin provides an additional link to the actin cytoskeleton, and profoundly affects actin cytoskeletal dynamics by recruiting important actin-regulators such as the GIT-Pix-PAK complex (Nayal et al., 2006; Nikolopoulos and Turner, 2000; Yamaji et al., 2004; Yoshimi et al., 2006). Additionally, α-Parvin also binds to the paxillin family member Hic-5 (Lorenz et al., 2008; Nikolopoulos and Turner, 2000). In contrast to α-Parvin, both β- and γ-Parvin directly bind to α-Pix through their CH1 domains (Mishima et al., 2004; Rosenberger et al., 2003; Yoshimi et al., 2006), and β-Parvin additionally can bind to β-PIX (Matsuda et al., 2008). Furthermore, through its CH1 domain, β-Parvin binds to dysferlin and both proteins co-localize at the sarcolemma of skeletal muscles, suggesting a potential function of β-Parvin in membrane repair (Matsuda et al., 2005). Based on luciferase-complementation assays, β-Parvin was recently suggested to interact with PKB/Akt, and thereby interfering with the

interaction between ILK and PKB/Akt (Kimura et al., 2010). Two additional binding partners for α-Parvin have been identified. However, it is not known, whether they also can bind to β- and/or γ-Parvin. The first one is CdGAP, a Cdc42- and Rac-specific GAP that concentrates at the ends of actin stress fibers. Binding to CdGAP is mediated by the N-terminus of α-Parvin and critically depends on residues 21-25 of α-Parvin (LaLonde et al., 2006).
The second one is the Ser/Thr kinase TESK1 (testicular protein kinase 1). Through its C-terminus α-Parvin directly binds to and thereby inhibits TESK1, which, when active, phosphorylates and inhibits cofilin. Interestingly, the binding of ILK and TESK1 to α-Parvin seems to be mutually exclusive and might be regulated through N-terminal phosphorylation of α-Parvin at Ser4 and Ser 8. Adhesion to FN promotes the phosphorylation of α-Parvin at these residues and negatively affects its association with TESK1, which finally results in inactivation of cofilin and stabilization of actin fibers (LaLonde et al., 2005).

8.3) Functions of the IPP complex

Many of the IPP interactors are involved in the linkage and/or regulation of the actin cytoskeleton. Accordingly it is not surprising that the IPP complex plays a fundamental role in the organization and regulation of the actin cytoskeleton and its dynamics, with profound effects on cell-adhesion, spreading and migration. Furthermore, several studies indicate an important function of the IPP complex for cell survival.

8.3.1) *In vitro* functions

In vitro studies in HeLa cells identified distinct functions of α- and β-Parvin in the regulation of the actin cytoskeleton, cell spreading, migration and survival. RNAi-mediated depletion of α-Parvin in HeLa cells stimulates Rac activation and cell spreading, whereas cell spreading is reduced upon knockdown of β-Parvin (Zhang et al., 2004). This antagonistic effect might be explained by the distinct binding partners of α- and β-Parvin. While α-Parvin binds to the Rac/Cdc42 GAP CdGAP, which negatively regulates cell spreading by inactivating Rac (LaLonde et al., 2006), β-Parvin stimulates Rac activity by binding to the Rac/Cdc42 GEF α-Pix (Mishima et al., 2004). However, Erk (extracellular signal-regulated protein kinase) dependent phosphorylation of α-Parvin or expression of a phosphomimetic mutant form of α-

Parvin (Ser4/8Asp), promote cell spreading and migration (Clarke et al., 2004). Interestingly, the phosphomimetic α-Parvin mutant exhibits impaired binding to TESK1, resulting in activation of TESK1, cofilin inhibition and stabilization of actin fibers (LaLonde et al., 2005). Additionally, α-Parvin is required to facilitate membrane translocation of PKB/Akt, and thus promotes cell survival by protecting cells from apoptosis (Fukuda et al., 2003b), whereas overexpression of β-Parvin promotes apoptosis (Zhang et al., 2004). Interestingly it has been reported, that β-Parvin negatively regulates the ILK-PKB/Akt interaction, which might explain the pro-apoptotic effect of β-Parvin in HeLa cells (Kimura et al., 2010). Although studies in HeLa cells have revealed distinct functions for α- and β-Parvin, it appears that the specific functions of the Parvin-isoforms critically depend on the cell context.

Overexpression of PINCH-2 in HeLa cells interferes with the PINCH-1-ILK interaction and results in reduced cell spreading and migration (Zhang et al., 2002a). Additionally, similar to α-Parvin, PINCH-1 is crucial for cell survival. Phosphorylation of PKB/Akt at both Ser473 and Thr308 is required for the activation of PKB/Akt and depletion of PINCH-1 results in reduced phosphorylation of both residues in HeLa cells. PINCH-1 might regulate the phosphorylation and activation of PKB/Akt indirectly by binding and inhibiting PP1α (Eke et al., 2010). Additionally, PINCH-1 has been shown to prevent activation of the intrinsic apoptosis pathway by negatively regulating Bim through Erk (Chen et al., 2008). Although increased expression of PINCH-2 is sufficient for stabilization and recruitment of ILK and α-Parvin to FAs, PINCH-2 fails to compensate for the defects in PKB/Akt phosphorylation, survival, spreading and migration in PINCH-1-depleted HeLa cells (Fukuda et al., 2003a). However, PINCH-2 can functionally compensate for the loss of PINCH-1 in cardiomyocytes (Liang et al., 2009; Liang et al., 2005) and in MEFs (Stanchi et al., 2005), indicating that the downstream signaling pathways of PINCH isoforms are also cell context dependent.

Similar to the depletion of PINCH-1, depletion of ILK in HeLa cells also impairs cell spreading, migration and survival. In contrast to the depletion of PINCH-1, depletion of ILK in these cells only reduced phosphorylation of PKB/Akt on Ser473, without affecting Thr308 phosphorylation (Fukuda et al., 2003a). Similarly in fibroblasts, deletion of ILK results in defects in cell adhesion, spreading and migration. Additionally, formation of stress fibers and maturation of FAs into fibrillar adhesions are impaired in ILK-deficient fibroblasts. Fibrillar adhesions are essential for the assembly and organization of the FN matrix. Accordingly, FN matrix assembly is hampered in ILK-deficient fibroblasts. Unlike in HeLa cells, deletion of ILK in fibroblasts diminishes their proliferation rate but does not result in altered phosphorylation of PKB/Akt and apoptosis (Sakai et al., 2003; Stanchi et al., 2009).

8.3.2) ILK is a pseudokinase

Although the C-terminal kinase-like domain of ILK has significant sequence homology to Ser/Thr protein kinases, essential residues required for kinase activity are not conserved in ILK (Hanks et al., 1988). Nevertheless, several studies showed Ser/Thr kinase activity towards several substrates including the cytoplasmic tail of β1 integrin (Hannigan et al., 1996), PKB/Akt, GSK3β (glycogen-synthase kinase-3β), MLC, MYPT1, α-NAC (nascent-polypeptide-associated complex and co-activator-α), Myelin basic protein, CPI-17 (protein kinase C-dependent phosphatase inhibitor of 17 kDa), PHI-1 (phosphatase holoenzyme inhibitor-1), and β-Parvin (Legate et al., 2006). However, the putative kinase activity of ILK has been a matter of controversy and there are striking arguments against ILK being a functional kinase under physiological conditions (Boudeau et al., 2006). First, in *Drosophila* and *Caenorhabditis*, the absence of ILK results in muscle detachment and embryonic lethality, but expression of kinase-dead versions of ILK are sufficient to completely restore the wild-type phenotype. This indicates that the putative kinase activity, at least in invertebrates, is dispensable *in vivo*. Furthermore, the phenotypes of PKB/Akt or GSK3β (or downstream signaling molecule β-catenin) deficient mutants, both suggested to be important downstream targets of ILK, differ strikingly from the phenotype of ILK deficient mutants (Mackinnon et al., 2002; Zervas et al., 2001). Second, although Ser473 phosphorylation of PKB/Akt is impaired in heart, skeletal muscle, Schwann cells, and macrophages in the absence of ILK (Pereira et al., 2009; Troussard et al., 2003; Wang et al., 2008; White et al., 2006), phosphorylation of PKB/Akt and/or GSK3β is not altered when ILK is deleted in fibroblasts, chondrocytes, or keratinocytes (Grashoff et al., 2003; Lorenz et al., 2007; Sakai et al., 2003). This indicates that the putative kinase activity of ILK is only required in certain cell-types, if at all. Third, mice carrying different point-mutations (R211A, S343A or S343D) in the ILK-gene, which have been reported to result in kinase-dead or hyperactive versions of ILK, are phenotypically normal and without obvious alterations in PKB/Akt or GSK-3β phosphorylation. This clearly excludes an essential function of the putative ILK-kinase activity *in vivo* (Lange et al., 2009). Only when the presumptive ATP-binding site is mutated (K220A/M) mice develop kidney agenesis/dysgenesis and die shortly after birth. However, neither phosphorylation of PKB/Akt or GSK-3β *in vivo* nor phosphorylation of MBP *in vitro* was impaired by these mutations. Thus, loss of the putative ILK-kinase activity cannot

explain the observed phenotype. Instead, the K220A/M mutations strikingly and specifically interfere with the binding of α-Parvin to ILK, suggesting that this interaction is crucial for kidney development. Indeed, loss of α-Parvin results in similar kidney defects (Lange et al., 2009; Montanez et al., 2009). Finally, the high-resolution crystal structure of the ILK kinase-like domain clearly demonstrates, that ILK is a pseudokinase unable to hydrolyze ATP, and that the pseudoactive site is essential for the ILK-α-Parvin interaction (Fukuda et al., 2009).
Collectively, these data identify ILK as a pseudokinase. ILK-associated changes in PKB/Akt phosphorylation and other potential substrates are likely indirect and depend on ILK's function as an essential scaffolding protein to locally orchestrate signaling networks downstream of integrins.

8.3.3) *In vivo* functions

8.3.3.1) Invertebrates

Studies in invertebrates, which only possess a limited number of integrins and IPP components, clearly demonstrate that the IPP complex is essential for the linkage of integrins to the actin cytoskeleton.
In *Caenorhabditis*, orthologues of β-integrin (PAT-3), ILK (PAT-4), PINCH (UNC-97), and Parvin (PAT-6) co-localize in FA-like structures known as dense bodies and M-lines. Dense bodies and M-lines are sites of muscle attachment, where actin- and myosin-filaments are connected to the basal sarcolemma and the underlying basement membrane (BM). Genetic deletion of β integrin or any of the IPP constituents impairs dense body and M-line assembly and results in a PAT (paralyzed and arrested at the twofold stage) phenotype, which is defined by muscle detachment from the body wall and embryonic lethality (Lin et al., 2003; Mackinnon et al., 2002; Norman et al., 2007).
The *Drosophila* orthologues of β integrin (βPS), ILK and PINCH, also co-localize at muscle attachment sites and at basal junctions of the wing epithelium. Loss-of-function mutations or deletion of either β integrin, ILK or PINCH, results in embryonic lethal muscle detachment from the body wall and blister formation in the wings of adult chimeras due to defective cell-adhesion (Brown, 1994; Clark et al., 2003; Zervas et al., 2001). However, the cell adhesion defects differ between β integrin and the ILK or PINCH mutants. Loss of β integrin impairs

the linkage between the cell membrane and the ECM, whereas loss of ILK or PINCH leads to the detachment of actin filaments from the plasma-membrane (Clark et al., 2003; Zervas et al., 2001). In contrast to ILK mutants, β integrin and PINCH mutants additionally exhibit defects in midgut morphogenesis and dorsal closure, which indicates that cell migration is compromised in these mutants, but not in ILK mutants (Brown, 1994; Kadrmas et al., 2004; Zervas et al., 2001). Interestingly, PINCH negatively regulates JNK activity by binding to RSU-1 and thereby integrates integrin and JNK signaling, which is essential for dorsal closure (Kadrmas et al., 2004). Thus in *Drosophila*, ILK might be dispensable for cell migration, whereas β integrin and PINCH are critically required, suggesting that β integrin and PINCH also can function in an ILK-independent manner. Indeed, in contrast to mammalian IPP complexes, individual IPP constituents can be recruited to adhesion complexes independently both in *Drosophila* and *Caenorhabditis* (Clark et al., 2003; Lin et al., 2003; Zhang et al., 2002b), suggesting IPP complex-independent functions in these organisms.

8.3.3.2) Vertebrates

8.3.3.2.1) Zebrafish

The essential function of the IPP complex *in vivo* has also been demonstrated in zebrafish (*Danio rerio*). In the zebrafish heart and skeletal muscle, ILK, PINCH and β-Parvin co-localize at sarcomeric Z-disks and costameres and are part of the cardiac mechanical stretch sensor. Antisense-mediated depletion either of ILK, PINCH-1, PINCH-2, or β-Parvin, phenocopies the embryonic lethal ILK-(L308P)-mutant *main squeeze* (MSQ), which is characterized by a progressive reduction of cardiac contractility. Although binding of β-Parvin to ILK is impaired in MSQ mutants, β-Parvin still localizes to costameres of cardiomyocytes and skeletal muscle, whereas depletion either of β-Parvin, PINCH-1, or PINCH-2 results in severe reduction of ILK protein levels. Loss of β-Parvin is accompanied by reduced phosphorylation of PKB/Akt at Ser 473 and the concomitant decrease of stretch-responsive atrial natriuretic factor (ANF) and vascular endothelial growth factor (VEGF) expression. Overexpression of VEGF or constitutively active PKB/Akt, reconstitutes the cardiac function, indicating that the β-Parvin- IPP complex-dependent regulation of PKB/Akt signaling plays an essential role in the zebrafish heart. Although it was reported in these

studies, that the cardiac and skeletal muscle ultrastructure was unaffected by the loss of ILK, PINCH-1/2 or β-Parvin (Bendig et al., 2006; Meder et al., 2011), a different study provided evidence for the detachment of the sarcolemma from the ECM following increased mechanical force in skeletal muscles of ILK- or β-Parvin-depleted zebrafish (Postel et al., 2008). Thus, the IPP complex not only regulates PKB/Akt-signaling but also is required to reinforce the integrin-actin linkage in this organism.

8.3.3.2.2) Mammals

In mice, genetic deletion either of β1 integrin (Fassler and Meyer, 1995; Stephens et al., 1995), ILK (Sakai et al., 2003), PINCH-1 (Li et al., 2005a; Liang et al., 2005), or α-Parvin (Montanez et al., 2009), results in embryonic lethality, whereas mice lacking PINCH-2, β-Parvin, γ-Parvin or both β- and γ-Parvin are phenotypically normal, likely due to compensation by PINCH-1 or α-Parvin, respectively (Chu et al., 2006; Montanez et al., 2009; Stanchi et al., 2005).

Embryos lacking β1 integrin and ILK die between day 5.5 and 6.5 of embryonic (E5.5-E6.5) development, whereas PINCH-1 deleted embryos survive slightly longer, until E6.5-E7.5. PINCH-2 is not expressed at this early developmental stage and thus cannot compensate for the loss of PINCH-1 (Braun et al., 2003).

The early lethality at the peri-implantation stage resulting from deletion of β1 integrin, ILK, or PINCH-1, complicates functional analyses. Embryoid bodies (EB) can recapitulate most aspects of early embryonic development and therefore have been employed to study the consequences of β1 integrin, ILK and PINCH-1 deletion. These studies revealed that β1 integrin is required for Ln secretion and assembly of the BM (Aumailley et al., 2000; Li et al., 2002), whereas secretion of Ln was unaffected in EBs lacking ILK or PINCH-1. However, in the absence of ILK or PINCH-1, EBs failed to form an amniotic cavity, displayed a distorted epiblast polarization, abnormal localization of F-actin, and impaired adhesion of primitive endoderm and epiblast to the BM (Li et al., 2005a; Sakai et al., 2003). These defects were more pronounced in EBs lacking ILK compared to PINCH-1, which may explain the slightly longer survival of PINCH-1 deficient embryos. Additionally, cell-cell adhesion and endoderm survival was impaired in EBs lacking PINCH-1 but not in EBs lacking ILK, suggesting an IPP-independent function of PINCH-1 in these processes (Li et al., 2005a).

While deletion of β1 integrin, ILK, or PINCH-1 results in death at the peri-implantation stage, embryos lacking α-Parvin develop normally until E9.5, but die between E11.5 and E14.5 from severe cardiovascular defects (Montanez et al., 2009). Additionally, these embryos show severe kidney agenesis/dysgenesis due to impaired ureteric bud (UB) invasion into the metanephric mesenchyme (MM) (Lange et al., 2009). This suggests that β-Parvin can compensate for the loss of α-Parvin during early mouse development, but fails to substitute for α-Parvin-specific functions at later stages.

Although β-Parvin is expressed in vSMCs and is sufficient to rescue protein-levels and adhesion recruitment of ILK and PINCH, β-Parvin cannot functionally compensate for the loss of α-Parvin in these cells. Without α-Parvin, vSMCs are hypercontractile and fail to establish a persistent leading edge due to elevated RhoA-ROCK signaling and enhanced phosphorylation of MLC. Although the random motility of these cells is increased, their directional migration is severely impaired, resulting in the inefficient recruitment of vSMCs to endothelial tubes and insufficient coverage of blood-vessels. Accordingly, remodeling of blood-vessels is rudimentary in the absence of α-Parvin, and is accompanied by the formation of micro-aneurysms, dilations and rupture of vessels, hemorrhages and edemas (Montanez et al., 2009).

Although constitutive deletion of β1 integrin, ILK, PINCH-1 and α-Parvin demonstrate that they are essential for mammalian development, analysis of their functions in adult animals and/or in specific organs is precluded due to embryonic lethality. Tissue-specific deletion and knock-in strategies to introduce specific point-mutations have been employed to overcome this limitation.

Similar to the constitutive ablation of α-Parvin, vSMC-specific deletion of ILK also results in increased RhoA activity and ROCK/MLC-dependent hypercontractility of vSMCs. Embryos lacking ILK specifically in vSMCs die around E18.5 due to severe vascular defects, including micro-aneurysms, inefficient recruitment of vSMCs to the vessel-wall, insufficient coverage and stabilization of blood-vessels, dilation and rupture of vessels as well as hemorrhages and edemas (Kogata et al., 2009).

Tissue-specific ablation of ILK in Schwann cells also leads to Rho-mediated hypercontractility in these cells, leading to defective myelination of axons (Pereira et al., 2009). Additionally, mice harboring a point mutation in the potential ATP-binding site of ILK, which specifically abrogates the ILK-α-Parvin interaction, develop kidney defects similar to mice lacking α-Parvin and die shortly after birth. Collecting duct epithelial cells from these mice display enhanced contractile properties and increased random migration but

are impaired in their directional migration, indicating that the ILK-α-Parvin interaction is required for persistent migration in these cells, possibly by negatively regulating RhoA activity (Lange et al., 2009). Collectively, these studies indicate that the α-Parvin plays an essential role in the regulation of RhoA activity in certain cell-types. Although vSMC-specific deletion of β1 integrin, similar to the loss of ILK and α-Parvin, also leads to vascular leakage and instability, these defects result from impaired differentiation and abnormal proliferation of vSMCs but are not due to increased MLC phosphorylation and hypercontractility of vSMCs (Abraham et al., 2008). This is consistent with a loss of the RhoA/ROCK-promoting function of α5β1 integrins upon deletion of β1 integrin. Thus, β1 integrin signaling promotes RhoA/ROCK signaling, whereas the α-Parvin is required to prevent excessive RhoA activity in certain cell types.

One possibility is that the α-Parvin directly counteracts the RhoA/ROCK promoting function of α5β1 integrins by recruiting or activating Rho GAPs and/or by displacing or inhibiting Rho GEFs. Alternatively, the α-Parvin-IPP complex could be required for αvβ3 integrin mediated signaling, which promotes Rac activation and negatively regulates β1 integrin recycling to the membrane (Danen et al., 2002; Danen et al., 2005).

Constitutive and conditional deletion of integrins and members of the IPP complex have identified important functions in a broad variety of tissues and cell-types, including skeleton/chondrocytes (Aszodi et al., 2003; Grashoff et al., 2003; Terpstra et al., 2003), heart/cardiomyocytes (Liang et al., 2009; Liang et al., 2005; Shai et al., 2002; White et al., 2006), vasculature/endothelial cells (Friedrich et al., 2004) and vSMCs (Abraham et al., 2008; Kogata et al., 2009; Montanez et al., 2009), skeletal muscle/myocytes (Gheyara et al., 2007; Wang et al., 2008), skin/keratinocytes (Brakebusch et al., 2000; Lorenz et al., 2007; Nakrieko et al., 2008; Raghavan et al., 2000), nervous system/neurons, Schwann cells and neural crest cells (Breau et al., 2006; Niewmierzycka et al., 2005; Pereira et al., 2009), kidney/podocytes and UB epithelial cells (Dai et al., 2006; El-Aouni et al., 2006; Kanasaki et al., 2008; Pozzi et al., 2008; Smeeton et al., 2010) and many more. A detailed discussion of integrin and IPP function in these numerous tissues and cell-types would exceed the scope of this introduction. Therefore, the focus will be on their function in the vascular system and in the skin.

9) Development of the vascular system

The first functional organ to form in vertebrate development is the cardiovascular system. It comprises the heart, blood vessels and lymphatic vessels. Blood vessels and lymphatic vessels are branched tubular networks, lined by endothelial cells (ECs). They facilitate the transport of gases, liquids, nutrients, signaling molecules and circulating cells. Insufficient blood supply results in ischaemia and severe pathological deficiencies, which are often fatal. In tumors, on the other hand, increased angiogenesis can promote tumor growth, and extravasation of tumor cells into lymphatic or blood vessels facilitates their metastatic spread, primarily into lymph nodes and the lung (Armulik et al., 2005).

9.1.1) Vasculogenesis

Early during embryogenesis, cells of mesodermal origin differentiate into endothelial precursor cells (EPCs), also referred to as angioblasts. EPCs then cluster into so-called blood islands, which coalesce to form a capillary network, known as primary capillary plexus. This process, which leads to the *de novo* formation of blood vessels, is referred to as vasculogenesis. The dorsal aorta, the cardinal vein and the yolk-sac vasculature are directly generated by vasculogenesis (Adams and Alitalo, 2007) (Figure 5).

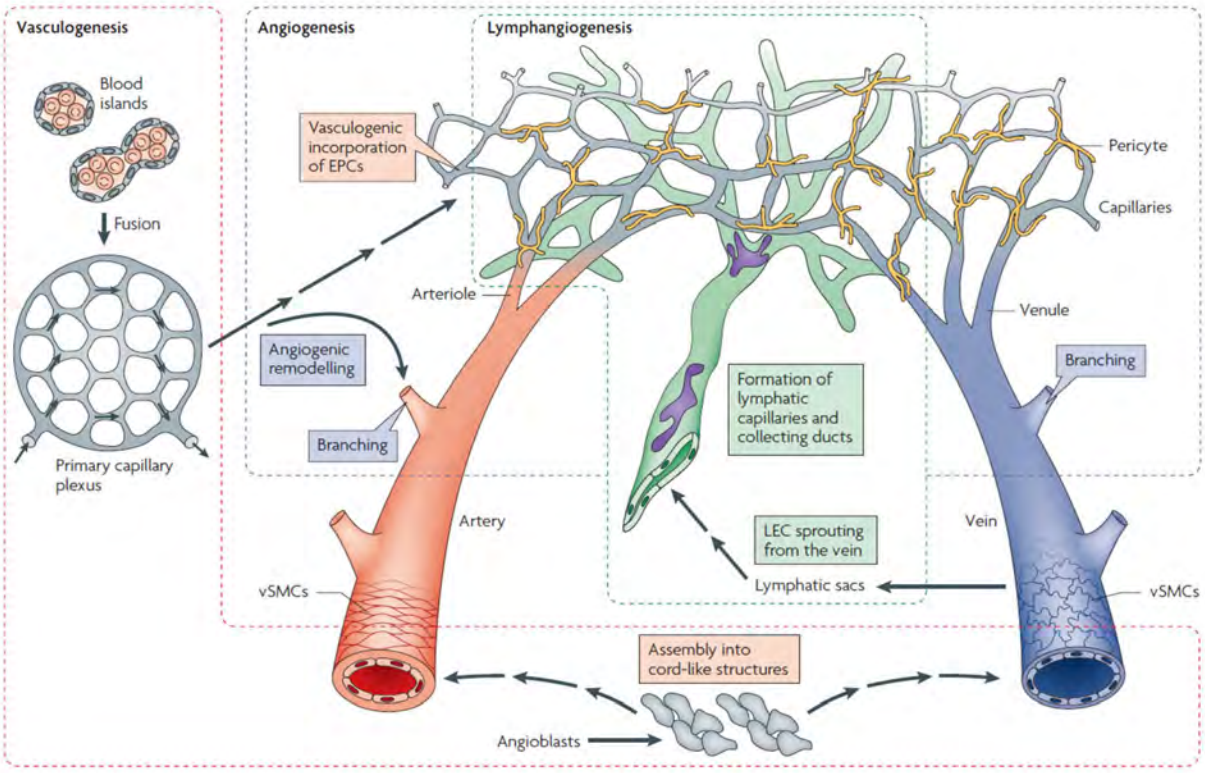

Figure 5: Vasculogenesis, angiogenesis and lymphangiogenesis

Vasculogenic fusion of blood islands results in the formation of a primary capillary plexus, which is extensively remodeled during angiogenesis. Pericytes associate with capillaries and immature blood vessels, whereas large and mature vessels are supported by vSMCs. Lymphangiogenesis is initiated by the sprouting of lymphatic ECs (LEC) from embryonic veins. For details see text. Figure taken from (Adams and Alitalo, 2007).

9.1.2) Angiogenesis

In a process known as angiogenesis, the primary vascular plexus extends and is extensively remodeled, which eventually results in the formation of a hierarchical system consisting of major arteries and veins, smaller (pre-capillary) arterioles and (post-capillary) venules, and a branched network of fine capillaries (Figure 5). During angiogenesis, new vessels can sprout from existing vessels (sprouting angiogenesis) or can be generated by splitting (intussusception) of vessels through the insertion of tissue pillars (Djonov and Makanya, 2005) (non-sprouting angiogenesis). While some vessels regress and become eliminated (pruned), others branch, fuse and/or mature. Maturation of vessels requires the recruitment of pericytes and vSMCs, collectively referred to as mural cells, to newly formed endothelial tubes. While capillaries and immature blood vessels are covered by pericytes, which are embedded in the BM and establish direct connections with ECs through focal cell-cell

contacts, larger vessels are ensheathed by vSMCs, which are separated from the endothelium by the vascular BM (Adams and Alitalo, 2007).
Mural cells not only mechanically stabilize vessels, but also prevent excessive proliferation and sprouting of EC and thus are required to facilitate a quiescent and mature vessel phenotype. In addition, vSMCs control the vascular tone through contraction or relaxation of their actomyosin cytoskeleton (Armulik et al., 2005; Bergers and Song, 2005).

9.1.3) Regulation of sprouting angiogenesis

Angiogenic sprouting is tightly regulated. ECs have to reverse their polarity and adopt motile and invasive features, which includes the activation and/or secretion of proteases to locally dissolve the vascular BM. Furthermore, endothelial sprouts have to elongate in a polarized, coordinated, and directional way.
Angiogenic sprouts are guided by ECs located at the front of the tip and referred to as tip cells. Similar to axonal growth cones, tip cells extend filopodia to sense attractive and repulsive guidance cues in their environment. Notch signaling is essential for the specification of tip cells and impaired Notch signaling increases tip cell formation, which leads to excessive sprouting, branching and fusion of endothelial tubes (Adams and Alitalo, 2007).
Expression of the Notch ligand DLL4 (Delta-like-4) in tip cells is induced by VEGFA (vascular endothelial growth factor A). This results in activation of Notch signaling in adjacent cells which suppresses the expression of VEGFR2 (VEGF receptor) (and VEGFR3 in zebrafish) in these cells and prevents the formation of additional sprouts.
Tip cells, in contrast, express high levels of VEGFR2 which help to guide the elongating sprout along a spatial concentration gradient of a ECM-anchored isoform of VEGFA (VEGF164 in mice, VEGF165 in humans) (Adams and Alitalo, 2007).
Interestingly, guidance of endothelial sprouts frequently parallels axonal path finding and similar guidance cues are used by ECs and neurons. Repulsive cues for ECs include the class 3 semaphorin SEMA3E and netrin-1, which prevent abnormal vascular patterning by interacting with their corresponding endothelial receptors Plexin D1 and UNC5B (uncoordinated-5B), respectively. Accurate vascular patterning also depends on the expression of ROBO4 (magic roundabout) on ECs, and SLIT proteins might function as repulsive cues for ROBO4 expressing ECs. However, it is not clear, if SLIT proteins can directly interact with ROBO4 (Adams and Alitalo, 2007).

Sprouting ends when elongating endothelial sprouts fuse with existing capillaries or the tips of other sprouts, which results in the formation of new vascular connections. Fusion requires the establishment of new cell-cell contacts between ECs. Furthermore, ECs have to revert to a non-migratory phenotype. However, inappropriate fusion may result in arteriovenous shunts, known as anastomoses, and thus fusion needs to be regulated. Repulsive cues are thought to prevent the inappropriate fusion of non-matching capillary sprouts (Adams and Alitalo, 2007).

9.1.4) Maturation of vessels

Maturation of vessels is supported by the onset of blood flow, which results in enhanced oxygen supply. This in turn lowers the local production of pro-angiogenic VEGFA, which results in pruning of immature vessels, whereas maturation prevents vessel regression in the absence of VEGFA. Assembly of a vascular BM and suppression of EC-proliferation and sprouting further promote the maturation of vessels. Additionally, recruitment of mural cells to nascent vessels is essential for vessel maturation. While ECs, in particular tip cells, secrete PDGFB (platelet-derived growth factor B), mural cells express the corresponding receptor PDGFRβ (PDGF receptor β), which is essential for proliferation and recruitment of mural cells to blood-vessels and for their association with the vessel-wall. Similar to VEGFA, spatial presentation of PDGFB is essential for the recruitment of mural cells. PDGFB contains a retention motif, which by binding to heparin sulfate proteoglycans facilitates the anchorage of PDGFB to the ECM. Consequently, mural cell coverage is impaired in mice that express a mutant form of PDGFB that lacks the retention motif (Armulik et al., 2005; Lindblom et al., 2003).

Interestingly, ECs also secrete sphingosine-1-phosphate (S1P), and PDGFRβ might cooperate with S1P-receptors, which belong to the G-protein coupled receptor family (Allende and Proia, 2002; Spiegel and Milstien, 2003). S1P1, S1P2 and S1P3 have been shown to be involved in vascular development, and deletion of S1P1 results in insufficient investment of blood-vessels by mural cells, which might be partially due to impaired Rac activation (Kono et al., 2004; Liu et al., 2000).

TGFβ (transforming growth factor β) signaling also plays an important role in mural cells. TGFβ and latency associated peptide (LAP) form a latent complex, which is anchored to the ECM through the latent TGFβ-binding protein (LTBP) (ten Dijke and Arthur, 2007). TGFβ signaling, together with serum response factor (SRF), myocardin, CRP1 and CRP2, and δEF1,

promotes differentiation of progenitor cells into mural cells (Adams and Alitalo, 2007). Deletion of TGFβ1, its receptors TGFβ receptor-2 (Tgfbr2) and activin-receptor-like kinase-1 and -5 (Alk1 and Alk5), the accessory receptor endoglin (Eng), or the downstream effector SMAD5, all result in severe cardiovascular defects and embryonic lethality (Armulik et al., 2005).

9.1.5) Integrins in the vascular system

Integrins play a major role in the development and homeostasis of the vascular system. ECs express several integrins, including the β1 integrins α1β1, α2β1, α3β1, α4β1, α5β1, α6β1 and α9β1, the αV integrins αvβ3 and αvβ5, as well as α6β4. The integrin repertoire of mural cells is less well characterized, although they were shown to express α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α9β1, α6β4 and, in addition, α7β1 and α8β1 (Avraamides et al., 2008; Silva et al., 2008). Genetic and pharmacological approaches revealed critical functions of several integrins in the vasculature. However, as shown for αV integrins, results between pharmacological inhibition and genetic ablation can differ significantly.

While the majority of mice lacking the αV integrin subunit die between E10.5 and E11.5 due to vascular defects in placental blood vessels, 20% complete embryonic development but die shortly after birth. These mice display enlarged and leaky vessels in the brain and intestine, accompanied by severe hemorrhages (Bader et al., 1998). However, deletion of αV specifically in ECs does not affect blood vessels in the brain, whereas specific deletion of αV in neuronal cells results in vascular defects in the brain that closely resemble those of mice completely lacking αV integrins (McCarty et al., 2005). Similar vascular defects in the brain are also observed in mice lacking the β8 integrin subunit, which is expressed by neither ECs nor mural cells, but instead is expressed by glia cells. This indicates, that the vascular defects in brain are due to the loss of αvβ8 in glia cells but not in ECs (McCarty et al., 2002; Zhu et al., 2002).

Studies using antagonists or blocking antibodies specific for the αV integrins αvβ3 and αvβ5 indicate an essential function for these integrins in pathological angiogenesis. Endothelial expression of αvβ3 increases during neovascularization in tumors, wounds and inflamed tissue and is promoted by angiogenic growth factors and cytokines, including bFGF (basic fibroblast growth factor), TNFα (tumor necrosis factor α) and IL8 (interleukin 8) (Brooks et al., 1994a). Furthermore, αvβ3 specific antagonists induce caspase 8-dependent apoptosis in

ECs (Stupack et al., 2001) and inhibit angiogenesis, indicating that this integrin is important for EC survival and migration (Brooks et al., 1994a; Brooks et al., 1994b; Brooks et al., 1995; Friedlander et al., 1995; Friedlander et al., 1996; Fu et al., 2007). However, genetic ablation of β3 and/or β5 (and hence αvβ3 and αvβ5) does not impair physiological angiogenesis but promotes tumor angiogenesis, possibly due to elevated levels of VEGFR-2 in the absence of αvβ3 (Reynolds et al., 2004; Reynolds et al., 2002). Moreover, migration and proliferation of αvβ3-deficient ECs is increased in response to VEGF (Reynolds et al., 2004). In contrast, tumor angiogenesis is impaired when both Tyrosine 747 and Tyrosine 759 in the β3 cytoplasmic tail are replaced by Phenylalanine (Mahabeleshwar et al., 2006). Consistently, adhesion, spreading, migration and capillary tube formation are compromised in the mutant ECs (Mahabeleshwar et al., 2006).

Genetic ablation of the β1 integrin subunit (and hence all β1 integrin heterodimers) demonstrates a key function for β1 integrins in vascular development. In the absence of β1 integrin, EC proliferation and branching of blood vessels is impaired in EBs and only host derived blood vessels form in β1-deficient teratomas (Bloch et al., 1997). Furthermore, endothelial-specific deletion of the β1 integrin subunit in mice results in embryonic lethality by E10.5 due to severe vascular defects. Although vasculogenesis and formation of larger vessels is unaffected, angiogenesis in the embryo and yolk sac is severely compromised in mice lacking β1 integrins in ECs (Tanjore et al., 2008). Similarly, endothelial-specific deletion of ILK results in severe vascular defects and embryonic lethality around E11.5 (Friedrich et al., 2004).

Specific deletion of the β1 integrin subunit in mural cells also results in severe vascular defects, which are characterized by insufficient investment of the vessel wall by mural cells. Although mural cell specific deletion of ILK and constitutive deletion of α-Parvin both lead to similar vascular defects, these defects are due to increased RhoA/ROCK mediated contractility, whereas β1 integrin-deficient mural cells are not hypercontractile. Instead, proliferation of mural cells is increased and their differentiation is compromised in the absence of β1 integrins (Abraham et al., 2008; Kogata et al., 2009; Montanez et al., 2009).

Constitutive deletion of the integrin α5 subunit results in embryonic lethality between E10 and E11. The mutant embryos lack posterior somites and have a truncated posterior. Furthermore, vascular development is severely impaired, demonstrating an essential role of the α5β1 integrin heterodimer in the development of the vasculature (Yang et al., 1993). Depletion of α5β1 also results in defects in the formation of the vasculature in EBs and

teratomas, which are similar to the defects in β1 deficient EBs and teratomas, albeit milder (Francis et al., 2002; Taverna and Hynes, 2001).

Consistent with the essential function of the α5β1 integrin in vascular development, mutation or constitutive deletion of its ligand FN in mice results in similar defects, including a severely compromised cardiovascular system and lack of somites (Astrof et al., 2007; Gettner et al., 1995; Takahashi et al., 2007).

In addition to αvβ3, αvβ5 and α5β1, the integrin heterodimers α4β1 and α9β1 are also FN receptors expressed by ECs and mural cells. Although genetic ablation of the integrin α9 subunit does not result in recognizable abnormalities in blood vessels, mice lacking α9β1 integrin fail to develop a normal lymphatic system and die between 6 and 12 days after birth due to respiratory failure caused by an accumulation of pleural fluid (Huang et al., 2000b). This suggests an essential function for α9β1 in the lymphatic vasculature.

Deletion of the α4 integrin subunit in mice results in embryonic lethality between E10 and E12, due to impaired chorio-allantoic fusion during placentation and defects in the development of the epicardium and coronary vessels, which lead to cardiac hemorrhages (Yang et al., 1995). Although it is not clear if α4 has a relevant function in physiological angiogenesis, its expression is increased during tumor angiogenesis and in response to angiogenic growth factors and cytokines. Furthermore, α4β1 can promote the association of VCAM-1 expressing vSMCs with ECs during neovascularization, whereas α4β1 antagonists impair survival of mural cells and ECs and inhibit tumor angiogenesis (Garmy-Susini et al., 2005). This suggests a pro-angiogenic function for α4β1.

Vascular development is normal in mice lacking the Col and LN-binding integrins α1β1 or α2β1. However, tumor angiogenesis is reduced in mice lacking α1β1 (Pozzi et al., 2000), whereas in the absence of α2β1, tumor angiogenesis is increased in melanomas and also in Lewis lung carcinomas (LLC), although only upon PLGF (placental growth factor) treatment (Zhang et al., 2008b). Thus, these integrins are dispensable for vascular development but might have modulatory functions during pathological angiogenesis.

Genetic ablation of the α6 integrin subunit, which prevents formation of α6β1 and α6β4, does not result in obvious vascular defects. Instead, mice lacking either the integrin subunit α6 or β4 die immediately after birth due to impaired hemidesmosome formation and severe detachment of the epidermis from the underlying dermis (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). However, integrin α6β1 is expressed on capillary ECs (Lee et al., 2006) and the β4 integrin subunit is present on the endothelium of tumors (Nikolopoulos et al., 2004), and blocking antibodies against the α6 integrin subunit

impair EC tube formation (Lee et al., 2006). Additionally RNAi-mediated reduction of the α6 integrin subunit impairs EC migration and tube formation in the brain microvasculature (Lee et al., 2006). Although a mutant form of the β4 subunit with a truncated cytoplasmic tail is sufficient to prevent the skin defects that result from the complete deletion of β4, tumor angiogenesis is reduced in mice expressing this truncated β4 subunit. While EC proliferation is normal, adhesion and migration are reduced in ECs expressing the truncated β4 subunit (Nikolopoulos et al., 2004). Thus, the α6 heterodimers α6β1 and α6β4 are dispensable for physiological angiogenesis but might negatively regulate tumor angiogenesis.

Integrins expressed in mural cells but not in ECs, are also implicated to play a role in vascular development. In mice, deletion of the α7 integrin subunit results in partial embryonic lethality, accompanied by cerebrovascular hemorrhages and vSMC hypoplasia as well as defects in the development of the placenta. Defects in vSMCs are also present in mice that continue to develop in the absence of the α7 integrin subunit. The vSMCs in the surviving mice are hyperplastic and hypertrophic (Flintoff-Dye et al., 2005; Welser et al., 2007). This suggests that the α7β1 integrin plays a role in mural cells thereby contributing to vascular development.

Finally, the integrin heterodimer α8β1 is also expressed in mural cells but not in ECs. With increasing number of passages, cultured vSMC convert from a contractile, low proliferative phenotype to a less contractile and more proliferative “synthetic” phenotype. Interestingly, while the expression of the integrin subunit α8 is reduced with increasing number of passages, overexpression of the integrin α8 subunit in cultured rat-vSMCs promotes the switch from the synthetic to the contractile phenotype in a Rho-dependent manner (Zargham et al., 2007). However, vSMC differentiation was not altered in vSMC of mice lacking the α8 integrin subunit (Marek et al., 2010).

In summary several integrins are implicated to play a role in the vasculature. Differences between pharmacological and genetic approaches, as well as between physiological and pathological angiogenesis indicate that, although some integrins (e.g. α5β1) are essential for vascular development, others are dispensable but might have specific modulatory functions, which, under physiological conditions, can be compensated by alternative integrins and/or signaling pathways. Future studies are required to resolve these discrepancies and to define the signaling specificities of different integrins. The IPP complex and in particular the α-Parvin is essential for vascular development by regulating actin cytoskeleton dynamics, migration and contractility of vSMC. Defining the signaling specificities of distinct IPP complexes will provide further insight into the underlying molecular mechanisms.

10) Development and architecture of the skin

The skin is the largest organ of the body and serves as a barrier that prevents dehydration and protects the organism against a variety of external threats of physical, chemical and biological nature including ultraviolet radiation, extreme temperatures, trauma, toxic or harmful substances and infectious organisms. Skin is composed of an outer stratified squamous epithelium, the epidermis, which is separated from the underlying mesenchymal dermis by a BM. (Fuchs and Raghavan, 2002; Lippens et al., 2009). Underneath the dermis lies the adipose subcutis or hypodermis. Additional components of skin are epidermal appendages such as hair follicles (HFs) and sweat glands, which derive from the epidermis.

10.1.1) Morphogenesis of the epidermis

During embryogenesis, the epidermis develops from a single layer of ectoderm. Wnt-signaling is required for the specification of the epidermis by repressing FGF signaling, which promotes a neural fate, and promoting BMP (bone morphogenetic proteins) signaling (Stern, 2005). From E9.5 to E12.5, the developing epidermis consists of a monolayer of multipotent epithelial progenitor cells. Their differentiation results in the formation of the periderm, a protective layer which initially covers the epithelial cells but is shed into the amniotic fluid when a functional epidermis has formed towards the end of embryonic development (M'Boneko and Merker, 1988). Starting at E12.5, mesenchymal cues induce a differentiation program in epithelial cells, which first leads to the formation of a mitotically active intermediate layer (stratum intermedium). The stratification of the epidermis successively increases through further differentiation and by E17.5 the epidermis consists of several transcriptionally, functionally, and morphologically distinct layers of keratinocytes, which are in consecutive stages of differentiation (Blanpain and Fuchs, 2006; Koster and Roop, 2007) (Figure 6).

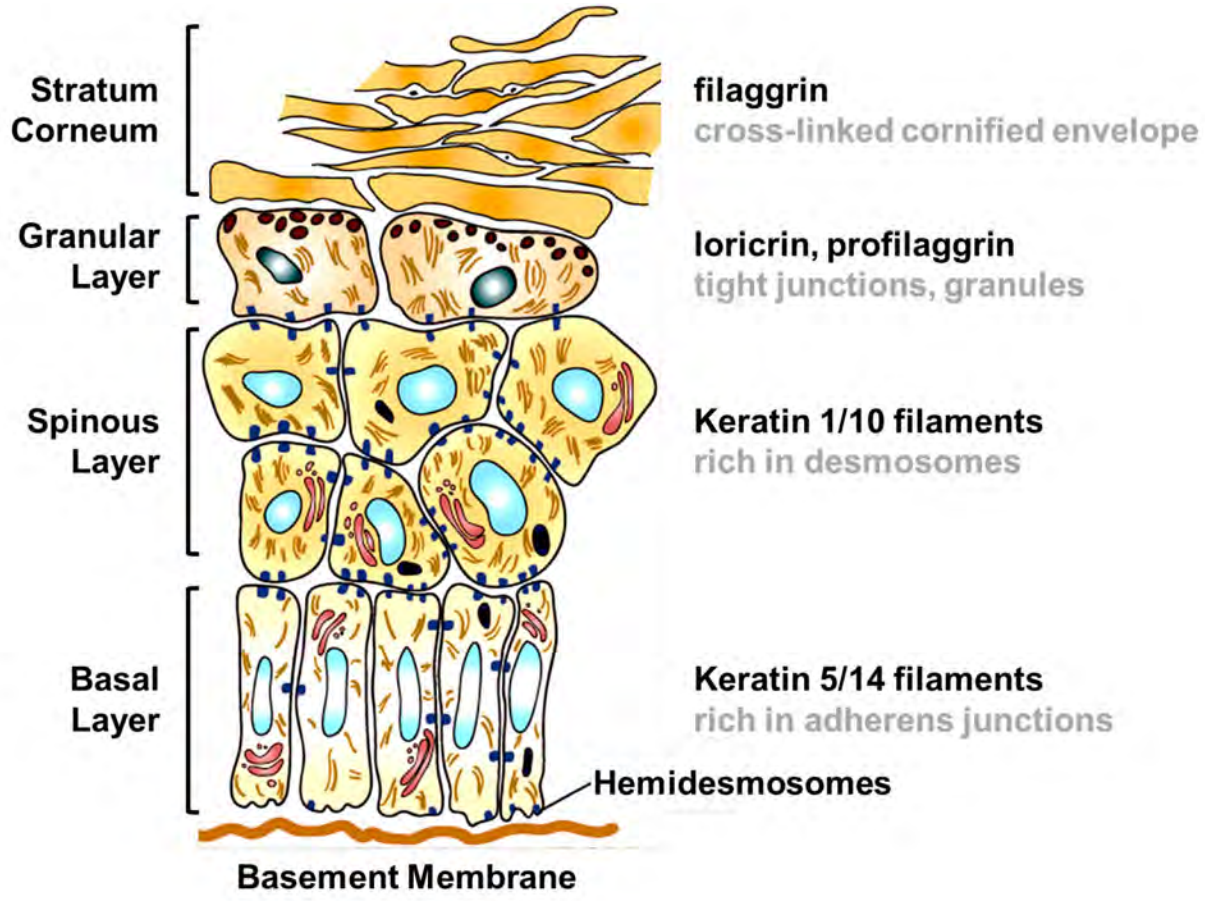

Figure 6: The epidermis is a stratified squamous epithelium

Schematic representation of the epidermis. Proliferation is restricted to keratinocytes in the basal layer of the epidermis, which firmly adhere to the BM through hemidesmosomes. Delamination of basal keratinocytes and sequential differentiation gives rise to the suprabasal layers of the epidermis, which are morphologically and functionally distinct and express different markers as indicated on the right side. See text for details. Figure is modified from (Fuchs, 2008).

Proliferation is confined to the basal layer of keratinocytes (basal keratinocytes), which are in direct contact with the underlying BM. The proliferating basal keratinocytes are characterized by the expression of keratin 5 and 14 as well as several integrins. Upon commitment to a terminal differentiation program, basal keratinocytes withdraw from the cell cycle, down regulate integrins, delaminate from the BM and translocate to the stratum spinosum, which is the first suprabasal layer. In contrast to basal keratinocytes, keratinocytes in the spinous layer are characterized by the expression of keratin 1 and 10 (Fuchs, 2008). Alternatively or additionally, asymmetric cell division, with a mitotic spindle oriented perpendicular to the BM, contributes to the formation of the spinous layer, at least during development (Lechler and Fuchs, 2005). However, in tail skin from adult mice, only 3% of dividing basal keratinocytes display spindle orientations perpendicular to the BM (Clayton et al., 2007).

Keratinocytes of the spinous layer further differentiate as they translocate to the stratum granulosum. Expression of loricrin is characteristic for keratinocytes in the granular layer. Finally, terminal differentiation of keratinocytes from the granular layer results in the formation of the stratum corneum, which consists of enucleated and dead keratinocytes, also referred to as corneocytes. Corneocytes are characterized by the presence of filaggrin and a cornified envelope of cross-linked lipids and proteins, which essentially contributes to the barrier function of the skin (Fuchs, 2008). During adult life, corneocytes are constantly shed off from the body surface in a process referred to as desquamation (Milstone, 2004). The shed corneocytes are replenished through the proliferation of basal keratinocytes and their successive terminal differentiation, which results in the continuous renewal of the epidermis throughout life.

The homeostasis of the epidermis and its remarkable ability to regenerate after injury depends on stem cells residing in the interfollicular epidermis (IFE), the bulge region of HFs and in sebaceous glands (SC). Stem cells are infrequently dividing cells with a high potential of self-renewal. In the epidermal proliferative unit (EPU) model, asymmetric lateral division of a single stem cell, positioned in the center of a discrete epidermal unit in the IFE, generates transit amplifying cells (TA), which in turn can undergo a limited number of divisions before being committed to terminally differentiate (Potten, 1981). Challenging the EPU model, a single progenitor model questions the existence of TAs. Instead, asymmetric lateral division of stem cells might directly result in the generation of post-mitotic cells committed for differentiation (Clayton et al., 2007).

10.1.2) Morphogenesis and cycling of the HF

Mammalian skin is characterized by the presence of hair, which physically protects the skin, contributes to thermoregulation, has sensory functions and plays an important role in social interactions (Botchkarev and Paus, 2003). Hair is formed by the HF, which together with the arrector pili muscle and the sebaceous gland is organized into a functional pilosebaceous unit (Schneider et al., 2009). In the HF, keratinocytes terminally differentiate into dead trichocytes, which condense into a fiber with high tensile strength, commonly known as hair, but more specifically referred to as the hair shaft (HS) (Schmidt-Ullrich and Paus, 2005). Morphogenesis of the HF is initiated during embryonic development and depends on the reciprocal interaction and signaling crosstalk between the epithelium and the underlying

mesenchyme. The involved signals include components of the Wnt, hedgehog, TGF-β/BMP, FGF and TNF signaling pathways (Schneider et al., 2009).

Inductive cues from the mesenchyme are thought to promote the proliferation of epithelial cells, which subsequently extend locally into the mesenchyme to form the hair placode. The hair placode first becomes morphologically recognizable by E14. In turn, signals derived from the epithelial cells in the placode induce the clustering of underlying mesenchymal fibroblasts into a dermal condensate, which is the precursor of the dermal papilla. Inductive cues from the dermal papilla promote further proliferation and differentiation of keratinocytes, which subsequently extend deeper into the mesenchyme to eventually encapsulate the dermal papilla in a structure referred to as the hair bulb. In the hair bulb, melanocytes derived from neural crest cells, form the pigmentary unit of the hair follicle. Melanocytes produce melanin, the pigment of the HS. Melanin is stored in melanosomes and is transferred to terminally differentiating keratinocytes of the HS, which in the end results in the pigmentation of the HS (Botchkarev and Paus, 2003). Highly proliferative keratinocytes in the hair bulb are in close proximity to the dermal papilla and are known as hair matrix (HM) keratinocytes. Proliferation and differentiation of HM keratinocytes critically depends on regulatory signals from the dermal papilla and results in the formation of six concentric layers of the HS (Medulla, Cortex and Cuticle) and the inner root sheath (IRS) (Huxley's layer, Henley's layer and Companion layer). The inner root sheath separates the hair shaft from the outermost layer of the HF, known as the outer root sheath (ORS) (Botchkarev and Paus, 2003). Keratinocytes from the ORS are continuous with the basal layer of the IFE, and as such express Keratin 5 and 14, and are in direct contact with the BM that surrounds the HF. HF development in mice is asynchronous. Vibrissa are induced at E12.5, whereas primary tylotrich guard hairs are initiated by E14.5, and secondary non-tylotrich intermediate (awl and auchene) and downy (zigzag) hairs start to develop between E17 and birth. By postnatal day 8 (P8), all HFs complete morphogenesis but HF elongation and maturation continues up to P14, by which time all HF bulbs reside deep in the subcutis and are in close vicinity to the panniculus carnosum muscle layer (Botchkarev and Paus, 2003; Schmidt-Ullrich and Paus, 2005).

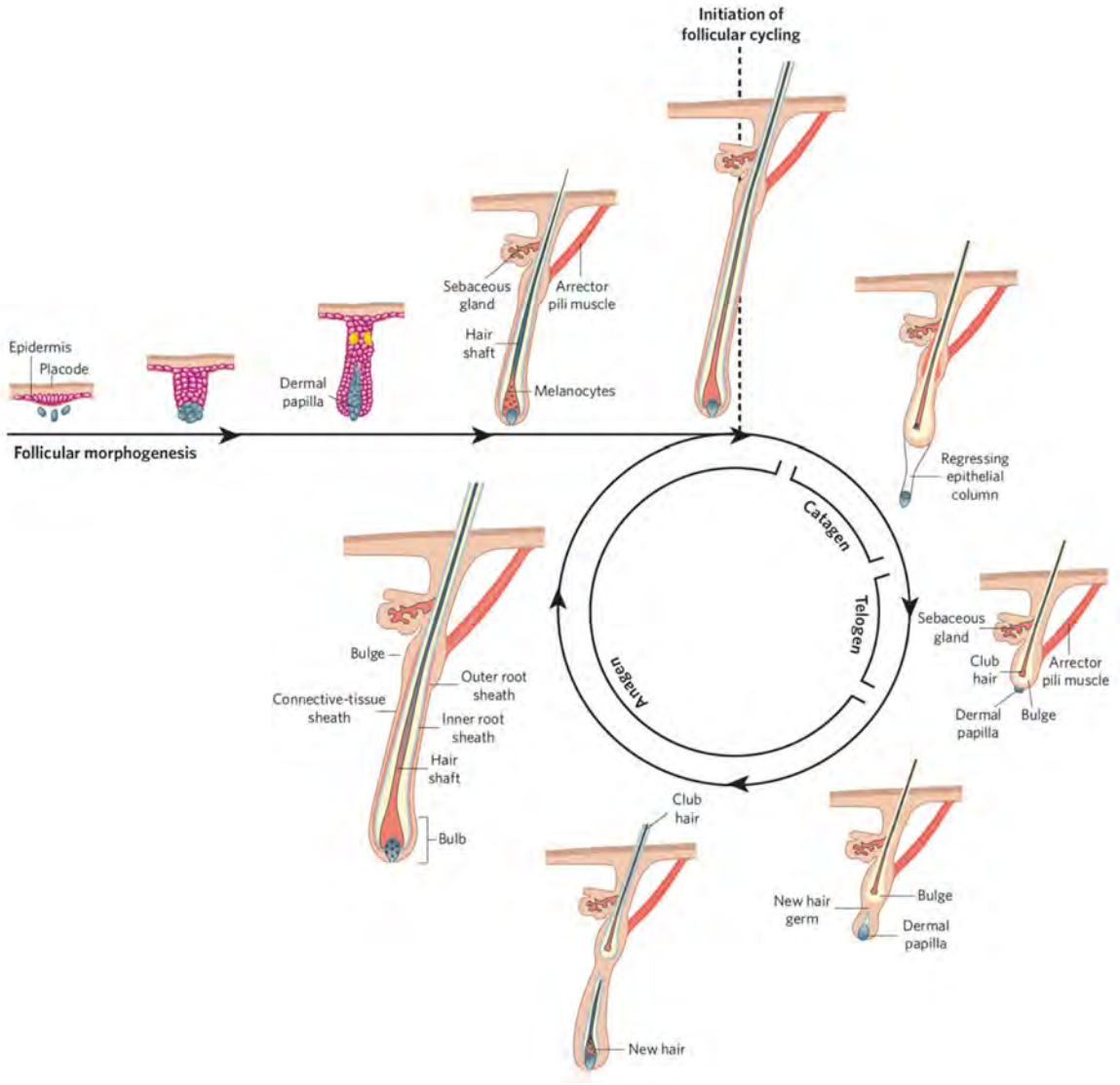

Figure 7: Hair follicle morphogenesis and cycling

Schematic representation of distinct stages of HF-morphogenesis and cycling. After HF-morphogenesis is completed, HF cycle between stages of apoptosis driven regression (catagen), relative quiescence (telogen) and active growth (anagen). See text for details. Figure taken from (Fuchs, 2007).

After HF morphogenesis is completed, HFs cycle between alternating phases of growth (anagen), rapid regression (catagen), and relative quiescence (telogen) throughout adult life (Schneider et al., 2009) (Figure 7). By P17 the HF cycle is initiated by the first catagen, which lasts about two to three days. Apoptosis of HM, IRS and ORS keratinocytes results in the degeneration of the non-permanent part of the HF, which comprises the region below the stem cell-containing HF bulge. Importantly, retraction of the remaining epithelial strand brings the dermal papilla into close proximity to the HF bulge. Following a resting telogen phase of several days, inductive signals from the dermal papilla promote the proliferation of stem cells in the bulge and thereby initiate the first anagen phase, which eventually results in the

regeneration of a mature HF. During the anagen phase, TA keratinocytes, derived from stem cells in the bulge, migrate along the ORS-underlying BM towards the bulb region, where they differentiate into the distinct HF lineages of the mature HF. Although the old HS, known as club hair, might be retained for several hair cycles, it eventually will be shed in a process referred to as exogen. In mice, the initial hair cycles are synchronous. However, synchronous cycling becomes confined to regional domains in aging animals and telogen phases successively get prolonged, leading to an increased duration of the hair cycle. Signals that regulate cycling include anagen promoting components of the Wnt, BMP and Shh signaling pathways, whereas FGF5 promotes the induction of catagen (Botchkarev and Paus, 2003; Schneider et al., 2009).

10.1.3) Integrins in the epidermis

Tight adhesion of basal and ORS keratinocytes to the underlying BM is essential for epidermal homeostasis. Integrins are the key mediators of this adhesion and play an essential structural and functional role in epidermal homeostasis, HF development/maintenance and wound healing. Conversely, abnormal expression of integrins or altered integrin signaling is involved in the development and progression of skin carcinomas and a psoriasis-like disease (Watt, 2002).

With the exception of αvβ8, which is expressed only in suprabasal layers, expression of the other epidermal integrins normally is confined to the basal layer of the epidermis and to keratinocytes of the ORS. Integrins constitutively expressed in the epidermis include the laminin receptors α3β1 and α6β4 as well as the collagen receptor α2β1 and the vitronectin receptor αvβ5 which is expressed at a low level. Additionally, the fibronectin receptor α5β1 and the fibronectin and tenascin receptors αvβ6 and α9β1 are induced or upregulated in cell culture and during pathological conditions such as wounding or tumorigenesis (Watt, 2002). Unlike other integrins, the α6β4 integrin heterodimer facilitates the linkage to intracellular keratin filaments and is a major component of hemidesmosomes, which tightly anchor keratinocytes to the BM. While α6β4 localization is restricted to the basal membrane, other integrins also localize to lateral and apical surfaces of basal keratinocytes. In mice, genetic ablation of either the α6 or the β4 subunit impairs hemidesmosome-formation and results in neonatal lethality due to severe epidermal detachment from the BM (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). However, differentiation of

keratinocytes is normal in regions of partial attachment to the BM, indicating that α6β4 is not essential for this process. Similar defects in humans are characteristic for the autosomal recessive disorder junctional epidermolysis bullosa, which is caused by mutations in the genes for the α6 or the β4 integrin subunits.

The constitutive deletion of the α3 integrin subunit also results in epidermal detachment. However these defects are less pronounced than the absence of α6β4 and are accompanied by a defective organization and rupture of the BM (DiPersio et al., 1997). Lack of α3 also affects the organization of the glomerular BM, impairs lung and kidney development and results in death shortly after birth (Kreidberg et al., 1996). Conclusively, the BM defects indicate that the α3β1 integrin heterodimer is essential for the organization and integrity of BMs. Genetic deletion of both α6β4 and α3β1 does not result in a more severe phenotype than the individual deletion of either heterodimer. Furthermore, even in the simultaneous absence of both heterodimers, proliferation, stratification and epidermal morphogenesis before blister formation are normal, indicating that both heterodimers are dispensable for epidermal development but are critically required for the organization of and attachment to the BM (DiPersio et al., 2000). Interestingly, in adult skin α3β1 is required for HF morphogenesis and maintenance. Growth and differentiation of HFs is severely impaired in skin-grafts from α3 deficient mice. Additionally, the BM of the IFE but not of the HF is disorganized in the α3-deficient skin-grafts (Conti et al., 2003).

Genetic ablations of the integrin subunits α2 (Chen et al., 2002; Holtkotter et al., 2002), α9 (Huang et al., 2000b), and β5 (Huang et al., 2000a) have not been reported to result in a skin-related phenotype, although migration is severely compromised in β5-deficient keratinocytes *in vitro* (Huang et al., 2000a) and α2β1 is critically required for the adhesion of keratinocytes to type I collagen (Zhang et al., 2006).

10.1.4) The function of β1 integrins in the epidermis

Conditional deletion of genes specifically in the epidermis and its appendages can be achieved by crossing mice in which the gene of interest has been modified by the insertion of two loxP-recombination sequences, with mice expressing the Cre-recombinase transgene under the control of the keratin-5 (K5) or keratin-14 (K14) promotor. Cre-mediated recombination results in the excision of the DNA-sequence between the two loxP-sites, leading to the disruption of the gene of interest. When controlled by the K5 or K14 promotor, Cre-

expression and Cre-mediated disruption of the gene of interest is restricted to basal and ORS keratinocytes, which are the progenitors for keratinocytes of the IRS, HS, and the suprabasal layers of the IFE. Consequently the gene of interest is disrupted in the progeny, resulting in conditional deletion in all keratinocytes of the epidermis and its appendages.

The keratinocyte-restricted deletion of the β1 integrin subunit, which prevents the formation of all epidermal β1 integrin heterodimers, results in severe defects in epidermal homeostasis, wound healing and HF morphogenesis (Brakebusch et al., 2000; Grose et al., 2002; Lopez-Rovira et al., 2005; Raghavan et al., 2000). Mice lacking β1 integrins in the epidermis and its appendages either die shortly after birth due to epidermal detachments and dehydration, when Cre-expression is driven by the K14-promotor (Raghavan et al., 2000), or within several weeks after birth, when Cre-expression is under the control of the K5-promotor (Brakebusch et al., 2000). This discrepancy can be explained by differences in the onset of Cre-transgene expression and its expression levels. In the absence of β1 integrins, assembly and organization of the IFE-underlying BM is severely impaired, whereas BM integrity around HFs is unaffected. BM defects are accompanied by reduced expression levels of the α6β4 integrin and a significant reduction/instability of hemidesmosomes leading to pronounced blister formation at the dermal-epidermal junction (DEJ). Although keratinocyte proliferation is reduced in the absence of β1 integrins, mutant epidermis eventually becomes hyperplastic due to a delay in terminal differentiation. However, differentiation *per se* is not impaired in the absence of β1 integrins, and basal keratinocytes lacking β1 integrins do not prematurely differentiate. This argues against β1 integrins being required to prevent premature differentiation of keratinocytes. HF morphogenesis is also distorted in the absence of β1 integrins and due to a progressive loss of hair mutant animals develop severe alopecia four to six weeks after birth. Defects associated with mutant HFs include reduced proliferation of HM keratinocytes, ORS hyperplasticity, stunted and prematurely arrested HF growth, and severe morphological HF abnormalities. Furthermore, from P9, macrophages and granulocytes infiltrate and accumulate around miss-formed mutant HFs, accompanied by an upregulation of pro-inflammatory cytokines. Due to an abnormal deposition of ECM components, the dermis of mutant mice becomes fibrotic at later stages. *In vitro,* β1-deficient keratinocytes are compromised in their adhesion to and spreading on various ECM components, as well as in their migratory capacity. Although proliferation of mutant keratinocytes is reduced *in vitro* and under physiological conditions *in vivo,* expansion of keratinocytes that escaped Cre-mediated deletion partially compensates for the proliferation defects during wound healing *in vivo* (Piwko-Czuchra et al., 2009), indicating that defects in

wound healing are due to impaired keratinocyte adhesion and migration rather than to a reduced proliferation rate (Grose et al., 2002).

10.1.5) The function of ILK in the epidermis

Similar to the conditional deletion of β1 integrin in keratinocytes, conditional ablation of ILK in the epidermis also results in severe defects in epidermal homeostasis and HF morphogenesis. Mice lacking ILK in keratinocytes die around P4, when Cre-expression is driven by the K14-promotor (ILK-K14) (Nakrieko et al., 2008). In these mice, keratinocyte proliferation is reduced in HFs and also *in vitro*. In contrast, mice with a K5-Cre-mediated deletion of ILK in the epidermis survive to adulthood (ILK-K5) (Lorenz et al., 2007). These mice are characterized by ectopic hyper-proliferation of integrin expressing keratinocytes in suprabasal layers of the epidermis from P7 and by the accumulation of proliferative keratinocytes in the ORS. Additionally, proliferation of ILK-K5 keratinocytes is increased *in vitro*. Irrespective of whether deletion is mediated by K5-Cre or by K14-Cre, keratinocyte-restricted deletion of ILK results in discontinuous distribution of the hemidesmosome integrin heterodimer α6β4, keratinocyte detachment from the BM and blister formation at the DEJ, disruption of the IFE-underlying BM, delayed terminal differentiation of keratinocytes and severe defects in hair follicle morphogenesis. These defects are highly reminiscent of the defects in mice lacking β1 integrin specifically in keratinocytes. Similarly, when ILK deletion in keratinocytes is mediated by K5-Cre, ORS and IFE become hyperplastic and macrophages infiltrate and accumulate around mutant HFs. Additionally, the morphology and polarity of ILK-deficient keratinocytes is severely compromised *in vivo* and *in vitro*. *In vitro*, adhesion, spreading and migration of ILK-deficient keratinocytes is severely compromised and directional migration and velocity is strongly reduced in these cells. Mutant keratinocytes fail to form stable lamellipodia and formation of FCs and FAs is reduced *in vitro*. Furthermore, actin cytoskeleton organization is severely distorted in ILK-deficient keratinocytes. While no change in Rac1 activity is detectable in ILK-K5 keratinocytes, activation of Rac1 is reduced and active Cdc42 distribution is altered in ILK-K14 keratinocytes (Lorenz et al., 2007; Nakrieko et al., 2008).

The striking similarities between the phenotypes of mice lacking β1 integrins or ILK in the epidermis demonstrate an essential function of ILK downstream of β1 integrins in epithelial cells. K5-mediated deletion of PINCH-1 in keratinocytes also results in a similar phenotype

(Lorenz-Baath, unpublished data). However, defects in cell-cell contacts might be more pronounced in the PINCH-1-deficient epidermis, although similar defects have been reported in the epidermis of ILK-K14 mice (Nakrieko et al., 2008). Differences between ILK- and PINCH-1-deficient keratinocytes suggest an IPP complex-independent function for PINCH-1. However, it is currently not clear whether IPP constituents can function in an IPP complex-independent manner in mammals. Alternatively, upon deletion of one IPP component, residual amounts of the remaining constituents could exert a dominant negative effect, which might result in additional cellular defects. Further studies are required to discern IPP-dependent and independent functions of its constituents and to identify and characterize potential PINCH and Parvin isoform-specific functions.

Aim of the thesis

The β1 integrins and members of the IPP complex are essential for mammalian development. Genetic ablation of either the β1 integrin subunit, or PINCH-1, or ILK results in embryonic lethality during the peri-implantation stage. The mammalian Parvin isoforms are essential components of the IPP complex. They connect the IPP complex to the actin cytoskeleton and are crucially involved in the regulation of actin cytoskeleton dynamics, cell adhesion, spreading, migration and survival. While β- and γ-Parvin are dispensable for mammalian development, the *in vivo* function of α-Parvin has not been analyzed.

Aim 1

Analysis of the *in vivo* consequences of the constitutive deletion of α-Parvin-gene in mice and characterization of the resulting alterations *in vitro*.

Constitutive deletion of α-Parvin results in embryonic lethality between E11.5 and E14.5, preventing the functional analysis of α-Parvin in adult animals. Furthermore, global gene-deletion complicates the discrimination between primary and secondary defects. The Cre-lox system enables gene disruption in a tissue specific and/or inducible manner and is a versatile tool to analyze the *in vivo* function of α-Parvin in various organs and cell types during development and in adult animals.

Aim 2

Generation of floxed α-Parvin mice by means of homologous recombination to enable the conditional deletion of α-Parvin-gene in specific cell types and organs.

Integrins are essential for skin homeostasis. They are involved in the deposition and organization of BM components, control keratinocyte adhesion, spreading and migration and influence proliferation, survival and differentiation of keratinocytes. How exactly integrins facilitate the spatiotemporal control of all this processes is unclear. Certainly it depends on the recruitment and assembly of signaling and adaptor molecules to the cytoplasmic tails of integrins. Among the numerous molecules recruited to the integrin cytoplasmic tails, the IPP complex has emerged to be a key component of integrin signaling. However, the underlying molecular mechanisms still remain largely elusive, necessitating the detailed functional analysis of its components *in vivo* and *in vitro*.

Aim 3

Analysis of the *in vivo* consequences of the K5-Cre mediated conditional deletion of α-Parvin in keratinocytes to identify the functional relevance of α-Parvin in epidermal morphogenesis and homeostasis.

Short summaries of publications

Publication 1: α-Parvin controls vascular mural cell recruitment to vessel wall by regulating RhoA/ROCK signalling

Together with ILK and PINCH, α-Parvin forms a ternary complex (IPP complex) whose assembly precedes its recruitment to FAs. The IPP complex is essential for the integration of integrin signaling with actin cytoskeleton organization and dynamics. To analyze the function of α-Parvin *in vivo,* we used homologous recombination to constitutively delete the α-Parvin gene in mice.

The constitutive deletion of α-Parvin-gene in mice resulted in embryonic lethality between E11.5 and E14.5 due to severe cardiovascular defects. Heart defects in α-Parvin-deficient embryos likely contributed to the early lethality and included a persistent single outflow tract due to defective septation of the *truncus arteriosus*, pericardial effusion, reduction of cardiomyofibrils and abnormal organization and shape of cardiomyocytes.

The vasculature of α-Parvin-deficient embryos and yolk-sacs was poorly remodeled due to compromised recruitment of mural cells to blood vessels. Micro-aneurysms and insufficient investment of the vessel wall by mural cells resulted in the dilation and rupture of blood vessels, leading to edemas and severe bleedings in α-Parvin-deficient embryos. Defective mural cell recruitment and vessel coverage was due elevated RhoA/ROCK/MLC2 signaling resulting in the hypercontractility of α-Parvin-deficient vSMCs, which failed to polarize their cytoskeleton to form stable membrane protrusions and were severely impaired in their directional migration.

Our studies demonstrate that α-Parvin is essential for vascular development *in vivo* by negatively regulating RhoA/ROCK/MLC2-mediated contractility in vSMCs. This function is specific for α-Parvin, since β-Parvin, although expressed in these cells, cannot compensate for the loss of α-Parvin.

Publication 2: α-Parvin controls epidermal homeostasis and hair follicle morphogenesis by regulating adhesion and migration of keratinocytes

Epidermal homeostasis and HF morphogenesis critically depend on β1 integrins and the FA-proteins ILK and PINCH-1, which together with α-Parvin form the IPP complex facilitating integrin signaling and linkage to the actin cytoskeleton. To analyze the *in vivo* function of α-Parvin in epidermal morphogenesis and homeostasis, we generated mice carrying a floxed α-Parvin-gene, allowing the conditional deletion of α-Parvin in keratinocytes.

In this manuscript we report that K5-Cre-mediated deletion of α-Parvin in keratinocytes severely impairs FA-formation, adhesion, spreading and migration of keratinocytes *in vitro*, resulting in local disruptions at the DEJ and severely compromised HF morphogenesis and cycling *in vivo*. Defects in integrin-dependent adhesion were accompanied by severe distortions in BM-integrity, compromised hemidesmosome formation, and displacement of proliferative basal keratinocytes into suprabasal layers of the epidermis. Ectopic hyperproliferation in suprabasal layers, suprabasal expression of integrins and delayed differentiation were observed in the mutant epidermis and likely contributed to the development of a severe epidermal hyperplasia, which clearly preceded the recruitment of inflammatory granulocytes and macrophages. *In vitro*, velocity and directionality of migration were severely impaired in α-Parvin-deficient keratinocytes due to defects in FA-formation and compromised actin cytoskeleton organization, resulting in inefficient migration of keratinocytes toward the dermal papilla *in vivo* and the accumulation of proliferative keratinocytes in the ORS during HF morphogenesis. HF-growth during anagen was completely inhibited, resulting in progressive hair loss and persistent alopecia.

We conclude that α-Parvin is crucially required for IPP-dependent integrin signaling and control of actin cytoskeletal dynamics in keratinocytes and thus is essential for epidermal homeostasis and hair follicle development *in vivo*.

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Appendix

Publication 1

α-Parvin controls vascular mural cell recruitment to vessel wall by regulating RhoA/ROCK signalling

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α-parvin controls vascular mural cell recruitment to vessel wall by regulating RhoA/ROCK signalling

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During blood vessel development, vascular smooth muscle cells (vSMCs) and pericytes (PCs) are recruited to nascent vessels to stabilize them and to guide further vessel remodelling. Here, we show that loss of the focal adhesion (FA) protein α-parvin (α-pv) in mice leads to embryonic lethality due to severe cardiovascular defects. The vascular abnormalities are characterized by poor vessel remodelling, impaired coverage of endothelial tubes with vSMC/PCs and defective association of the recruited vSMC/PCs with endothelial cells (ECs). α-pv-deficient vSMCs are round and hypercontractile leading either to their accumulation in the tissue or to local vessel constrictions. Because of the high contractility, α-pv-deficient vSMCs fail to polarize their cytoskeleton resulting in loss of persistent and directed migration. Mechanistically, the absence of α-pv leads to increased RhoA and Rho-kinase (ROCK)-mediated signalling, activation of myosin II and actomyosin hypercontraction in vSMCs. Our findings show that α-pv represents an essential adhesion checkpoint that controls RhoA/ROCK-mediated contractility in vSMCs.

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Introduction

The cardiovascular system is the first functional organ that develops in vertebrate embryos. Vascular development starts with the differentiation and expansion of endothelial cell (EC) precursors that coalesce into a primitive vascular network. This vascular plexus is then extended and remodelled by a process called angiogenesis, which involves sprouting, branching and fusion (Risau, 1997). Once the ECs are assembled into vascular tubes, they become surrounded by mural cells (MCs) of the smooth muscle cell lineage, referred to as pericytes (PCs) and vascular smooth muscle cells (vSMCs). PCs are associated with capillaries, small venules and immature blood vessels in which they are enclosed by a single basement membrane (BM). In contrast, vSMCs are associated with mature and large blood vessels in which they form one or several sheets around the BM of ECs. The vSMC/PCs are essential for blood vessel development, as they provide mechanical support required to counterbalance the increasing blood pressure, control ECs proliferation, limit further sprouting and regulate the vascular tone with their highly contractile actomyosin cytoskeleton (Adams and Alitalo, 2007).

The vSMC/PCs differentiate from the mesenchyme and migrate around the growing blood vessels. The migration to and spreading on developing blood vessels are tightly regulated by growth factors and extracellular matrix (ECM) proteins, and their receptors such as integrins. Integrins are heterodimeric cell adhesion molecules composed of α and β subunits. When bound to the ECM they cluster and form focal adhesions (FAs), through which they relay signals into cells (Hynes, 2002; Legate *et al*, 2009). *In vitro* and *in vivo* studies have shown that integrin adhesion has essential functions in angiogenesis and vascular remodelling. For instance, deletion of the β1 integrin gene in ECs impairs angiogenesis (Carlson *et al*, 2008; Tanjore *et al*, 2008) and deletion in MCs impairs their ability to spread, differentiate and support vessel wall stability (Abraham *et al*, 2008). Conversely, ablation of genes encoding for integrin ligands such as fibronectin (FN), laminin α4 or collagen 4a1/2 has also a fatal effect on blood vessel formation (George *et al*, 1993; Thyboll *et al*, 2002; Pöschl *et al*, 2004). How integrins execute these properties is less clear.

The cytoplasmic domains of integrins are short and lack enzymatic activity, and therefore they trigger signalling by recruiting kinases such as FAK and src, and adaptor proteins such as integrin-linked kinase (ILK), PINCH and parvins. Parvins are a family of adaptor proteins consisting of three members; α-parvin/actopaxin/CH-ILKBP (α-pv), which is ubiquitously expressed; β-parvin/affixin (β-pv), which is enriched in heart and skeletal muscle; and γ-parvin (γ-pv) whose expression is restricted to haematopoietic cells (Nikolopoulos and Turner, 2000; Olski *et al*, 2001; Tu *et al*, 2001; Yamaji *et al*, 2001; Chu *et al*, 2006). Together with ILK and PINCH they form a ternary protein complex (IPP complex) that localizes to FAs. Parvins consist of an N-terminal polypeptide stretch followed by a single actin-binding domain (ABD) that consists of two in tandem arranged calponin homology (CH) domains. The ABD domain enables parvins to recruit F-actin to FAs and associate with stress fibres. Additional parvin-binding partners are actin binding and regulatory proteins including paxillin, Hic5, α-actinin, CdGAP and α-PIX, which explains the prominent functions of parvins in integrin-mediated adhesion and actin-dependent processes such as cell shape regulation and cell migration (Legate *et al*, 2006).

Vascular tone, which is regulated by the actomyosin-mediated contractility of the vSMCs, controls the blood

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pressure and tissue perfusion. The contraction and relaxation of smooth muscle actin (SMA) filaments in vSMCs are controlled through phosphorylation of the myosin light chain (MLC). The phosphorylation of MLC is mediated by myosin light chain kinase (MLCK) and reversed by myosin light chain phosphatase (MLCP). Vasodilators activate MLCP, which causes MLC dephosphorylation and relaxation, whereas vasoconstrictors induce phosphorylation and inhibition of MLCP activity, which in turn stabilizes MLC phosphorylation and leads to contraction (Karnam, 2006). Vasoconstrictor agonists promote contractility of vSMCs through RhoA-mediated activation of ROCK, which in turn can either indirectly activate MLC through phosphorylation and inactivation of MLCP, or by directly phosphorylating MLC (Amano *et al*, 1996; Kimura *et al*, 1996). The parvin-binding partner ILK has also been shown to directly phosphorylate MLC and thereby modulate vSMC contraction (Wilson *et al*, 2005). A basal RhoA activity is required for cardiovascular homeostasis, whereas a sustained hyperactivation of RhoA is a common feature of several cardiovascular pathologies. Furthermore, RhoA signalling is also critical for cell polarity and directed cell migration (Danen *et al*, 2005) by promoting the maturation of integrin adhesion sites, formation of stress fibres and cell contraction at the rear (Xu *et al*, 2003). Therefore, a tight regulation of RhoA in vSMCs is crucial for the mature vascular system but may be equally important during the recruitment of vSMCs to developing vessels.

To directly address the function of α-pv *in vivo*, we generated mice and cells lacking α-pv expression. Our findings indicate that α-pv controls vSMC/PC recruitment to developing vessels and vessel wall stability by regulating RhoA/ROCK signalling in vSMCs.

Results

Loss of α-pv leads to severe cardiovascular defects

To explore the functions of α-pv *in vivo*, we disrupted the *α-pv* gene by homologous recombination in embryonic stem (ES) cells and generated α-pv mutant mice (Supplementary Figure 1A and B). Mice heterozygous for the α-pv null mutation (α-pv$^{+/-}$) were viable and phenotypically normal (data not shown). α-pv$^{+/-}$ intercrosses failed to yield newborn α-pv homozygous mutant (α-pv$^{-/-}$) mice. Timed mating of α-pv$^{+/-}$ intercrosses showed that α-pv$^{-/-}$ mice were present at the expected Mendelian ratio up to embryonic day (E) 11.5 (Supplementary Table 1). Lethality of α-pv$^{-/-}$ mice commenced at around E10.5 and no alive α-pv$^{-/-}$ mice were found later than E14.5 (Supplementary Table 1). Western blot analysis of E9.5 α-pv$^{-/-}$ embryo and yolk sac (YS) lysates showed loss of α-pv expression, unaltered or increased levels of β-pv and slightly decreased levels of ILK and PINCH1 when compared with wild-type (wt) lysates (Supplementary Figure 1C and D).

Development of α-pv$^{-/-}$ embryos was normal until E9.5 (data not shown). Growth retardation was first evident in E10.5 α-pv$^{-/-}$ embryos (Figure 1A). α-pv$^{-/-}$ embryos displayed different degrees of cardiovascular abnormalities including aberrant vascular beds with dilated blood vessels and pericardial effusion (Figure 1A and B; Supplementary Figure 2A). By E12.5, α-pv$^{-/-}$ embryos showed whole-body edema and severe bleedings due to vessel rupture (Figure 1A and B; Supplementary Figure 2B).

Aberrant cardiac morphogenesis and disrupted sarcomeric integrity in α-pv$^{-/-}$ embryos

To examine the heart abnormalities, we performed histological analysis of serial sections of wt and α-pv$^{-/-}$ embryonic hearts. Heart chambers developed normally in α-pv$^{-/-}$ embryos (data not shown). However, although E12.5 wt embryos showed septation of the truncus arteriosus resulting in an ascending aorta and pulmonary trunk (Figure 1C), α-pv$^{-/-}$ embryos showed a defective septation of the truncus arteriosus leading to a persistent single outflow tract (OFT) (Figure 1C).

Cell-ECM adhesion mediated by integrins is required to stabilize myofibrils (Fässler *et al*, 1996). Immunostaining of α-actinin and desmin, important Z-disc proteins that cross-link sarcomeric actin and connect Z-discs with adjacent myofibrils, showed reduced numbers of cardiomyofribrils in α-pv$^{-/-}$ embryos compared with wt littermates (Figure 1D and data not shown). Moreover, wt cardiomyocytes were elongated and aligned in a parallel manner, while α-pv$^{-/-}$ cardiomyocytes were round and distributed in a random pattern (Figure 1D). In line with earlier reports (Chen *et al*, 2005), we found that α-pv is localized at FAs as well as at Z-disc of the sarcomeres in normal cardiomyocytes (data not shown). Together, these data indicate that α-pv is required for the development of the OFT of the heart and for maintaining the structure and stability of sarcomeres, but not for initiating cardiomyofibrillogenesis. Because of the well-known functions of integrins, ILK and parvins for cardiac muscle morphogenesis and function (Fässler *et al*, 1996; Chen *et al*, 2005; Bendig *et al*, 2006), we focused our further analysis on the vascular defects.

Defective MC coverage in α-pv$^{-/-}$ embryos

To study the vascular abnormalities of α-pv$^{-/-}$ embryos in more detail, we performed whole mount immunostaining of wt and α-pv$^{-/-}$ embryos and YSs using antibodies against CD31, αSMA and anti-neuron glial 2 (NG2) to visualize ECs and MCs, respectively. Immunostaining for CD31 revealed the presence of a vascular plexus with microvessel and macrovessel containing blood cells in E10.5 and E11.5 α-pv$^{-/-}$ embryos and YSs (Figure 2A; Supplementary Figure 2C). In addition, isolated primary ECs from α-pv$^{-/-}$ YSs did not show differences in their ability to adhere and spread on collagen I (Col I) and FN (Supplementary Figure 2D and E and data not shown). Furthermore, ECs differentiated from α-pv$^{-/-}$ ES cells were able to migrate and form blood vessel-like structures comparable to wt ECs (Supplementary Figure 2F), suggesting that the vascular defects do not arise from defects in ECs.

However, α-pv$^{-/-}$ vessels were frequently enlarged and exhibited multiple microaneurysms, whereas in other areas they were constricted (Figure 2A–C; Supplementary Figure 3A). Vessel enlargement was associated with reduced MC coverage (Figure 2A; Supplementary Figure 3B). MCs directly interact with ECs and regulate vessel sprouting. Consistently with this function, the number of vascular sprouts was significantly increased in the vascular plexus of α-pv$^{-/-}$ embryos (Supplementary Figure 3C and D).

To assess the defect in MC coverage, we analysed the hindbrain vasculature, which is particularly rich in these cells (Abramsson *et al*, 2007). Quantitative analysis of EC coverage by PCs revealed that in wt embryos, the PCs were

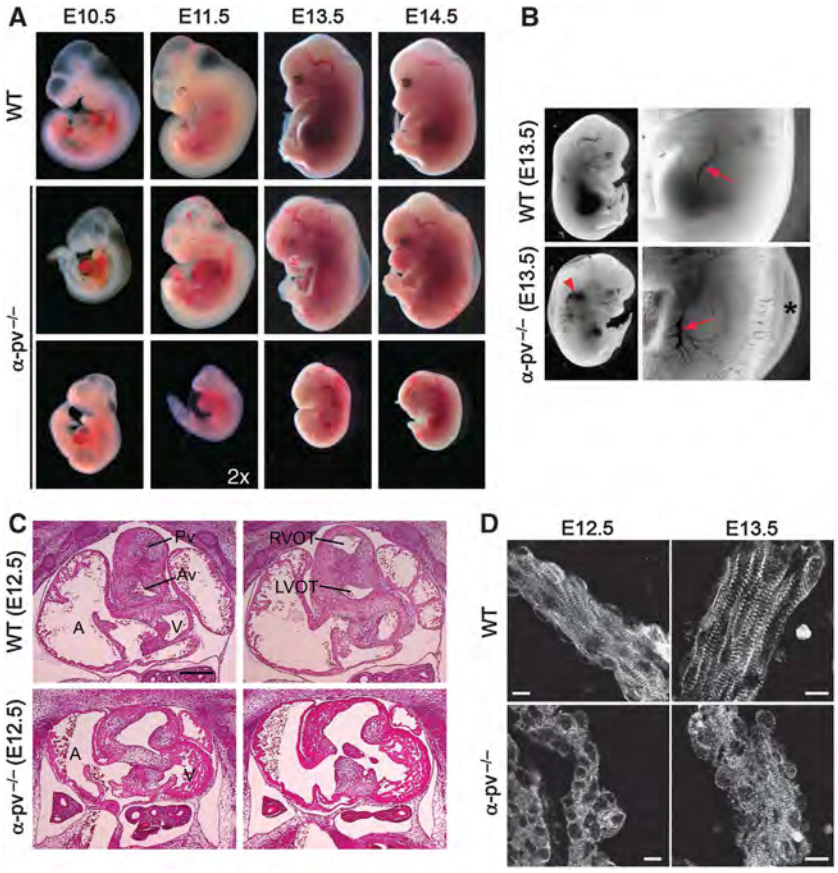

Figure 1 Embryonic lethality and cardiovascular defects in the absence of α-pv expression. (**A**) Gross examination of wt and α-pv$^{-/-}$ embryos. (**B**) α-pv$^{-/-}$ embryos display bleedings (arrowhead), whole-body edema (asterisks) and enlarged vessels (arrows). (**C**) Hematoxylin and eosin staining of frontal sections through the heart outflow tract (OFT) of E12.5 wt and α-pv$^{-/-}$ embryos. Bar: 50 μm. (**D**) Confocal sections of E12.5-13.5 hearts from wt and α-pv$^{-/-}$ embryos. Cardiomyocytes were labelled with α-actinin. Bar: 10 μm. A, atrium; V, ventricle; Av, aortic valve; Pv, pulmonary valve; RVOT, right ventricular OFT; LVOT, left ventricular OFT.

tightly associated with ECs and the relative proportion of endothelial staining (CD31) overlapping with PC staining (NG2) per total vessel area was 58% (±9.6) (Figure 2D and E). In contrast, the PCs of α-pv$^{-/-}$ embryos frequently stretched away from the endothelium, and covered only 23% (±2.4) of the endothelial area (Figure 2D and E). Furthermore, analysis of the distance from the growing endothelial edge to the closest PCs revealed that in E10.5 wt embryos, the PCs arrived to the growing front of the vascular plexus marked by the tip cells, whereas in α-pv$^{-/-}$ littermates PCs failed to reach the growing vascular front (Figure 2F). No differences could be detected in E11.5 embryos, indicating that lack of α-pv is a result of a delay in PC recruitment *in vivo* (Figure 2F). Poor MC coverage and impaired MC/EC association were also observed in blood vessels of α-pv$^{-/-}$ YSs and placentas (Supplementary Figure 4A and B). Collectively, these results indicate that α-pv is dispensable for vasculogenesis, but required for vascular maturation and MC investment into vessel walls.

α-pv regulates spreading and polarity of MCs

Successful MC coverage of the vascular bed depends on a number of factors including proliferation, survival, spreading and migration (Beck and D'Amore, 1997). We found no apparent defects in MC proliferation or increased apoptosis of E9.5, E10.5, E11.5 and E12.5 α-pv$^{-/-}$ embryos (Supplementary Figure 5 and data not shown). However, in contrast to wt vSMCs/PCs, which spread and showed strong and often punctate αSMA staining at the plasma membrane and thin filamentous staining in the cytoplasm (Figure 3A and B), α-pv$^{-/-}$ vSMC/PCs displayed a round shape and showed thick αSMA-positive actin bundles traversing the cytoplasm in a criss-cross manner (Figure 3A and B; Supplementary Figure 6).

To analyse this defect in more detail, we isolated SMA-positive cells from wt and α-pv$^{-/-}$ YSs and tested their ability to adhere and spread on ECM proteins. No difference in the ability of wt and α-pv$^{-/-}$ cells to adhere to laminin-111 (LN111), FN or Col I was observed (Figure 3C). In addition, the cell surface expression pattern of integrins was also unaltered (data not shown). However, despite their ability to adhere to ECM substrates, α-pv$^{-/-}$ cells remained round even after an overnight culture and developed multiple membrane protrusions, whereas wt cells spread within 60 min after plating (Figure 3D and E). Live video microscopy showed that wt cells were able to extend lamellipodia and spread to adopt a flattened shape. In contrast, α-pv$^{-/-}$ cells displayed continuous, highly dynamic and instable membrane ruffling at the cell cortex, leading to the formation of multiple retraction fibres (Supplementary Movies 1 and 2). To further analyse the morphology of the cells, we performed computational analysis of the cell shape (shape factor;

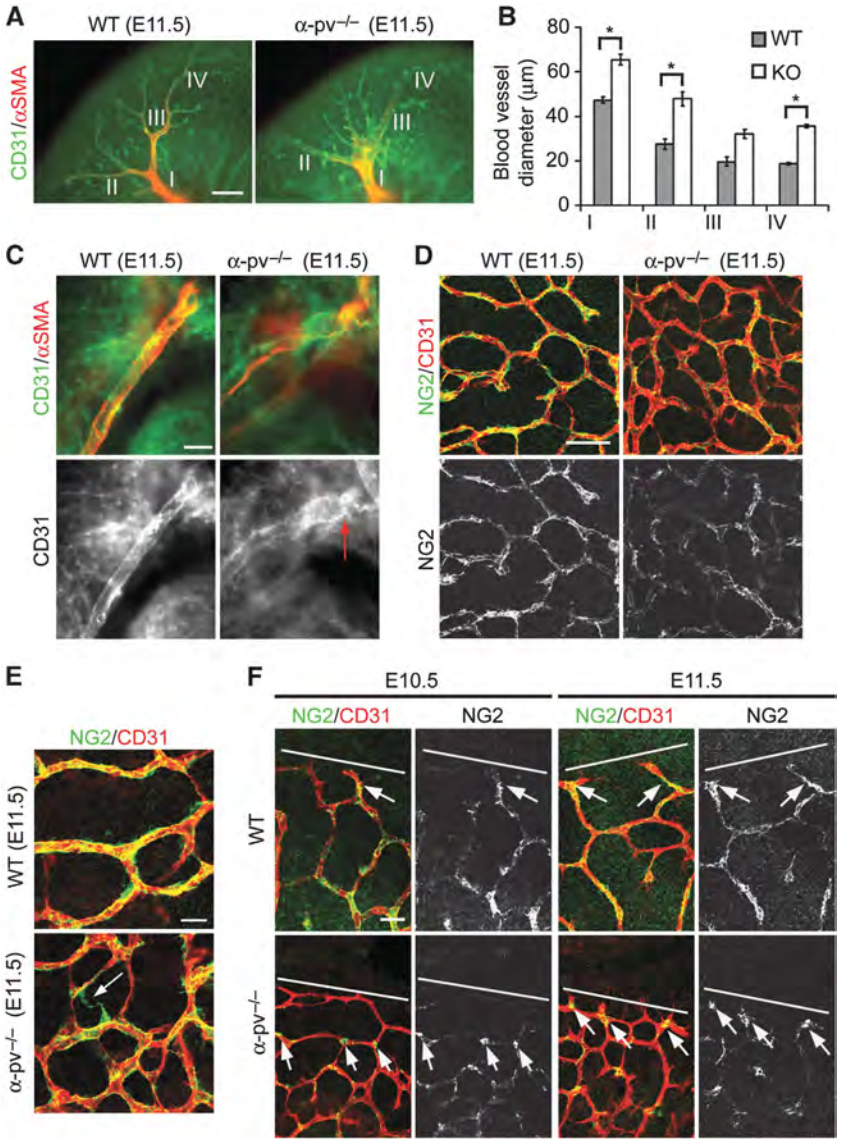

Figure 2 Reduced MC coverage and defective MC/EC association in the absence of α-pv$^{-/-}$. (**A**) Whole mount immunostaining of the head vasculature. ECs were labelled with CD31 (green) and vSMC with αSMA (red). Bar: 0.2 mm. (**B**) Quantification of the diameter of the macrovessels (I) and microvessel (II, III, IV) from the head vasculature. Values are mean ± s.e.m.; *P = 0.012 (I); *P = 0.039 (II); P = 0.091 (III); *P = 0.01 (IV). (**C**) CD31 and αSMA whole mount immunostaining of the right subclavian artery. Note the defective vSMC coverage and the presence of local constriction (red arrows) of α-pv$^{-/-}$ vasculature. Bar: 50 μm. (D–F) Whole mount immunostaining of E10.5-115 hindbrain endothelium CD31 (red) and associated PCs (green) from wt and α-pv$^{-/-}$ embryos. Bar: 50 μm (D) and 20 μm (E, F). (**D**) Reduced MC coverage in the peripheral region of the hindbrain of α-pv$^{-/-}$ embryos. (**E**) Defective MC/EC association in α-pv$^{-/-}$ vasculature (arrow). (**F**) Delayed PC recruitment in α-pv$^{-/-}$ embryos. The distance of the endothelial edge (line) to the closest PC (arrows) is labelled.

described in detail in Materials and methods), of individual cells plated for 3 h on FN. We found that >90% of the wt cells reached a shape factor value between 0.25 and 0.75, indicating that they contain a single broad lamella (Figure 3F). In contrast, more than half of the α-pv$^{-/-}$ cells adopted a value below 0.25, which corresponds to an unpolarized cell with either an elongated or a highly complex outline of the plasma membrane (Figure 3F). Importantly, the spreading and shape defects were rescued by re-expressing α-pv-GFP in α-pv$^{-/-}$ SMA-positive cells (Figure 3G–I).

Sustained cell contraction leads to retraction, followed by cell rounding and formation of retraction fibres, which are, unlike stress fibres, not associated with myosin (M) (Cramer and Mitchison, 1995). To examine whether cell spreading, shape and polarity defects of the α-pv$^{-/-}$ cells were a consequence of abnormal cell contraction, we performed double immunostaining of wt and α-pv$^{-/-}$ cells using anti-αSMA and anti-M-II antibodies and found that the actin protrusions of α-pv$^{-/-}$ cells lacked M-II (Figure 4A). The majority of the M-II signal was observed in the thick cortical actin bundles of the α-pv$^{-/-}$ cells, whereas stress fibres were positive in wt cells (Figure 4A). These data further indicate that the actin protrusions of α-pv$^{-/-}$ cells are retraction fibres.

α-pv controls RhoA/ROCK-mediated cell contraction

The morphological analysis suggested that the α-pv$^{-/-}$ SMA-positive cells were highly contractile. Sustained contraction

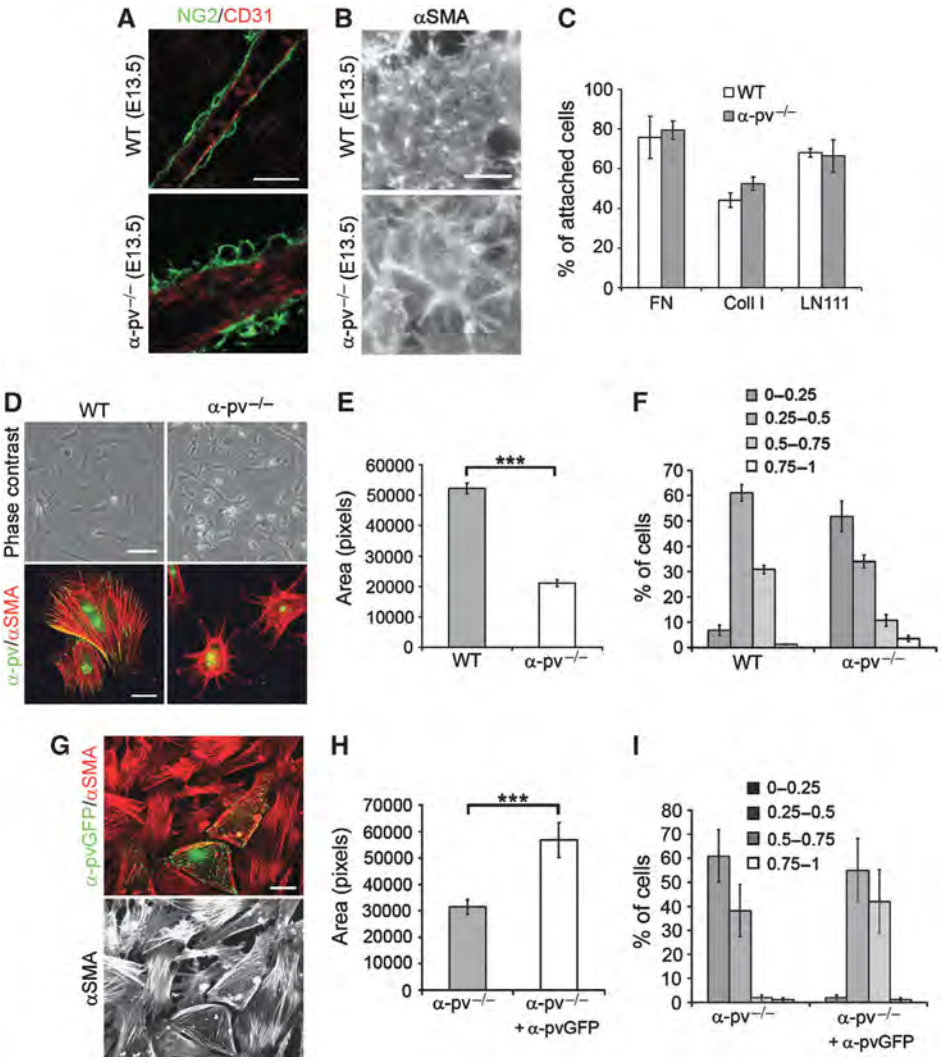

Figure 3 Impaired cell spreading and cell shape of MCs in the absence of α-pv$^{-/-}$. (**A**) Whole mount immunostaining of vasculature of E13.5 YSs. ECs were labelled with CD31 and MCs with NG2. Bar: 20 μm. (**B**) Whole mount immunostaining of vasculature of E13.5 YSs. MCs were labelled with αSMA. Bar: 10 μm. (**C**) Adhesion assay of isolated wt and α-pv$^{-/-}$ SMA-positive cells on different ECM substrates. Values are mean ± s.e.m. (**D**) Brightfield images and immunofluorescence staining for αSMA (red) and α-pv (green) of wt and α-pv $^{-/-}$ SMA-positive cells seeded of FN (10 μg/ml). Bar: 50 and 10 μm, respectively. (**E**) Quantification of cell area and (**F**) shape factor of wt and α-pv$^{-/-}$ SMA-positive cells. Values are mean ± s.e.m; ***$P = 0.0001$ (**G–I**) Re-expression of α-pv-GFP restores size and shape of α-pv$^{-/-}$ SMA-positive cells. Green represents the EGFP signal and the cytoskeleton is visualized with αSMA (red). Bar: 20 μm. Values are mean ± s.e.m; ***$P = 0.0001$.

of vSMCs can be mediated by the RhoA/ROCK/MLC2 signalling pathway (Karnam, 2006). To determine RhoA activity in primary vSMCs, we measured Rho-GTP binding to the Rhotekin-Rho binding domain and found a significant increase in RhoA-GTP levels in primary α-pv$^{-/-}$ cells (Figure 4B). Consistent with the increased RhoA activity, α-pv$^{-/-}$ cells showed higher levels of phospho-MLC2 than wt cells (Figure 4C). Increased levels of RhoA-GTP and elevated levels of phospho-MLC2 were also observed in immortalized SMA-positive cells derived from E9.5 α-pv$^{-/-}$ embryos (Figure 4D and E; Supplementary Figure 7A and B). Re-expression of α-pv in the immortalized cells induced normal cell spreading, accompanied by loss of retraction fibres and reduced RhoA-GTP and phospho-MLC2 levels (Figure 4D and E; Supplementary Figure 7A–D). Importantly, immunostaining of tissue sections of YSs also revealed elevated MLC2 phosphorylation in vSMC/PCs in the vascular plexus of E13.5 α-pv$^{-/-}$ embryos (Figure 4F), indicating that activation of the RhoA/ROCK/MLC2 signalling pathway is also elevated *in vivo*.

To test whether increased RhoA and MLC2 activity leads to increased contractile properties of vSMCs, we seeded wt or α-pv$^{-/-}$ cells in 3D collagen gels and observed gel contraction over a time period of 48 h. Indeed, α-pv$^{-/-}$ cells showed a two-fold increase in gel contraction compared with wt cells (Figure 5A and B).

Consistent with increased RhoA/ROCK signalling, addition of Y-27632, an inhibitor of ROCK, to collagen gels normalized the collagen gel contraction (Figure 5B). Interestingly, treatment of α-pv$^{-/-}$ cells with ML-9, an inhibitor of MLCK, did not change the contractile properties of α-pv$^{-/-}$ cells (data not shown). Finally, when cells were cultured in the presence of Y-27632, wt and α-pv$^{-/-}$ cells displayed similar morphology (Figure 5C and D). Collectively, these results indicate that α-pv controls vSMC contraction through negatively regulating the RhoA/ROCK/MLC2 signalling pathway.

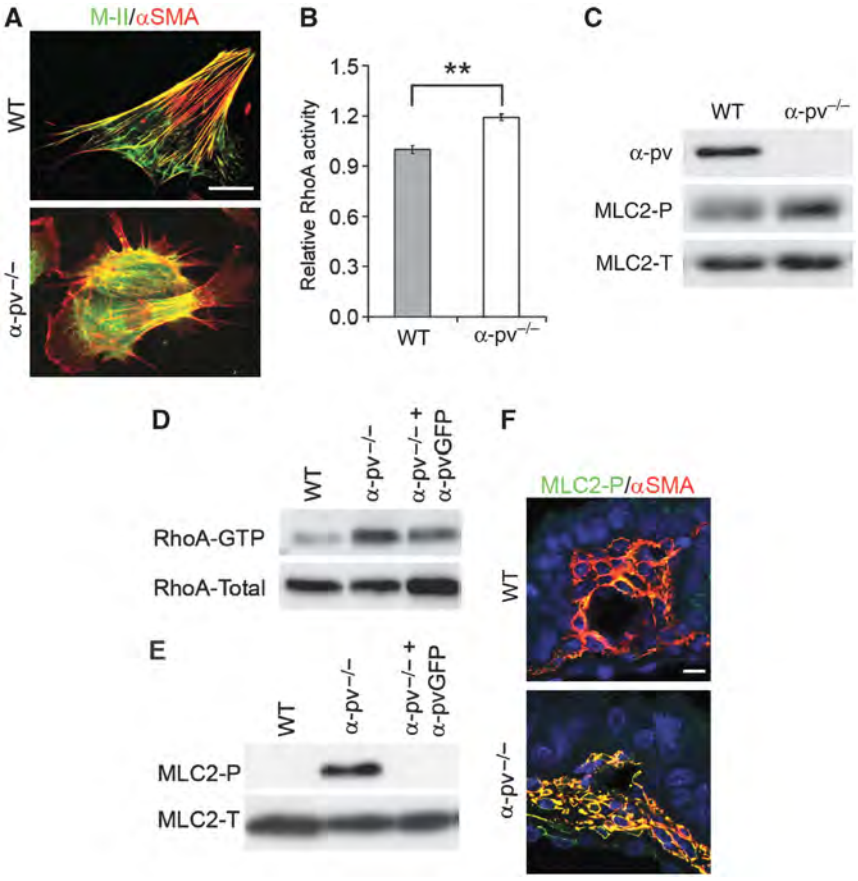

Figure 4 Elevated RhoA activity in the absence of α-pv. (**A**) Immunofluorescence staining for αSMA (red) and M-II (green) of wt and α-pv$^{-/-}$ SMA-positive cells. Note that actin protrusions of α-pv$^{-/-}$ cells lack M-II staining. Bar: 25 μm. Freshly isolated α-pv$^{-/-}$ SMA-positive cells show increased RhoA activity. Values are mean +/− s.e.m.; $^{**}P = 0.0012$ (**B**) and increased phosphorylation of MLC2 (**C**). Total MLC2 served as loading control. Immortalized α-pv$^{-/-}$ SMA-positive cells show increased RhoA activity (**D**) and increased phosphorylation of MLC2 (**E**). (**F**) Immunofluorescence analysis of E13.5 YS sections with αSMA (red) and phospho-MLC2 (green) shows increased phosphorylation of MLC2 *in vivo*. Bar: 20 μm.

α-pv controls MC recruitment and directed cell migration

MC recruitment to angiogenic vessels depends on platelet-derived growth factor BB (PDGF-BB)-mediated directed migration (Hellström *et al*, 1999; Abramsson *et al*, 2007). As we observed defects in this process, we next determined whether α-pv$^{-/-}$ SMA-positive cells were able to respond to PDGF-BB. To this end, we stimulated serum-starved primary wt and α-pv$^{-/-}$ cells with 100 ng/ml PDGF-BB and measured the phosphorylation levels of downstream signalling molecules. PDGF-BB treatment triggered a comparable increase in Erk and Akt phosphorylation in wt and α-pv$^{-/-}$ cells, indicating that loss of α-pv did not impair PDGFR signalling *per se* (Figure 6A and data not shown). To test whether α-pv$^{-/-}$ cells were still capable of migrating towards a source of PDGF-BB, we performed both chemokinesis and chemotaxis assays using Transwell motility chambers and found that α-pv$^{-/-}$ cells exhibited accelerated rates of random chemokinetic migration, but migrated less efficiently towards a PDGF-BB gradient compared with wt cells (Figure 6B and C). Similar results were observed in cells migrating to a source of serum (data not shown). These data indicate that α-pv is required for persistent directed migration.

Cell polarity is essential for directed migration. Live video microscopy over a period of 12 h showed that wt cells extended stable lamellipodia in the direction of the movement, while α-pv$^{-/-}$ cells continuously formed lamellipodia-like protrusions that were highly instable and appeared randomly at different parts of the cells, causing continuous changes in the direction of cell movement (Supplementary Movies 3 and 4). Similar migration behaviour was observed in the immortalized cells, whereas re-expression of α-pv restored normal cell motility (Supplementary Movies 5, 6 and 7). Tracking of individual cells combined with statistical analysis confirmed that α-pv$^{-/-}$ cells moved significantly less persistently than wt cells (Figure 6D). In the presence of Y-27632, α-pv$^{-/-}$ cells formed stable lamellipodia, and the persistence of cell motility was restored to the level of wt cells (Figure 6D; Supplementary Movies 8 and 9).

Rac activity is essential for the establishment of lamellipodia and directed cell migration. It has been shown that α-pv can regulate Rac activity in HeLa and osteosarcoma cells by controlling the activity of the Rac/Cdc42 GAP CdGAP (LaLonde *et al*, 2006). To assess whether dysregulation of Rac activity is also involved in the altered lamellipodial dynamics of α-pv$^{-/-}$ SMA-positive cells, we determined Rac activity in immortalized cells during the first 90 min of spreading on FN. Adhesion to FN induced a transient increase in Rac-GTP levels that peaked after 10 min of spreading and was comparable in wt and α-pv$^{-/-}$ cells (Supplementary

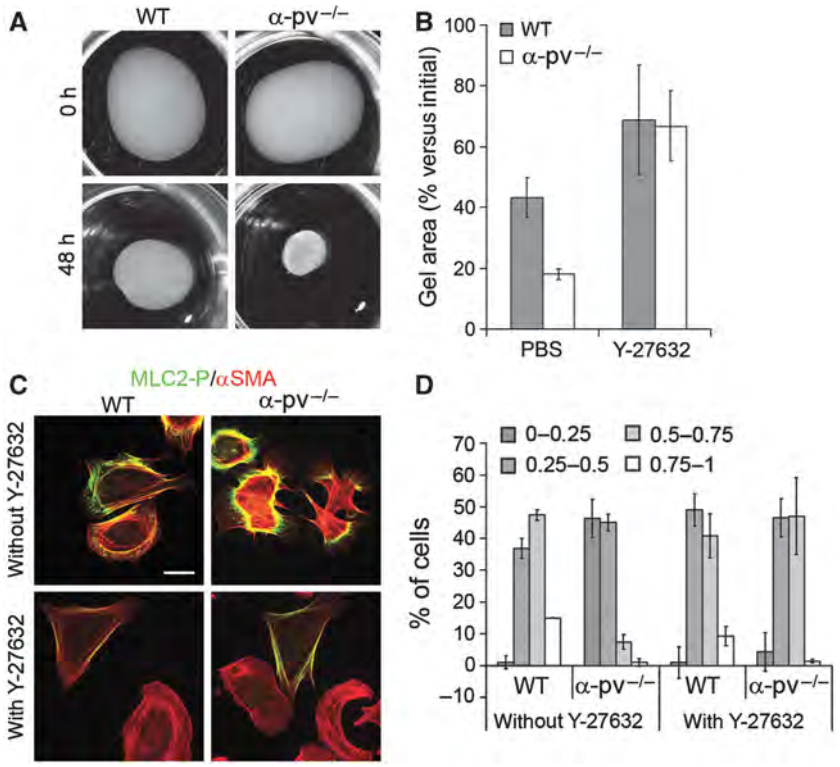

Figure 5 Increased collagen matrix contraction in the absence of α-pv. (**A**) Three-dimensional collagen gel containing wt and α-pv$^{-/-}$ SMA-positive cells. (**B**) Quantification of collagen gel area. α-pv$^{-/-}$ SMA-positive cells display higher collagen matrix contraction capacity than wt cells. Note that in the presence of ROCK inhibitor Y-27632 (1.5 μM) wt and α-pv$^{-/-}$ cells show similar contraction capacity. (**C**) Wt and α-pv$^{-/-}$ SMA-positive cells seeded on FN for 15 min in the absence and presence of Y-27632 (1.5 μM) and stained with αSMA (red) and phospho-MLC2 (green). Bar: 25 μm. (**D**) Quantification of shape factor of wt and α-pv$^{-/-}$ SMA-positive cells in the absence and presence of Y-27632. Values are mean ± s.e.m.

Figure 7E and F). Although in wt cells Rac-GTP levels remained stable for 30 min and gradually decreased to basal levels after 90 min of spreading, α-pv$^{-/-}$ cells showed a rapid decrease in Rac-GTP levels to baseline levels already after 10 min (Supplementary Figure 7E and F). The early decrease of Rac-GTP levels in α-pv$^{-/-}$ cells correlated with a strong increase in RhoA activity at this time point (Supplementary Figure 7G and H). Moreover, Y-27632-mediated inhibition of ROCK normalized Rac activity in α-pv$^{-/-}$ cells (Supplementary Figure 7I). These results indicate that Rac activation occurs normally in α-pv$^{-/-}$ cells, but its sustained activation is suppressed through elevated RhoA/ROCK activity.

α-pv-mediated regulation of RhoA is specific to MCs and is not compensated by β-pv

The interaction of parvins with ILK is necessary to target both proteins to FAs and to prevent degradation of the IPP complex (Legate *et al*, 2006). As the IPP complex is an important regulator of cell shape and cell migration, we tested whether loss of α-pv affects the stability and subcellular localization of the other members of the complex. WB analysis revealed normal levels of ILK and PINCH1 in α-pv$^{-/-}$ SMA-positive cells, whereas the levels of β-pv were increased when compared with wt cells (Figure 7A). Immunostaining showed normal ILK localization in FAs (Figure 7B). In addition, immunoprecipitation experiments from wt and α-pv$^{-/-}$ cells showed that ILK co-precipitated with β-pv both in the presence and absence of α-pv (Figure 7C). Furthermore, in wt cells both α- and β-pv associated with ILK, but not with each other (Figure 7C).

To assess whether the function of α-pv in regulating cell contractility is specific for vSMCs, we isolated and investigated wt and α-pv$^{-/-}$ fibroblasts (Supplementary Figure 8A). Like ECs, fibroblast lacking α-pv did not show apparent defects in spreading and actin cytoskeleton organization. In addition, there was no apparent difference in the levels of MLC2 phosphorylation in these cells (Supplementary Figure 8B and data not shown).

It has been suggested that ILK modulates vSMC contractility by directly phosphorylating MLC (Wilson *et al*, 2005). To test whether loss of α-pv could lead to hyperphosphorylation of MLC through ILK, we depleted ILK in wt cells using siRNA and found increased levels of MLC2 phosphorylation and reduced levels of α-pv (Figure 7D). To further confirm that the elevated RhoA/ROCK signalling was due to the loss of α-pv and not due to an upregulation of β-pv, we depleted β-pv in wt and α-pv$^{-/-}$ cells. Depletion of β-pv had no effect on MLC2 phosphorylation (Figure 7E), whereas depletion of α-pv in wt cells increased the levels of MLC2 phosphorylation (Figure 7F). Together, these data suggest that (i) β-pv can stabilize ILK and PINCH1 protein levels and localize them into FAs in the absence of α-pv, (ii) α- and β-pv exist in separate complexes containing ILK, (iii) the spreading and shape defects are exclusively due to the absence of α-pv and (iv) the ILK/α-pv complex is a negative regulator of MLC2 phosphorylation in these cells.

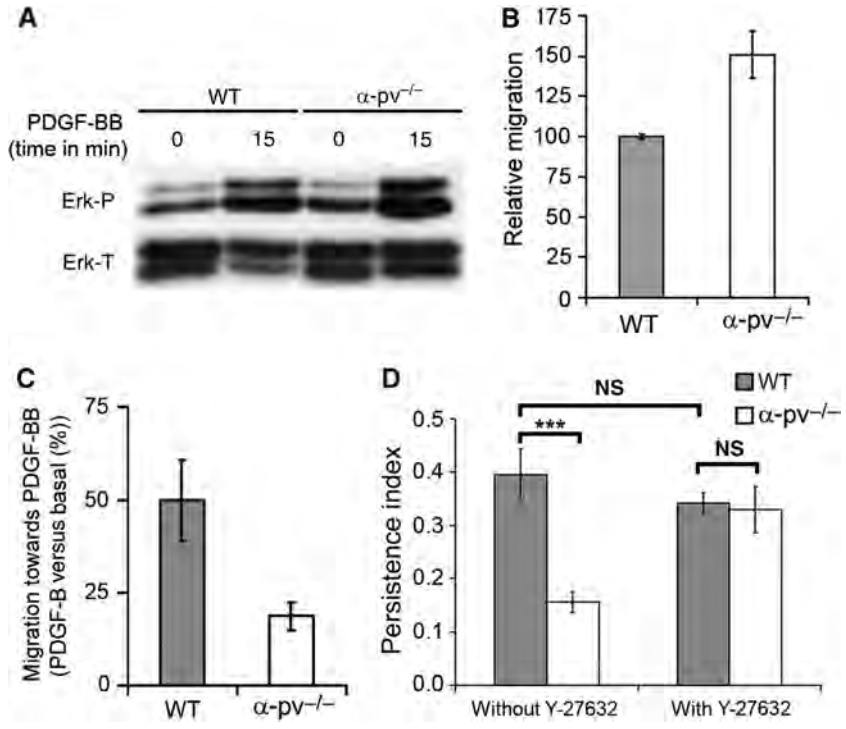

Figure 6 Normal PDGF-BB signalling but impaired directed cell migration of α-pv$^{-/-}$ SMA-positive cells. (**A**) Wt and α-pv$^{-/-}$ SMA-positive cells stimulated for 15 min with 100 ng/ml PDGF-BB show similar phosphorylation of Erk. Total Erk served as loading control. (**B**) Quantification of chemokinetic migration. Note that α-pv$^{-/-}$ cells display higher rates of random migration compared with wt cells. (**C**) Quantification of chemotactic migration using 20 ng/ml PDGF-BB as chemoattractant (24 h). Control medium without PDGF-BB was used to assess baseline migration. Note that although all cells migrated towards PDGF-BB, the ratio of stimulated/unstimulated migration is reduced in α-pv$^{-/-}$ cells. (**D**) Quantification of persistent motility of wt and α-pv$^{-/-}$ vSMCs (12 h) seeded on FN. Note that α-pv$^{-/-}$ cells move less persistently than wt cells, whereas in the presence of Y-27632 (0.5 μM), wt and cells display similar persistent motility. Values are mean ± s.e.m; ***$P = 0.0001$; NS, not significant.

Discussion

The results of our study show that α-pv is a central regulator of vascular maturation and blood vessel stability. Disruption of the α-pv gene in mice results in embryonic lethality due to severe cardiovascular defects. In the absence of α-pv, vascular beds are aberrantly organized and abnormally covered by MCs. α-pv$^{-/-}$ vSMCs display defects in cell spreading, polarity and directed migration. These cellular defects are caused by increased RhoA/ROCK activity that leads to elevated MLC2 phosphorylation and aberrant cell contractility.

The pump function of the heart has a critical role in embryonic development, growth and survival by transporting oxygen and nutrients through the vascular network and by promoting organogenesis such as vascular remodelling and formation of haematopoietic stem cells (Lucitti *et al*, 2007; Adamo *et al*, 2009; North *et al*, 2009). Therefore, severely impaired heart function caused either by defects during heart morphogenesis or by abnormal cardiomyocyte organization and sarcomere assembly can lead to embryonic lethality as early as E10.5-12.5 (Huang *et al*, 2003). Integrin-mediated cell adhesion has an essential function during cardiac development (Sengbusch *et al*, 2002). In this study, we report that α-pv is required for the remodelling of the OFT and formation and/or stability of cardiomyofribrils, which likely contributes to the early embryonic lethality of the α-pv$^{-/-}$ mice. These observations are in line with earlier studies reporting that β1 integrins, ILK and parvins have an essential function in heart development, cardiomyocyte contraction and integrity in different model organisms (Fässler *et al*, 1996; Chen *et al*, 2005; Bendig *et al*, 2006).

Deletion of α-pv also leads to severe vascular defects, including impaired vascular remodelling, vessel dilatation, formation of microaneurysms and vessel rupture. As cell type-specific ablation of the ILK gene in mice revealed an important function for ILK in vascular development and EC survival (Friedrich *et al*, 2004), it was important to determine whether α-pv would act in co-operation with ILK to regulate the endothelium. Interestingly, we found no increase in apoptosis of α-pv$^{-/-}$ ECs. Furthermore, α-pv$^{-/-}$ ECs adhere, spread and are able to migrate and form vascular networks comparable to wt ECs. This suggests that the defects in the vascular endothelium of α-pv$^{-/-}$ embryos are likely cell non-autonomous and arise from cells that regulate vascular remodelling and stability. In line with this hypothesis, we observed vascular abnormalities already in E10.5 embryos, a developmental stage when flow-dependent remodelling defects are still of minor importance. Although this observation indicates that vascular abnormalities develop independent of the heart defect, we need to determine the contribution of the defective heart function on the abnormal vascular remodelling (Lucitti *et al*, 2007) at later developmental stages by deleting the α-pv gene exclusively in cardiomyocytes using the Cre/loxP system. To this end, we have generated α-pv floxed mice.

Functional blood vessels consist of endothelial tubes surrounded by tightly associated and organized vSMC/PCs that provide stability to vessels. Dysfunction of vSMC/PCs is

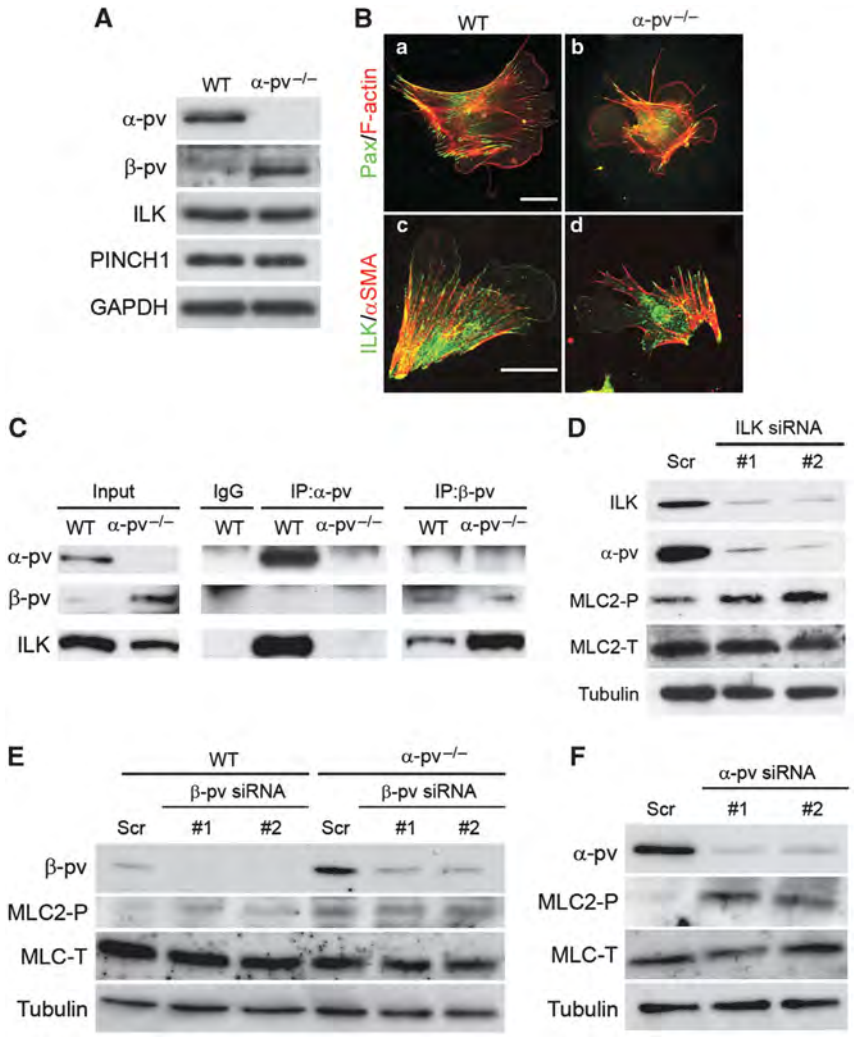

Figure 7 ILK/α-pv complex is a negative regulator of MC contractility. (**A**) Western blot analysis of IPP proteins in wt and α-pv$^{-/-}$ SMA-positive cells. Note that α-pv$^{-/-}$ cells show similar protein levels of ILK and PINCH1, and increased protein levels of β-pv compared with wt. (**B**) Immunofluorescence staining for paxillin (green) and F-actin (red) (a, b) and ILK (green) and αSMA (red) (c, d) of wt and α-pv$^{-/-}$ SMA-positive cells seeded of FN. Bar: 25 μm. (**C**) Immunoprecipitation experiments from wt and α-pv$^{-/-}$ SMA-positive cells. Note that both α- and β-pv co-immunoprecipitate with ILK but not with each other. (**D**) ILK depletion by two siRNA duplexes in wt cells induces increased MLC2 phosphorylation accompanied by reduced levels of α-pv. (**E**) β-pv depletion by two siRNA duplexes in wt and α-pv$^{-/-}$ cells does not change the levels of MLC2 phosphorylation. (**F**) α-pv depletion by two siRNA duplexes in wt cells induces increased MLC2 phosphorylation.

associated with dilated vessels, formation of microaneurysms and vessel wall disruption (Hellström *et al*, 2001; Boucher *et al*, 2003). The same defects were also observed in α-pv$^{-/-}$ embryos. Consistently, the endothelial tubes of α-pv$^{-/-}$ embryos are poorly covered with vSMC/PCs, and the few recruited cells fail to properly spread around the endothelial tube. To surround and stabilize the vascular endothelium, MCs have to proliferate and migrate to vessels. Integrin-mediated adhesion has an important function in both of these functions. This was shown by deleting the β1 integrin gene in MCs, which leads to aberrant proliferation of these cells and deficient vessel wall stability (Abraham *et al*, 2008), and by ablating the α4 integrin subunit, which impairs migration of vSMCs/PCs to developing blood vessels (Grazioli *et al*, 2006). We found no requirement of α-pv for proliferation or survival of vSMC/PCs indicating that the poor vSMC/PCs investment is not caused by a deficiency of vSMC/PCs. This finding also suggests that β1 integrin function is not completely compromised in the absence of α-pv, a notion further supported by the findings that α-pv$^{-/-}$ cells adhere normally to various ECM substrates and that ILK is normally recruited to integrin adhesion sites. Moreover, the restricted phenotype of the α-pv$^{-/-}$ mice to the cardiovascular system indicates that α-pv is indispensable for only a specific subset of signals downstream of β1 integrin.

Cell migration is directed by gradients of chemoattractants and/or repulsive molecules as well as by ECM molecules that serve as haptotactic tracks. During vascular development, MCs are attracted by a gradient of PDGF-BB produced by ECs to migrate directionally towards newly formed vessels (Hellström *et al*, 1999; Abramsson *et al*, 2007). Our results show that α-pv$^{-/-}$ MCs are improperly recruited to the vascular endothelium and that α-pv$^{-/-}$ vSMC-like cells display accelerated random motility but migrate less efficiently towards PDGF-BB or towards a serum source. Interestingly, α-pv$^{-/-}$ vSMCs are still capable of responding to PDGF-BB stimulation by activating mitogenic signalling pathways. These findings suggest that α-pv does not control recruitment

of MCs through regulating PDGFR signalling *per se*, but rather by regulating the molecular machinery required for efficient directional motility of MCs.

In response to a pro-migratory stimulus, cells polarize in the direction of the chemotactic gradient by extending a lamellipodium at the leading edge. Integrin-mediated adhesions then form directly behind the lamellipodium to stabilize the lamella and to exert a force between the ECM and the cytoskeleton required for the advance of the leading edge. To move the cell body forward, adhesions are subsequently disassembled at the cell rear, which is then pulled in the direction of motility by the contractile machinery of the actomyosin network (Ridley *et al*, 2003). Integrins and Rac establish a positive feedback loop within the leading edge that forms and maintains the lamella, whereas RhoA is necessary for the generation of contractile forces and tail retraction (Burridge and Wennerberg, 2004). Our results show that α-pv$^{-/-}$ vSMC fail to spread due to enhanced RhoA/ROCK activity leading to elevated MLC2 phosphorylation and increased cell contractility. This finding is consistent with earlier observations showing that cell spreading requires a transient downregulation of RhoA activity (Ren *et al*, 1999). Suppression of RhoA activity is necessary to promote lamellipodial protrusion during migration (Arthur *et al*, 2000; Arthur and Burridge, 2001). This is partly achieved by negative regulation of RhoA activity by Rac, which is transiently activated on cell adhesion to induce lamellipodia formation (Sander *et al*, 1999; del Pozo *et al*, 2000). On the other hand, RhoA activation restricts lamellae formation by inhibiting Rac activity (Tsuji *et al*, 2002; Worthylake and Burridge, 2003). We could also show that α-pv is not required for Rac activation induced by cell adhesion in vSMC-like cells. However, Rac activity is rapidly suppressed during the spreading of α-pv$^{-/-}$ SMA-positive cells. This downregulation occurs concomitantly with a robust upregulation of RhoA activity. These observations together with the finding that defects in cell spreading and in directional motility can be rescued with ROCK inhibitors and that the ROCK-inhibited cells are able to establish lamellipodia and normalize their Rac activity strongly suggests that the aberrant RhoA activity is the primary defect in α-pv$^{-/-}$ SMA-positive cells. ROCK-mediated activation of MLC2, which is located both at the leading edge and at the rear of the cell, leads to a global contraction of the cells and loss of cell polarity (Matsumura and Hartsthorne, 2008). Thus, as a consequence of the elevated RhoA activity and the subsequent suppression of Rac activity, α-pv$^{-/-}$ vSMC are not able to properly establish a stable leading edge and a cell rear, resulting in highly inefficient and non-directional cell motility.

The mechanisms by which integrin adhesion regulates RhoA activity are complex, and it seems that distinct integrin heterodimers use different strategies to regulate this activity, even in response to the same extracellular ligand (Danen *et al*, 2005). The parvin-binding partner ILK has been shown to negatively regulate RhoA activity (Yamazaki *et al*, 2009), but also to positively regulate vSMC contraction through direct phosphorylation of MLC2 (Wilson *et al*, 2005). The interaction of parvins with ILK is necessary to target both proteins to FAs and to prevent degradation of the IPP complex (Legate *et al*, 2006). However, ILK levels and localization to FAs are apparently not altered in α-pv$^{-/-}$ vSMCs. In addition, β-pv levels are upregulated in these cells, and β-pv, which also localizes to FAs, binds ILK also in the absence of α-pv. These results suggest that the β-pv upregulation acts to stabilize the IPP complex in the absence of α-pv, and is sufficient to localize this complex to FAs. However, we also show that α- and β-pv exist in separate complexes, and that the β-pv/ILK complex is unable to compensate for the loss of α-pv as a negative regulator of vSMC contractility. Furthermore, we found that depletion of ILK in wt SMA-positive cells triggers MLC2 phosphorylation accompanied by a dramatic reduction in α-pv levels, whereas the depletion of β-pv has no effect on MLC2 phosphorylation. Moreover, it has been observed that a vSMC/PCs specific-deletion of ILK leads to MCs dysfunction also associated with a RhoA-dependent hypercontractility phenotype (Kogata *et al*, 2009). This clearly indicates that ILK does not contribute to the hyperphosphorylation of MLC2 through directly phosphorylating MLC2 in α-pv$^{-/-}$ cells and that the recruitment of α-pv and ILK form a mechanosensory complex at FAs of contractile cell types such as vSMCs. This complex has an essential function in downregulating RhoA-dependent contractility to allow efficient cell spreading and migration. Consistent with our observations, a similar mechanism for ILK has been recently observed in the nervous system (Pereira *et al*, 2009). How the ILK/α-pv complex downregulates RhoA is not known. This might occur through α-pv-dependent recruitment of negative regulator(s) of the RhoA/ROCK/MLC2 signalling pathway to this complex. As we observed the RhoA downregulation only in certain cell types, the negative regulators recruited by the ILK/α-pv complex are likely to be expressed in a cell type-specific manner.

Materials and methods

Generation of α-parvin-deficient mice

A 400 bp α-parvin cDNA fragment derived from EST clone AI006605 was used to screen a 129/Sv mouse P1-derived artificial chromosome library. Five positive PAC clones were identified and used to generate the α-parvin targeting construct. To abolish α-parvin gene function, an IRES-β-galactosidase cassette and a neomycin resistance gene was inserted into exon 2. Genotyping of wt and recombinant alleles was performed by Southern blot using an external probe after PstI digestion of genomic DNA. Wt and mutant mice were genotyped by PCR using a three-primer system; forward 1 (F1) 5′-GGAATGAACGCCATCAACCT-3′, F2 5′-GATTAGATAAATGC CTGCTC-3′, reverse (R) 5′-TTGCGTGAGTTTGGATCGAC-3′.

Antibodies

The following antibodies were used: rabbit antibody against α-parvin (Chu *et al*, 2006); rabbit antibody against β-parvin (Chu *et al*, 2006); rat antibody against CD31 (PharMingen); rabbit antibody against desmin (Oldberg); Cy3-conjugated mouse antibody against αSMA (Sigma); rabbit antibody against anti-NG2 (Chemicon); mouse antibody against GAPDH (Calbiochem); rabbit antibody against ILK (Cell Signalling Technology); rabbit antibody against myosin light chain (MLC2) (Santa Cruz); rabbit antibody against phospho-MLC2 (Cell Signaling); mouse antibody against paxillin (Transduction Laboratories); Cy3-and FITC-conjugated antibodies specific for mouse IgG, rabbit IgG and rat IgG (Jackson Immunochemicals Laboratories Inc.; West Grove, PA, USA) were used as secondary antibodies. TRITC-conjugated phalloidin was used to detect F-actin (Molecular Probes; Eugene, OR, USA).

Isolation of SMA-positive cells

Embryos and YSs were harvested and washed in PBS. For each embryo, the tail was removed and used for genotyping. YSs were treated with Type I collagenase (2 mg/ml) (Invitrogen) in PBS at

37°C for 45 min. The tissue was then passed through a fine-tip Pasteur pipette with a 2-μm diameter. Cells were plated on tissue culture plates coated with 10 μg/ml FN and cultured in DMEM medium containing 10% fetal bovine serum and antibiotics. After 60 min of culture, the SMA-positive cells had attached to dish, and the medium containing visceral endoderm cells and ECs was carefully aspirated. After 2 days in culture, SMA-positive cells were used for experiments.

The same protocol was used to isolate SMA-positive cells from E9.5 embryos, which were subsequently immortalized using the SV40 T-large oncogene. SMA-positive cells were isolated and characterized by western blot and immunofluorescence analyses. To avoid changes in the contractile properties of the cells, experiments were performed with low (8–10) cell passages.

Transient expression of α-pv

α-pv-GFP was generated by cloning the murine α-pv cDNA (gift from Dr A Noegel, University of Cologne) into the pEGFP-C1 vector (Clontech). Primary cells were transiently transfected with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol.

Adhesion assay

Cells (1×10^5 cells/well) were plated onto 96-well plates coated with FN, LN or collagen I. After 45 min incubation, cells were lysed in a substrate buffer (7.5 mM NPAG (Sigma), 0.1 M Na citrate pH 5, 0.5% Triton X-100) over night at 37°C. The reaction was stopped by adding 50 mM Glycine pH 10.4, 5 mM EDTA after which OD 405 was measured.

Immunoprecipitation

Cells were lysed in lysis buffer (in 150 mM NaCl, 50 mM Tris pH 8, 10 mM EDTA, 1% Triton X-100, 0,05% sodium deoxycholate supplemented with protein inhibitors (Roche) and phosphatase inhibitors (Sigma)) and 0.5 mg of cell lysate was incubated with anti-α-parvin rabbit polyclonal or anti-β-parvin rabbit polyclonal antibodies for 30 min on ice. Immunocomplexes were then bound to protein G-beads (Sigma) for 1 h, washed in lysis buffer, resuspended in SDS sample buffer (Invitrogen) and analysed by SDS–PAGE.

RhoA activation assay and affinity precipitation of cellular GTP-Rho and Rac

Freshly isolated cells from the YSs were seeded on FN (10 μg/ml) for 10 min and a quantitative assay for RhoA activity was performed using G-LISA RhoA Activation Assay Biochem Kit following the manufacturer's instructions (Cytoskeleton, Inc., CO).

For affinity precipitation of GTP-Rho and Rac, immortalized cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, supplemented with protease inhibitors (Roche)). Cell lysates were clarified by centrifugation at 900 r.p.m. at 4°C for 10 min, and equal volumes of lysates were incubated with GST–Rhotekin (Rho) beads or GST-PAK-CRIB beads at 4°C for 60 min. The beads were washed four times with buffer B (50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, supplemented with protease inhibitors (Roche)). Bound Rho and Rac proteins were detected by western blotting using monoclonal antibodies against RhoA and Rac1 (Santa Cruz Biotechnology).

siRNA-mediated ILK and parvin depletions

siRNA duplexes for ILK, α-parvin, β-parvin and scrambled control were purchased from Sigma. Two siRNA duplexes (5′-CAGUGUAAU CGAUCGAUGAATT-3′ and 5′-CCAUAUGGAUCUCUCUACATT-3′ for ILK; 5′-CGACAAUGGUCGAUCCAAA-3′ and 5′-GAACAAGCAUCUGA AUAAA-3′ for α-parvin; 5′-CAAACACCUGAAUAAGCUA-3′ and 5′-CC UGACUCCUGACAGCUUU-3′ for β-parvin) were transfected into SMA-positive cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Experiments were carried out 48 h after transfection.

SDS–PAGE and immunoblotting

Cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris pH 7,4, 1 mM EDTA, 1% Triton X-100 supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma)), homogenized in Laemmli sample buffer and boiled for 5 min. Cell lysates were resolved by SDS–PAGE gels. Proteins were then electrophoretically transferred from gels onto nitrocellulose membranes, followed by incubation with antibodies. Bound antibodies were detected using enhanced chemiluminescence (Millipore Corporation, Billerica, USA).

Whole embryo immunohistochemistry

Staged embryos were dissected in PBS and genotyped by PCR. YSs and embryos were fixed overnight in fixation buffer (80% methanol, 20% DMSO). Samples were rehydrated in 0.1% Tween-20 in PBS, incubated in blocking buffer (10% goat serum, 5% BSA in PBS) for 2 h, and exposed to primary antibody overnight at 4°C. After 5–7 h wash with 0.1% Tween-20 in PBS, samples were incubated with secondary antibodies overnight at 4°C.

Histology of tissue sections, immunostaining and morphological analysis

Immunohistochemistry and immunofluorescence studies of embryos, YSs and cells were performed as described earlier in Montanez *et al* (2008). Analysis of vessel diameter was performed as described by Grazioli *et al* (2006). Three embryos of each genotype were analysed. The area and the shape factor (4πarea/perimeter^2) of cells were analysed using the MetaMorph software. The data represent three independent assays (150 cells/experiment).

Migration assay

Chemotactic and chemokinetic migration assays were performed in 3-μm pore size chamber inserts (BD Falcon). For chemotaxis assays, 4×10^4 cells were plated into the chamber and transferred into 24-well plates containing serum-free medium with or without 20 ng/ml PDGF-BB. For chemokinesis assays, 4×10^4 cells in serum-free medium with or without 20 ng/ml PDGF-BB were plated into the chamber and transferred into 24-well plates containing serum-free medium.

After overnight incubation, the cells in the bottom part of the chamber were stained with a crystal violet solution and counted. Five microscopic fields per chamber were analysed. Data are represented as percentage of total cell number/field ± s.d. The assay was performed in triplicate in three independent assays.

Collagen matrix contraction assay

Cells were suspended in a collagen mixture (1.6 mg/ml collagen I (INAMED), 7.5% NaHCO3 in cell culture medium (MEM)) with a final concentration of 4×10^5 cells/ml. A measure of 100 μl of the collagen-cell mixture was placed in a 24-well suspension culture plate (Greiner) and incubated for 1 h under standard cell culture conditions causing the polymerization of the collagen. Finally, 2 ml of cell culture medium (DMEM + 10% serum) was applied on top of the gel. The area of the collagen lattices was calculated after 24 and 48 h of culture. The assay was performed in triplicate in three independent assays.

Statistical analysis

The statistical analysis was performed using the Mann–Whitney test. The values are presented as mean + s.e.m. At least three independent experiments were performed.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Publication 2 in preparation

α-Parvin controls epidermal homeostasis and hair follicle morphogenesis by regulating adhesion and migration of keratinocytes

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α-Parvin controls epidermal homeostasis and hair follicle morphogenesis by regulating adhesion and migration of keratinocytes

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Running title: Epidermal homeostasis and hair follicle morphogenesis depend on α-Parvin

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Abstract

The focal adhesion (FA) protein α-Parvin (αPv) has been shown to regulate integrin signaling and integrin-actin linkage. Here we report that the conditional deletion of the *αPv* gene in keratinocytes leads to impaired hair follicle (HF) morphogenesis, epidermal hyperplasia and micro-blistering. Expression of integrin α6 was reduced on αPv-deficient keratinocytes, leading to defects in hemidesmosome (HD) formation and integrin-dependent adhesion associated with rupture of the basement membrane and the presence of proliferating keratinocytes in suprabasal layers of the epidermis. Suprabasal expression of integrins was accompanied by disturbed and delayed terminal differentiation of αPv-deficient keratinocytes. The defects in FA formation/maturation and actin cytoskeleton organization resulted in impaired migration of primary αPv-deficient keratinocytes which is likely the cause for the impaired HF morphogenesis and cycling and progressive loss of hair. Our data demonstrate that αPv is required for epidermal homeostasis and HF development by facilitating integrin signaling and actin cytoskeleton organization in keratinocytes.

Introduction

Skin constitutes the interface to the external environment. It prevents dehydration and protects against environmental threads of physical, chemical and biological nature. These vital functions depend on the establishment and maintenance of a tight barrier, which is formed by the outermost compartment of skin, the epidermis. The epidermis is a stratified squamous epithelium, consisting of multiple layers of keratinocytes that are separated from the mesenchyme-derived dermis by a basement membrane (BM). Proliferation is restricted to the basal layer, where keratinocytes adhere to the BM. Once basal keratinocytes commit to a program of terminal differentiation, they exit the cell cycle, detach from the BM and translocate sequentially from the basal layer to the spinous layers, to the granular layers and finally to the cornified layers of the epidermis. Keratinocyte transition is accompanied by the successive differentiation of basal keratinocytes into terminally differentiated corneocytes, which are shed from the skin surface (Fuchs and Raghavan, 2002). Throughout life the epidermis is continuously renewed by the proliferation and differentiation of basal keratinocytes, which are replenished by stem cells that reside in the basal layer of the epidermis, the sebaceous gland and the HF bulge (Fuchs, 2008). The HF and sebaceous gland are epidermal appendages that form a pilosebaceous unit. Its major function is the production of the hair shaft (HS), which is enveloped by the inner-root-sheath (IRS) and the outer-root-sheath (ORS) of the HF. The ORS is contiguous with the basal layer of the interfollicular epidermis (IFE) and is surrounded by the BM. HF morphogenesis is initiated during embryogenesis by epithelial progenitors in response to inductive cues from the underlying mesenchyme and is completed by postnatal day 14 (P14). Thereafter, HFs cycle between phases of apoptosis-dependent regression (catagen), quiescence (telogen), and growth (anagen) (Paus and Cotsarelis, 1999). During HF growth, bulge-derived keratinocytes of the ORS migrate along the BM towards the distal HF bulb, where they come in close proximity with the dermal papilla (DP), a specialized mesenchymal compartment enclosed by the hair bulb. In the hair bulb, ORS keratinocytes differentiate into highly proliferative hair matrix (HM) keratinocytes. Terminal differentiation of HM keratinocytes results in the specification of the distinct HF linages of the IRS and the HS. Keratinocyte differentiation as well as HF morphogenesis and cycling critically depend on epithelial-mesenchymal signaling crosstalk.

Epidermal homeostasis, keratinocyte differentiation, and HF morphogenesis depend on the ability of keratinocytes to adhere to and migrate along the extracellular matrix (ECM). This is to a large extent facilitated by integrins. They are cell-ECM receptors composed of an α- and

a β-subunit. Integrins switch from an inactive conformation with low ligand affinity to an active conformation with high ligand affinity. Integrin "activation" is regulated by cytoplasmic proteins such as talin and kindlin in a process referred to as inside-out signaling (Moser et al., 2009). Basal and ORS keratinocytes express the α2β1, α3β1 and α6β4 integrins (Commo and Bernard, 1997; Watt, 2002). Additionally, basal keratinocytes express αvβ5 and upregulate α5β1, α9β1 and αvβ6 under pathological conditions such as wound healing and in cell culture (Watt, 2002). Adhesion to the BM is mediated by the laminin (Ln) binding α6β4 integrin heterodimer, which is a central component of HDs and connects the BM to keratin intermediate filaments. The β1 integrins also contribute to the adhesion of basal keratinocytes to the BM and are required for deposition and integrity of the BM, keratinocyte proliferation and differentiation, HF morphogenesis and cycling and wound healing (Brakebusch et al., 2000; Grose et al., 2002; Raghavan et al., 2000).

The short cytoplasmic tails of integrins lack intrinsic enzymatic activity. Instead integrins depend on the recruitment of signaling and adaptor molecules to accomplish downstream signaling events (outside-in signaling) and to enforce the linkage to the actin cytoskeleton. The integrin linked kinase (ILK), which directly binds to β1-integrin cytoplasmic tails, has been identified as a key component required for integrin functions. Through its N-terminal ankyrin repeats ILK binds to the LIM-only proteins PINCH-1 and PINCH-2, and through its C-terminal kinase-like domain ILK binds to the CH (calponin homology) domain-containing proteins α-, β-, or γ-Parvin (**α**Pv, **β**Pv and **γ**Pv). Together ILK, PINCH and Parvin form a ternary complex (IPP-complex), whose assembly in the cytosol precedes its recruitment to integrin adhesions. Assembly of the ternary IPP-complex is required for the stability of its constituents and genetic ablation of one component results in a proteasome dependent downregulation of the remaining constituents (Fukuda et al., 2003; Grashoff et al., 2004; Legate et al., 2006).

Genetic ablation of ILK in the epidermis and its appendages alters BM integrity, epidermal homeostasis and HF development/maintenance and results in epidermal detachments from the BM and the progressive loss of hair (Lorenz et al., 2007; Nakrieko et al., 2008).

Parvins regulate cell adhesion, spreading, migration and survival by controlling actin cytoskeleton dynamics via direct binding to actin and modulating the activity of actin regulators (Legate et al., 2006). αPv is widely expressed, βPv expression is enriched in heart and skeletal muscle and γPv expression is restricted to the hematopoietic system (Chu et al., 2006; Nikolopoulos and Turner, 2000; Olski et al., 2001; Tu et al., 2001; Yamaji et al., 2001).

Deletion of αPv in mice leads to embryonic lethality due to cardiovascular defects (Montanez et al., 2009). The functions of αPv in epidermal homeostasis, however, are not known.

To analyze the function of αPv in epidermal homeostasis we conditionally deleted *αPv* gene in the epidermis. Here we show that αPv is required for epidermal homeostasis and HF morphogenesis and maintenance. Mice lacking αPv in the epidermis progressively loose hair and develop a persistent alopecia. This is accompanied by epidermal detachments from the BM and hyperplasticity of the epidermis and the ORS due to the accumulation of ectopically proliferating keratinocytes in suprabasal layers of the epidermis and in the ORS. Surface expression of integrin α6 is reduced on αPv-deficient keratinocytes, which display disorganized actin cytoskeleton organization and impaired cell adhesion, spreading, migration and differentiation. Our data indicate that αPv regulates HF morphogenesis and epidermal homeostasis by controlling keratinocyte adhesion and migration.

Results

Keratinocyte restricted deletion of αPv leads to progressive hair loss

To analyze the function of αPv in the epidermis and its appendages we deleted the *αPv* gene in basal keratinocytes by generating αPv-floxed mice ($\alpha Pv^{fl/fl}$) (Sup. Fig. 1) and intercrossing them with mice expressing *Cre* recombinase under the control of the *K5* promotor (K5-Cre) (Ramirez et al., 2004). K5-Cre-expressing $\alpha Pv^{fl/+\ K5Cre}$ control (αPv Ctrl) and $\alpha Pv^{fl/fl\ K5Cre}$ mutant mice (αPv K5) were born at the expected Mendelian ratio. No macroscopic differences were observed until skin pigmentation set in at around postnatal day (P) 3 to 4. From P4 on, αPv K5 mice showed irregular skin pigmentation. By P7, control mice developed a homogenous hair coat, whereas αPv K5 mice displayed a patchy hair coat. Sparse hair with partial alopecia persisted for about 4 weeks (Sup. Fig. 2A). Thereafter αPv K5 gradually lost hair, resulting in complete and persistent alopecia at around 8 weeks of age (Fig. 1A). Western-blot (WB) analysis of protein lysates of keratinocytes isolated from one week old mice showed loss of αPv protein and reduced ILK and Pinch protein levels in αPv K5 mice compared to control mice (Fig. 1B). We did not detected βPv expression in keratinocyte lysates.

To characterize the expression of αPv in skin, we performed immunostaining using an antibody specific for αPv (Chu et al., 2006) on back-skin sections from 2-week-old mice and tail-skin from 2-month-old mice. In the skin of control mice, αPv was found highly expressed in basal keratinocytes of interfollicular epidermis (IFE) and outer-root-sheath (ORS) (Fig. 1C). αPv was evenly distributed around the cortex of basal keratinocytes in control IFE (Fig. 1D). No αPv-specific signal was detected in the epidermis of αPv K5 mice, except for some locally restricted areas adjacent to intense integrin α6 staining and in a few suprabasal cells (Fig. 1C). ILK expression was absent in IFE of αPv K5 mice while clearly present in IFE of control mice (Fig. 1E). Taken together, these data showed that the efficient deletion of αPv in keratinocytes destabilizes the IPP-complex and results in gradual hair loss followed by persistent alopecia.

Deletion of αPv impairs HF development and epidermal homeostasis

To morphologically characterize the consequences of keratinocyte-restricted αPv-deletion, we performed hematoxylin and eosin (H&E) staining on back-skin sections of control and αPv K5 mice. No differences in epidermal morphology were found between control and αPv K5 mice before P3. At later stages, in contrast to control skin, progressive epidermal hyperplasia and locally confined epidermal detachments at the dermal-epidermal junction (DEJ) were observed in the skin of αPv K5 mice (Fig. 2C, E, F and 3A). From P3 on, αPv K5 mice also displayed impaired HF morphogenesis (Fig. 2A-F and 3A, B). At P14, HFs of control mice reached an average length of 1100µm. In contrast, 68% of HFs of αPv K5 mice were shorter than 400µm with an average length of 270µm and thus entirely remaining in the dermal compartment (Fig. 2B, C and 3A, B). HS and dermal papilla were either severely distorted or completely absent in those prematurely growth arrested HFs (Fig. 3A) and only 32% of HFs of αPv K5 mice reached into the subcutaneous fat layer (Fig. 2B, C and 3A, B). At P23, HFs of both control and αPv K5 mice were in telogen, indicating that HF regression was not impaired in the absence of αPv (Fig. 2D). HF of control mice elongated during the following anagen phase and by P28 reached into the subcutis. In contrast, HFs of αPv K5 mice remained confined to the dermis (Fig. 2E). By P56, complete resorption of HFs resulted in aberrant melanin depositions in the dermis of αPv K5 mice (Fig. 2F). These results showed that αPv is required for HF morphogenesis and epidermal homeostasis.

Deletion of αPv results in decreased surface expression of integrin α6

The IPP complex is a major mediator of integrin signaling (Legate et al., 2009). Next, we analyzed surface expression of integrins on control and αPv-null keratinocytes by FACS analysis using antibodies against several integrin subunits. Surface levels of integrins β1, β4, α2, αv and α5 were not altered in αPv-null keratinocytes compared to control cells (Fig. 4A, C). However, surface expression of integrin α6 on αPv-null keratinocytes was significantly reduced compared to control cells (Fig. 4A, C). To investigate whether integrin localization was affected upon deletion of αPv, immunohistochemistry using antibodies against β1, β4 and α6 integrins was performed on back-skin sections from 2-week-old mice. While in the epidermis of control mice integrin β1 expression was restricted to basal keratinocytes, in the epidermis of αPv K5 mice β1 integrin expressing cells were also found in suprabasal cell-

clusters (Fig. 5A). In control mice, basal keratinocytes showed a continuous integrin β4 and α6 staining that was restricted to the basal membrane (Fig. 1C and Fig. 5A, B). In contrast, basal keratinocytes of αPv K5 mice showed a discontinuous integrin β4 and α6 staining (Fig. 1C and Fig. 5A, B) and suprabasal cells expressing β4 integrin on their entire surface were observed in areas where the BM was disrupted (Fig. 5B). These data showed that deletion of αPv results in decreased surface expression of integrin α6 and ectopic expression of β1 and β4 integrins in suprabasal layers of the epidermis.

Abnormal cell-polarity, impaired HD formation and compromised adhesion to and organization of the BM in the absence of αPv

Discontinuous integrin α6 and β4 staining and reduced integrin α6 surface expression suggested defective HD formation. To investigate, whether HD formation was compromised in the absence of αPv, electron microscopy (EM) was performed on back-skin sections from 2-week-old control and αPv K5 littermates. Ultrastructural analyzes showed the absence of HDs in areas where basal keratinocytes had detached from the BM in the skin of αPv K5 mice (Fig. 5C, D and Sup. Fig. 5). In the skin of control mice the BM was continuous and was located between the epidermis and the dermis. In contrast, in the skin of αPv K5 mice the BM was frequently disrupted or displaced into the dermis. (Fig. 5D and Sup. Fig 5). EM analyzes also revealed a complete lack of caveolae in αPv-deficient BM-detached basal keratinocytes (Fig. 5D). Additionally, edemas and lose cell-cell contacts were observed in the epidermis of αPv K5 mice (Sup. Fig. 5).

Cell adhesion, shape and polarity as well as cell-cell contacts depend on the correct organization of the actin cytoskeleton. In the epidermis of control mice, actin localized at the apical and lateral side of basal keratinocytes. In contrast, in the epidermis of αPv K5 mice actin was frequently localized at the basal side of basal keratinocytes and actin cables were observed between loosely connected adjacent cells (Fig. 5E). Furthermore, in contrast to control mice, in αPv K5 mice β-catenin (Fig. 5F) as well as E-cadherin (Fig. 5G) were found at the basal side of basal keratinocytes that were detached from the BM. Together, these data indicated that αPv is required for adhesion and polarization of keratinocytes.

Deletion of αPv compromises adhesion and impairs recruitment of the IPP complex and paxillin into FAs of keratinocytes

To analyze actin organization and subcellular distribution of the IPP complex *in vitro*, phalloidin-staining to visualize filamentous actin and immunostaining using antibodies against αPv, ILK, Pinch and paxillin were performed on primary keratinocytes isolated from control and αPv K5 mice. While αPv, ILK, Pinch and paxillin were colocalizing at FAs in control keratinocytes, in αPv-null keratinocytes paxillin-containing FAs were either missing or strongly reduced in size without detectable αPv, ILK and Pinch signals (Fig. 6B-D, Sup. Fig. 4B-D). αPv-null keratinocytes displayed a roundish or spiky cell morphology and were less spread than control keratinocytes (Fig. 6A-D and Sup. Fig. 4A-D). αPv was localizing to the tip of actin stress-fibers in control keratinocytes. In contrast, formation of stress-fibers was impaired in αPv-null keratinocytes (Fig. 6A and Sup. Fig. 4A).
To analyze whether the *in vivo* detachment of keratinocytes primarily resulted from abnormal BM organization or defective integrin-mediated adhesion to the BM, adhesion of primary keratinocytes to ECM components was analyzed *in vitro*. While integrin-independent binding to poly-L-Lysine was not affected by the deletion of αPv, integrin-dependent binding of αPv-null keratinocytes to fibronectin (FN), collagen type-I (Col-I) and laminin 332 (Ln332) was significantly reduced compared to control cells (Fig. 6E).

Ectopic hyperproliferation upon loss of αPv

To investigate whether the hyperplastic epidermis and ORS observed in αPv K5 mice were a consequence of increased proliferation, immunostaining using the proliferation markers Ki67 and phospho-Histone 3 was performed on skin sections from 2-week-old animals. In HFs of control mice, proliferation was mainly observed in HM-keratinocytes surrounding the DP (Fig. 7A). In contrast, in HFs of αPv K5 mice, proliferative cells accumulated in the ORS and partially were absent from the hair bulb (Fig. 7A). In the epidermis of control mice, proliferative cells were found in the basal layer of the epidermis (Fig. 7B-D). In contrast in the epidermis of αPv K5 mice, about 30% of Ki67-positive keratinocytes were observed in suprabasal layers (Fig.7 B, C). However, the percentage of proliferating cells in the basal layer of the epidermis was not different between control and αPv K5 mice (Fig. 7B-D). These

data suggested that hyperplasia in αPv-deficient epidermis and ORS is due, at least in part to ectopic proliferation of keratinocytes in suprabasal layers of the epidermis and in the ORS.

Deletion of αPv results in delayed keratinocyte-differentiation

Ectopic proliferation and expression of integrin β1 and β4 by suprabasal cells could be a consequence of delayed differentiation and/or abnormal translocation of undifferentiated basal keratinocytes to suprabasal layers. To assess whether αPv regulates keratinocyte differentiation, immunohistochemistry using epidermal differentiation markers was performed on back skin sections of 2-week-old mice. In the epidermis of control mice, expression of K5 was restricted to the basal layer (Fig. 8A), K10 was found to be expressed in the first suprabasal cell-layer (spinous layer) (Fig. 8B) and loricrin expression was confined to the stratum granulosum (Fig. 8C). In contrast, in the epidermis of αPv K5 mice expression of K5 was detected in the basal layer and additionally in up to five suprabasal layers (Fig. 8A) and K10 (Fig. 8B) and loricrin (Fig. 8C) were expressed in all suprabasal layers. Together these data suggested that αPv controls differentiation of keratinocytes.
Wound-healing responses and recruitment of inflammatory cells upon blister-formation at the DEJ could additionally contribute to the hyperproliferation. To analyze whether loss of αPv in the epidermis results in the recruitment of inflammatory cells, we performed immunostaining on back-skin sections of control and αPv K5 mice using markers for granulocytes (gr1) and macrophages (mac1). At P14, both granulocyte and macrophage infiltrates were found in areas with disrupted or displaced BM and around distorted HFs in the skin of αPv K5 mice, whereas no signs of inflammation could be observed in the skin of control mice (Fig. 8D, E). Although epidermal hyperplasia, abnormal polarity of keratinocytes and defects in BM-integrity were already obvious in skin of αPv K5 mice at P7 (Sup. Fig. 3A-D), no signs of inflammation were detected in back-skin sections from 1-week-old control and αPv K5 animals (Sup. Fig. 3E). These data provided evidence that hyperproliferation precedes the inflammatory response rather than being its consequence.

Impaired directional migration upon αPv deletion

The accumulation of proliferative cells in the ORS and their partial absence in the hair bulb indicate compromised downward migration of keratinocytes from the bulge region. To

analyze whether αPv regulates migration of keratinocytes, we performed *in vitro* wound-healing migration assays with primary keratinocytes isolated from control and αPv K5 animals. Control keratinocytes closed the wound within 24h, whereas αPv-null keratinocytes failed to efficiently migrate into the wound (Fig. 9A). Single-cell tracking revealed two reasons for the impaired wound closure (Fig. 9B). First, whereas control keratinocytes migrated with an average speed of 0.56 ±0.19 µm/min, αPv-null keratinocytes were significantly slower, only migrating with an average speed of 0.34 ±0.16 µm/min. Therefore the accumulated distance migrated by αPv-null keratinocytes (481.92 ± 227.85 µm) was significantly shorter than that of control keratinocytes (808.52 ± 275.12 µm). Second, αPv-null keratinocytes were impaired in their directionality. The directionality index determined for control keratinocytes was 0.34 ± 0.14, whereas it was only 0.14 ± 0.09 for αPv-null keratinocytes. Both defects together resulted in a significantly reduced euclidean migration distance of αPv-null (62.59 ± 40.76 µm) compared to control (262.23 ± 107.13 µm) keratinocytes and thus in the impaired wound-closure.

Discussion

In the present manuscript, we reported that αPv is essential for epidermal homeostasis and HF morphogenesis and cycling. Conditional deletion of the *αPv* gene in keratinocytes in mice resulted in locally confined epidermal detachments at the DEJ, abnormal BM organization, epidermal hyperplasia and progressive and persistent alopecia due to impaired HF development and maintenance. Loss of αPv also resulted in delayed keratinocyte-differentiation and accumulation of proliferating keratinocytes in suprabasal layers of the epidermis and in the ORS of HFs. We reported that loss of αPv is required for the proper expression and distribution of integrins, actin cytoskeleton organization and polarity of keratinocytes

Epidermal detachments from the BM upon keratinocyte-restricted deletion of αPv indicate that αPv is essential for the adhesion of keratinocytes to the BM. The adhesion of basal keratinocytes to the BM largely is mediated by the hemidesmosomal integrin α6β4 (Watt, 2002). Here we show that in the absence of αPv the surface levels of the α6 integrin subunit are reduced. Moreover, our analyses showed absence of HDs in αPv K5 mice, indicating that αPv is required for HD formation/maintenance. Additionally, deletion of αPv in keratinocytes resulted in splitting of the interfollicular BM and its displacement into the dermis, indicating that αPv is required for BM-integrity. These results are in line with previous data showing that β1 integrins and ILK are required for HD formation/maintenance and BM-integrity (Brakebusch et al., 2000; Lorenz et al., 2007; Nakrieko et al., 2008; Raghavan et al., 2000). A direct interaction of the IPP-complex with the α6β4 integrin heterodimer has not been reported, suggesting that loss of αPv might indirectly affect HD assembly/stability. One possibility is that defects in BM integrity compromise the assembly/stability of HDs (Litjens et al., 2006). Additionally, the integrin heterodimer α3β1 has been suggested to be involved in the nucleation of HDs (Litjens et al., 2006; Sterk et al., 2000). However, although BM integrity is severely compromised in α3β1-deficient mice, HDs are not affected (DiPersio et al., 1997). This might be due to a compensatory function of other β1 integrins and suggests that impaired BM integrity is not the primary cause for the defective HD assembly/maintenance in αPv-deficient epidermis, that αPv facilitates BM integrity downstream of integrin α3β1 and that αPv is required to facilitate β1 integrin-dependent HD assembly/stability, possibly by affecting surface expression and/or localization of the integrin α6 subunit.

Integrin-dependent adhesion of αPv-deficient keratinocytes to ECM-components was also severely compromised *in vitro*, suggesting that epidermal detachments are due to impaired integrin-mediated adhesion. Although reduced surface expression of the integrin α6 subunit in αPv-deficient keratinocytes likely contributes to the compromised adhesion to Ln332, it cannot explain the defects in the adhesion to Col-I and FN. This indicates, that αPv additionally facilitates adhesion through β1 integrins such as the collagen receptor α2β1, the fibronectin receptor α5β1 and the laminin receptor α3β1.

Adhesion to the ECM depends on the clustering of integrins into adhesive structures such as FAs. Maturation and strengthening of FAs in turn requires the linkage of integrins to the actin cytoskeleton. The IPP-complex facilitates the linkage of integrins to the actin cytoskeleton through Parvins. Although small paxillin-containing FAs could form in the absence of αPv, ILK and Pinch were absent from FAs and αPv-deficient keratinocytes displayed a poorly organized actin cytoskeleton with an almost complete lack of actin stress fibers. This indicates that actin cytoskeleton organization and recruitment of ILK and Pinch-1 to FAs critically depend on αPv and suggests that the adhesion defects of αPv-deficient keratinocytes are due to a combination of reduced surface expression of integrin α6, defective organization of the actin cytoskeleton and compromised FA formation/maturation.

Rho GTPases are major regulators of actin cytoskeleton dynamics and although Rac1 activation was normal during spreading on Ln332 in the absence of ILK (Lorenz et al., 2007), Rac1 activation was reduced during *in vitro* wound healing assays in ILK-deficient keratinocytes (Nakrieko et al., 2008), indicating a critical function of the IPP complex in the regulation of Rho GTPases during keratinocyte migration. Additionally, αPv negatively regulates RhoA activity in vascular smooth muscle cells (Montanez et al., 2009), suggesting that αPv might regulate keratinocyte-migration by controlling the organization of the actin cytoskeleton via Rho GTPase signaling. Whether αPv regulates Rho GTPases in keratinocytes remains to be elucidated.

In addition to the adhesion defects, filamentous (f)-actin, β-catenin and E-cadherin localized to the basal membrane of αPv-deficient basal keratinocytes *in vivo*, indicating that αPv is required to maintain cell-polarity. E-cadherin and β-catenin are core components of adherens junctions, which facilitate cell-cell adhesion and are linked to the actin cytoskeleton (Yonemura, 2011). Loosely connected cells and edemas (Sup. Fig. 5) in the epidermis of αPv-deficient animals suggest that αPv facilitates formation or stabilization of cell-cell contacts. Cell polarity and adherens junctions depend on the correct organization of the actin cytoskeleton, suggesting that αPv might indirectly affect polarity and cell-cell adhesion by

controlling the organization of the actin cytoskeleton. Defects in cell-cell adhesion also have been reported in embryoid bodies in the absence of Pinch-1 but not in the absence of ILK and it has been suggested that these differences could be due to an IPP complex-independent functions of Pinch-1 in cell-cell adhesion (Li et al., 2005). However, how Pinch-1 and αPv can control cell-cell adhesion in an IPP-independent manner currently is not known and needs to be elucidated.

Keratinocyte-restricted deletion of ILK results in suprabasal expression of integrins, inflammation, delayed terminal differentiation, ectopic proliferation in suprabasal layers of the epidermis and epidermal hyperplasia (Lorenz et al., 2007). In line with these findings, we observed strikingly similar defects in the epidermis of αPv-deficient mice. It has been suggested that inflammation is required to trigger the epidermal hyperproliferation in mice expressing integrins in suprabasal layers (Romero et al., 1999). However, hyperthickening of αPv-deficient epidermis clearly preceded the accumulation of inflammatory granulocytes and macrophages, indicating that inflammation is not the primary cause for hyperproliferation in αPv-deficient epidermis but rather results from disturbed epidermal homeostasis in the absence of αPv.

Loss of αPv resulted in severely compromised HF morphogenesis and progressive loss of hair. About one-third of the HFs in αPv K5 mice completed morphogenesis by P14, whereas the remaining two-thirds were severely distorted and prematurely growth-arrested. Asynchronous initiation and morphogenesis of distinct types of HFs together with a perinatal loss of αPv-protein can explain the development of both prematurely growth arrested and mature HFs in αPv K5 mice (Paus et al., 1999; Schmidt-Ullrich and Paus, 2005). Thus, in prematurely growth arrested HFs αPv had been deleted in early phases of HF-morphogenesis, leading to their premature growth arrest, whereas mature HFs had lost αPv in late stages of morphogenesis. However, also mature HFs displayed several morphological abnormalities, including a markedly reduced length, a hyperplastic ORS, interrupted HSs and a distorted DP. Although catagen and telogen appeared to proceed normal in HFs of αPv K5 mice, all HFs failed outgrowth during the following anagen phase. HF growth depends on the proliferation and directional migration of stem cell-derived keratinocytes from the HF bulge region towards the HF bulb, where they differentiate into HM keratinocytes. Directed migration is impaired in αPv-deficient keratinocytes, suggesting that these migration defects account for the accumulation of proliferative keratinocytes in the ORS of mature αPv-K5 HFs, the impaired development of prematurely growth arrested αPv-K5 HFs and the impaired HF outgrowth during anagen in the absence of αPv.

Materials and methods

Generation of floxed αPv mice and intercrossing with K5-Cre mice

A 129/Sv mouse P1-derived artificial chromosome (PAC) library was screened with cDNA-probes to identify PAC-clones containing αPv-genomic DNA. Positive PAC clones were used to generate the αPv targeting construct, encompassing a 2.7 kb 5'-homology arm and a 5.9 kb 3'-homology arm, targeting a region from intron 1 to intron 4 of the *αPv* gene. A neomycin-resistance cassette flanked by two frt-recognition sequences was inserted into intron 2 to allow the selection of neomycin-resistant ES cell clones. The first loxP sequences was introduced 5' of the neomycin cassette into intron 2, the second loxP sequences was introduced into intron 3, thus flanking exon 3 of the *αPv* gene. Genotyping of wt and recombinant alleles was performed by Southern blot using an external probe after EcoRI digestion of genomic DNA. The neomycin-resistance cassette was removed by intercrossing αPv-floxed mice with FLP recombinase-expressing mice (Farley et al., 2000). Efficient removal of the neomycin-resistance cassette was confirmed by PCR. Homozygous αPv-floxed mice lacking the neomycin-resistance cassette were viable and phenotypically normal. For the genotyping of αPv-floxed mice, a PCR with the three primers APE2f (forward) 5'-GAAGGAATGAACGCCATCAAC-3', APloxPf (forward) 5'-CTGAGTGACATGGAGTTTGAG-3' and APloxPr (reverse) 5'-GGACTTGTGGACTAGTTAG AC-3' was used, allowing the discrimination of wt (1.1kb and 186bp), floxed (240bp) and Cre-mediated-recombined (595bp) alleles. For the keratinocyte-restricted deletion of the floxed αPv-gene, homozygous floxed αPv-females were mated with heterozygous floxed αPv-males, expressing the Cre-recombinase under the control of the keratin 5 promotor (Ramirez et al., 2004). Presence of the K5-Cre transgene in littermates was determined by PCR using the primers CreF (forward) 5'-AACATGCTTCATCGTCGG-3' and CreR (reverse) 5'-TTCGGATCAGCTACACC-3'.

Isolation and culture of primary keratinocytes

Primary keratinocytes were isolated and cultured as previously described (Montanez et al., 2007). Keratinocyte growth medium (KGM) was prepared from Minimal Essential Medium (Spinner Modification; Sigma-Aldrich), complemented with 8% chelated FCS, 45µM $CaCl_2$,

5mg/ml insulin (Sigma-Aldrich), 10ng/ml EGF (Sigma-Aldrich), 10mg/ml transferrin (Sigma-Aldrich), 10mM phosphorylethanolamine (Sigma-Aldrich), 10mM ethanolamine (Sigma-Aldrich), 0.36mg/ml hydrocortisone (Calbiochem), 2mM L-Glutamine (Invitrogen) and 1x Penicillin/Streptomycin (PAA). Keratinocytes were cultured on tissue-culture dishes coated with 10µg/ml Col-I (INAMED) and 10µg/ml FN (Merck).

Adhesion assay

Primary keratinocytes ($1*10^5$ cells/well) were plated onto 96-well plates coated with poly-L-lysine (Sigma-Aldrich), Col-I (INAMED), FN (Merck) or Ln332 (Dr. Monique Aumailley, University of Cologne, Germany). After 30 min incubation, cells were lysed in substrate buffer (7.5mM NPAG (Sigma-Aldrich), 0.1M Na citrate pH 5, 0.5% Triton X-100) over night at 37°C. The reaction was stopped by adding 50mM Glycine pH 10.4, 5mM EDTA, and the OD 405 was measured.

***In vitro* wound healing assay**

After 4h incubation in KGM supplemented with 4µg/ml Mitomycin C (Sigma-Aldrich), keratinocyte monolayers were gently scratched with the tip of a cell-scraper. Subsequently, images were captured every 10 min for 24h at 37°C and 5% CO_2 using a Zeiss Axiovert microscope equipped with 10× NA 0.3, 20× NA 0.4, 40× NA 0.6, and 100× NA 1.3 objectives, a motorized scanning table (Märzhäuser), a stage incubator (EMBL Precision Engineering) and a CCD camera (Roper Scientific MicroMAX). MetaMorph (Universal Imaging Corp.) software was used for microscope control and data acquisition. For single-cell tracking and data analyzes, the ImageJ software-plugins “Manual Tracking” (Fabrice P. Cordelières) and “Chemotaxis and Migration Tool” (ibidi) were used. At least three independent experiments were performed and more than 20 individual cells were tracked for each experiment.

FACS analyses

FACS analyses were performed as previously described (Montanez et al., 2007). Primary keratinocytes were cultured three to four days prior to FACS analyses, to exclude suprabasal

keratinocytes from the analyses. Antibodies used for FACS analyzes were biotinylated hamster antibody against integrin β1; rat antibody against integrin β4; FITC-conjugated rat antibody against integrin α6; biotinylated rat antibody against integrin αv; biotinylated rat antibody against integrin α5 (all from PharMingen, San Diego, CA); FITC-conjugated hamster antibody against integrin α2 (BD Biosciences); Cy5-conjugated Streptavidin (Jackson Immunochemicals Laboratories Inc.; West Grove, PA, USA); Anti-Rat-Alexa488 (Invitrogen); FITC-conjugated IgG2a,k (rat) (NatuTec); biotinylated IgG2a,k (rat) (eBioscience); FITC-conjugated IgG2 (hamster) (PharMingen, San Diego, CA); biotinylated IgM (hamster) (BioLegend); biotinylated IgG2a,k (rat) (eBioscience).

Epidermal lysates and cell lysates

Primary keratinocytes, either directly after isolation or after cultivation for up to five days, were lysed in lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP40, 1% SDS, 1% Na-deoxycholate) supplemented with protease inhibitor cocktail tablets (Complete Mini, EDTA-free; Roche). Proteins were separated by SDS-PAGE and blotted onto PVDF-membranes. For subsequent immuno-detection, the following antibodies were used: rabbit antibody against αPv (Chu et al., 2006); rabbit antibody against βPv (Chu et al., 2006); rabbit antibody against ILK (Cell Signaling Technology); mouse antibody against Pinch (BD Biosciences); mouse antibody against GAPDH (Calbiochem); goat anti–mouse HRP; and goat anti–rabbit HRP (Bio-Rad Laboratories).

Histology and immunohistochemistry

Histology and immunohistochemistry on skin sections was performed as previously described (Montanez et al., 2007). Back-skin was fixed overnight at 4°C in 4% PFA in PBS, dehydrated in a graded alcohol series and embedded in paraffin (Paraplast X-tra; Sigma-Aldrich) using a embedding machine (Shandon). For cryo-sections, unfixed back-skin was embedded in OCT (Shandon Cryomatrix, Thermo) and rapidly frozen. Prior to staining, cryo-sections were fixed with PFA, methanol or Zn-fixative (40mM $ZnCl_2$, 3mM Calcium acetate monohydrate, 10mM Zinc trifluoroacetate hydrate, 100mM Tris pH 6.8). For immunostaining, primary keratinocytes were plated overnight onto glass-coverslips coated with 10µg/ml FN and 10µg/ml Col-I. Cells were fixed in 3% PFA, permeabilized with 0.1% Triton-X100 and

blocked with 3% BSA (all in PBS). The following antibodies were used for immunohistochemistry: rabbit antibody against αPv (Chu et al., 2006); mouse antibody against ILK, Pinch and Paxillin (all BD Biosciences); TRITC-conjugated phalloidin and rabbit antibody against β-catenin (all Sigma-Aldrich); rat antibody against β4-Integrin, FITC-conjugated rat antibody against α6-Integrin, Biotin-conjugated rat antibody against Mac-1 (integrin αM), and PE-conjugated rat antibody against Gr1 (all PharMingen, San Diego, CA); Alexa488-conjugated goat antibody against rabbit, Alexa546-conjugated goat antibody against rabbit (Invitrogen) and Alexa546-conjugated goat antibody against mouse (all Invitrogen); Cy3-conjugated goat antibody against rat and Cy3-conjugated streptavidin (all Jackson Immunochemicals Laboratories Inc.; West Grove, PA, USA); rat antibody against β1 integrin (Chemicon); rat antibody against E-cadherin (zymed); rabbit antibody against Ln332 (Dr. Monique Aumailley, University of Cologne, Germany); rat antibody against Ki67 (Tec3) (DakoCytomation); biotin-conjugated rabbit antibody against phospho-Histone H3 (Ser10) (Upstate); rabbit antibodies against keratin 5 and 10 and loricrin (all Covance). Confocal images were acquired with a Leica TCS SP5 microscope (Leica Microsystems CMS, Mannheim, Germany), equipped with 20.0x NA 0.70, 40x NA 1.25, 63x NA 1.4 and 63x NA 1.2 objectives, using Leica Application Suite Advanced Fluorescence (LAS AF) software version 1.6.2. build 1110.

Epidermal whole mounts

Whole mounts from tail-skin were prepared as previously described (Braun et al., 2003). Small pieces of tail-skin were incubated in 5mM EDTA in PBS at 37°C for four hours. Subsequently, the epidermis was carefully peeled from the dermis and fixed in Zn-fixative (40mM $ZnCl_2$, 3mM Calcium acetate monohydrate, 10mM Zinc trifluoroacetate hydrate, 100mM Tris pH 6.8) at 4°C overnight. For immunohistochemistry, rabbit antibody against αPv (Chu et al., 2006), mouse antibody against ILK (BD Biosciences) and FITC-conjugated rat antibody against α6-Integrin (PharMingen, San Diego, CA) were used.

Electron microscopy

Samples from back-skin were processed as described (Hess et al., 2010) by using immersion fixation with glutaraldehyde and OsO_4, followed by epoxy resin embedding.

Statistical analysis

Statistical analyses were performed using a two-tailed T-test. Values are presented as mean plus standard error of the mean. P-values lower than 0.05 (*), 0.01 (**) or 0.001 (***) were regarded as significant.

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Abbreviations

αPv, βPv and γPv: α-, β-, and γ-Parvin; BM: basement membrane; CH: calponin homology; Col-I: collagen-I; D: dermis; DEJ: dermal-epidermal junction; DP: dermal papilla; E: epidermis; ECM: extracellular matrix; EGF: epidermal growth factor; EM: electron microscope FA: focal adhesion; FACS: fluorescence activated cell sorting; f-actin: filamentous actin; FCS: fetal calf serum; FN fibronectin h: hour; HD: hemidesmosome; H&E: hematoxylin and eosin; HF, hair follicle; HM: hair matrix; HRP: horse radish peroxidase; HS hair shaft; IFE: interfollicular epidermis; IL-1α: interleukin-1α; ILK: integrin linked kinase; IPP-complex: ILK-Pinch-Parvin-complex; IRS: inner-root-sheath; K: keratin; kb: kilobase; KGM: keratinocyte growth medium; LIM: Lin11, Isl1, Mec3; Ln332: Laminin 5, Laminin 332; min: minute; µm: micrometer; ORS: outer-root-sheath; P: postnatal day; PAC: P1-derived artificial chromosome; PC: panniculus carnosum; PFA: paraformaldehyde; Pinch: particularly interesting Cys-His-rich protein; SC: subcutis; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; WB: Western-blot.

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Figure Legends

Figure 1: Keratinocyte-restricted deletion of αPv results in complete and persistent alopecia

(A) αPv Ctrl and αPv K5 mice at four month of age. (B) αPv, βPv, Pinch and ILK protein levels in αPv-null kidney-derived fibroblasts (αPv -/-) and primary keratinocytes from one-week-old Ctrl and αPv K5 mice. (C) Immunostaining of αPv (red) and integrin α6 (green) on back-skin sections from Ctrl and αPv K5 mice at P14. Arrows indicate residual cells in αPv K5 skin that escaped Cre-mediated deletion. Scale bar: 20µm. (D) Whole mount staining of αPv (red) and integrin α6 (green) of tail-skin from two-month-old Ctrl and αPv K5 mice. Scale bar: 40µm. A higher magnification of the αPv-staining (white) of the boxed region is shown below. αPv localizes to the cell cortex of basal keratinocytes of the IFE. Scale bar: 20µm. (E) Whole mount staining of ILK (red) and integrin α6 (green) of tail-skin from two-month-old Ctrl and αPv K5 mice. Scale bar: 40µm. A higher magnification of the ILK-staining (white) of the boxed region is shown below. Scale bar: 20µm.

Figure 2: Keratinocyte-restricted deletion of αPv affects HF morphogenesis and results in epidermal hyperplasia and regional detachments from the DEJ

H&E staining of back-skin from Ctrl and αPv K5 mice at (A) P3, (B) P7, (C) P14, (D) P23, (E) P28 and (F) P56. Arrows indicate areas of epidermal detachments at the DEJ; arrowheads indicate abnormal melanin-deposits, triangles (▲) indicate short and prematurely growth-arrested HFs and rectangles (■) indicate long HFs. E: epidermis; D: dermis; SC: subcutis; PC: panniculus carnosum. Scale bar: 200µm.

Figure 3: Impaired HF-cycling, epidermal hyperplasia and local detachments at the DEJ upon keratinocyte-restricted deletion of αPv

H&E staining of back-skin from Ctrl and αPv K5 mice at P14. (A) Epidermis of Ctrl and αPv K5 mice. Asterisks indicates epidermal hyperplasia; arrows indicate epidermal detachments at the DEJ; triangles (▲) indicate short and prematurely growth-arrested HFs. Scale bar: 100µm. (B) HFs of Ctrl and αPv K5 mice. Star indicates hyper-thickened ORS; rectangle (■) indicates long HF. Note the interrupted HS and the abnormal dermal papilla in the mutant HF. Scale bar: 50µm. (C) Histogram of HF length distributions at distinct stages of the HF-

cycle. The length of a minimum of 100 HFs was measured on H&E stained back-skin from Ctrl and αPv K5 mice.

Figure 4: Reduced surface expression of α6 integrin on αPv-deficient primary keratinocytes

(A) Integrin-profile of primary keratinocytes isolated from Ctrl (blue) and αPv K5 (red) mice. The isotype-control is shown in grey. (B) Quantification of at least three FACS integrin-profile experiments of αPv Ctrl (blue) and αPv K5 (red) primary keratinocytes. Values of integrin-expression on αPv K5 were normalized to the respective integrin-expression on αPv Ctrl cells.

Figure 5: Keratinocyte-restricted deletion of αPv impairs BM-integrity, disturbs keratinocyte-polarity and results in the translocation of integrin-expressing cells to suprabasal layers

(A) Immunostaining of Ln332 (green) and β1 integrin (red) of back skin from Ctrl and αPv K5 mice at P14. Arrow indicates β1 integrin expressing suprabasal cells. Scale bar: 20µm. (B) Immunostaining of Ln332 (green) and β4 integrin (red) of back skin from P14 Ctrl and αPv K5 mice. Arrow indicates β4 integrin expressing suprabasal cells. Scale bar: 20µm. (C and D) EM analysis of back skin from Ctrl and αPv K5 mice at P14. Long arrows indicate HDs. Short arrows indicate the presence of the BM. Arrowheads indicate the presence of caveoli in basal keratinocytes. Scale bar: 500 nm. (E) Immunostaining of Ln332 (green) and f-actin (red) of back-skin from Ctrl and αPv K5 mice at P14. Arrow indicates basal f-actin localization in basal keratinocytes. Asterisks indicate intercellular spaces and spiky protrusions of f-actin. Scale bar: 10µm. (F) Immunostaining of Ln332 (green) and β-Catenin (red) of back-skin from Ctrl and αPv K5 mice at P14. Arrow indicates basal localization of β-Catenin in basal keratinocytes. Asterisks indicate intercellular spaces. Scale bar: 10µm. (G) Immunostaining of Ln332 (green) and E-Cadherin (red) of from back-skin from Ctrl and αPv K5 mice at P14. Arrow indicates basal localization of E-Cadherin in basal keratinocytes. Asterisks indicate intercellular spaces and disruption of the BM. Scale bar: 10µm.

Figure 6: Compromised FA-formation, abnormal organization of the actin-cytoskeleton and reduced spreading and adhesion of αPv-deficient primary keratinocytes

Immunostaining of (A) f-actin (red) and αPv (green) (B) ILK (red) and αPv (green) (C) Pinch (red) and αPv (green) (D) Paxillin (red) and αPv (green) of primary keratinocytes isolated from Ctrl and αPv K5 mice. Scale bars: 20µm. (E) Quantification of adhesion to PLL, Ln332, Col-I and FN of αPv Ctrl and αPv K5 primary keratinocytes. Mean values of the percentage of adhering cells plus standard error of the mean are represented. At least three independent adhesion assays were performed.

Figure 7: Keratinocyte-restricted deletion of αPv results in the accumulation of ectopically proliferating cells in suprabasal layers of the epidermis and in the ORS

Immunostaining of (A and B) Ki67 (brown) (C) integrin α6 (green) and phospho-histone 3 (red) of back-skin from Ctrl and αPv K5 mice at P14. (A) Ki67-positive cells are located in the bulb of αPv Ctrl HFs, whereas they accumulate in the ORS of αPv K5 HFs and fail to replenish proliferating cells in the bulb. Arrow indicates proliferative cells in the ORS of αPv K5 HFs. Asterisks indicate a region in the αPv K5 HF-bulb that is devoid of proliferative cells. Scale bar: 50µm. (B) Ectopic proliferation in suprabasal layers of αPv K5 epidermis. Arrows indicate Ki67-positive cells in suprabasal layers of αPv K5 epidermis. Scale bar: 50µm. (C) Ectopic proliferation in suprabasal layers of αPv K5 epidermis. Arrows indicate phospho-histone 3-positive cells in suprabasal layers of αPv K5 epidermis. Scale bar: 20µm. (D) Quantification of Ki67-positive cells in basal and suprabasal layers of αPv Ctrl and αPv K5 epidermis. The percentage of proliferative to total cells in the basal layer of αPv Ctrl and αPv K5 is not significantly different, whereas proliferative cells in suprabasal layers are almost exclusively observed in αPv K5 epidermis, comprising approximately 30% of Ki67-positive cells in αPv K5 epidermis. Depicted are the mean values plus standard error of the mean.

Figure 8: Keratinocyte-restricted deletion of αPv results in delayed differentiation and the recruitment of inflammatory cells.

Immunostaining of (A) integrin α6 (green) and K5 (red); (B) integrin α6 (green) and K10 (red); (C) Ln332 (green) and loricrin (red); (D) Ln332 (green) and Mac1 (red); (E) Ln332 (green) and Gr1 (red) of back-skin from Ctrl and αPv K5 mice at P14. Scale bars: 20µm. (A) Expression of keratin 5 is restricted to the basal layer of αPv Ctrl epidermis, whereas keratin

5 additionally is expressed in suprabasal layers in αPv K5 epidermis. (B) Expression of keratin 10 is restricted to the first suprabasal layer of αPv Ctrl epidermis, whereas keratin 10 is expressed in additional suprabasal layers of αPv K5 epidermis but not in the basal layer. (C) Expression of loricrin is restricted to the stratum granulosum in αPv Ctrl epidermis, whereas loricrin is expressed in all suprabasal layers of αPv K5 epidermis but not in the basal layer. (D) Macrophages are recruited to αPv K5 dermis in areas where the BM is disrupted, whereas no signs of inflammation can be recognized in αPv Ctrl dermis. Arrow indicates Mac1-positive macrophages in αPv K5 dermis. Star indicates the splitting and disruption of the BM in αPv K5 skin. (E) Granulocytes are recruited to αPv K5 dermis in areas where the BM is disrupted and to distorted αPv K5 HFs, whereas no signs of inflammation can be recognized in αPv Ctrl epidermis and around αPv Ctrl HFs. Arrows indicates Gr1-positive granulocytes in αPv K5 dermis and adjacent to αPv K5 HFs. Asterisk indicates the splitting and disruption of the BM in αPv K5 epidermis.

Figure 9: Reduced migration-velocity and directionality of αPv-deficient primary keratinocytes

(A) *In vitro* wound healing assay with αPv Ctrl and αPv K5 primary keratinocytes. While αPv Ctrl keratinocytes close the artificial wound within 24 hours, αPv K5 keratinocytes are unable to migrate efficiently into the wounded area. Colored lines represent the migration-tracks of individual keratinocytes. (B) Quantification of the migration-parameters of αPv Ctrl and αPv K5 primary keratinocytes during *in vitro* wound healing assays. Migration-velocity and directionality of αPv keratinocytes are significantly reduced compared to αPv Ctrl keratinocytes, resulting in a severe reduction in the accumulated distance (Acc. D.) and in the Euclidean distance (Euc. D.) of αPv keratinocytes. Mean values plus standard error of the mean are represented. At least three independent scratch assay experiments were performed and more than 20 individual cells were tracked in randomly chosen regions in each experiment.

Supplementary figure 1: Targeting strategy for the generation of αPv floxed animals

The wild type (WT) αPv locus was targeted with a construct containing homology arms of 2.7 kb and 5.9 kb (indicated by grey lines). A neomycin-resistance cassette (Neo; grey box) flanked by two frt-sites (grey triangles) was introduced into intron 2. The first loxP-site (black triangle) was inserted into intron 2 and the second loxP-site was inserted into intron 3,

thus flanking exon 3 of the *αPv* gene. Binding sites for the primers a: APE2f, b: APloxPf and c: APloxPr are indicated by bent arrows, pointing into the direction of polymerization. The resulting PCR-products are indicated by black lines and their respective product size above. Southern blot was used to identify ES-cell clones after homologous recombination. EcoRI-restriction-sites are indicated by grey lines and the position of the 3' external probe is indicated by a black line. The genomic-fragment resulting from EcoRI digestion is indicated by a black line and its expected size is below. The neomycin-resistance cassette was removed by crossing heterozygous floxed αPv mice (fl n+) with FLP-recombinase-expressing mice (Farley et al., 2000), resulting in αPv floxed mice without neomycin-resistance cassette (fl n-). Cre-recombinase mediated recombination results in the excision of exon 3 and deletion of the αPv gene (fl-).

Supplementary figure 2: Keratinocyte-restricted deletion of αPv results in progressive loss of hair

(A) αPv Ctrl and αPv K5 mice at the age of one week, two weeks, four weeks and four month (as in Fig.1A). Reduction of hair can be clearly recognized one week after birth and αPv K5 mice are characterized by a complete and persistent alopecia by two month of age. (B) Southern-blot of ES-cell-clones after homologous recombination. Genomic DNA from ES-cell-clones in lane 3, 8 and 10 indicates successful homologous recombination of the targeting vector and the αPv-gene. (C) PCR with DNA from a litter of αPv homozygous floxed female mated to an αPv heterozygous male, expressing the Cre-recombinase under the control of the K5-promotor. LoxP: PCR to distinguish the wild type (WT), floxed (fl) and recombined (fl-) *αPv* gene. CRE: PCR to determine the presence of the K5-Cre transgene. The first two mice (lanes 1-4) are αPv homozygous floxed and carry the K5-Cre transgene (fl/fl K5+). Mice of this genotype are referred to as αPv K5 in this study. The third mouse (lanes 5-6) is αPv homozygous floxed but does not carry the K5-Cre transgene (fl/fl K5-). The fourth mouse (lanes 7-8) is αPv heterozygous floxed and also does not carry the K5-Cre transgene (fl/+ K5-). The fifth mouse (lanes 9-10) is αPv heterozygous floxed and carries the K5-Cre transgene (fl/+ K5+). Mice of this genotype are referred to as αPv Ctrl and served as control animals in this study.

Supplementary figure 3: Defects in HF morphogenesis and epidermal homeostasis precede the inflammatory response

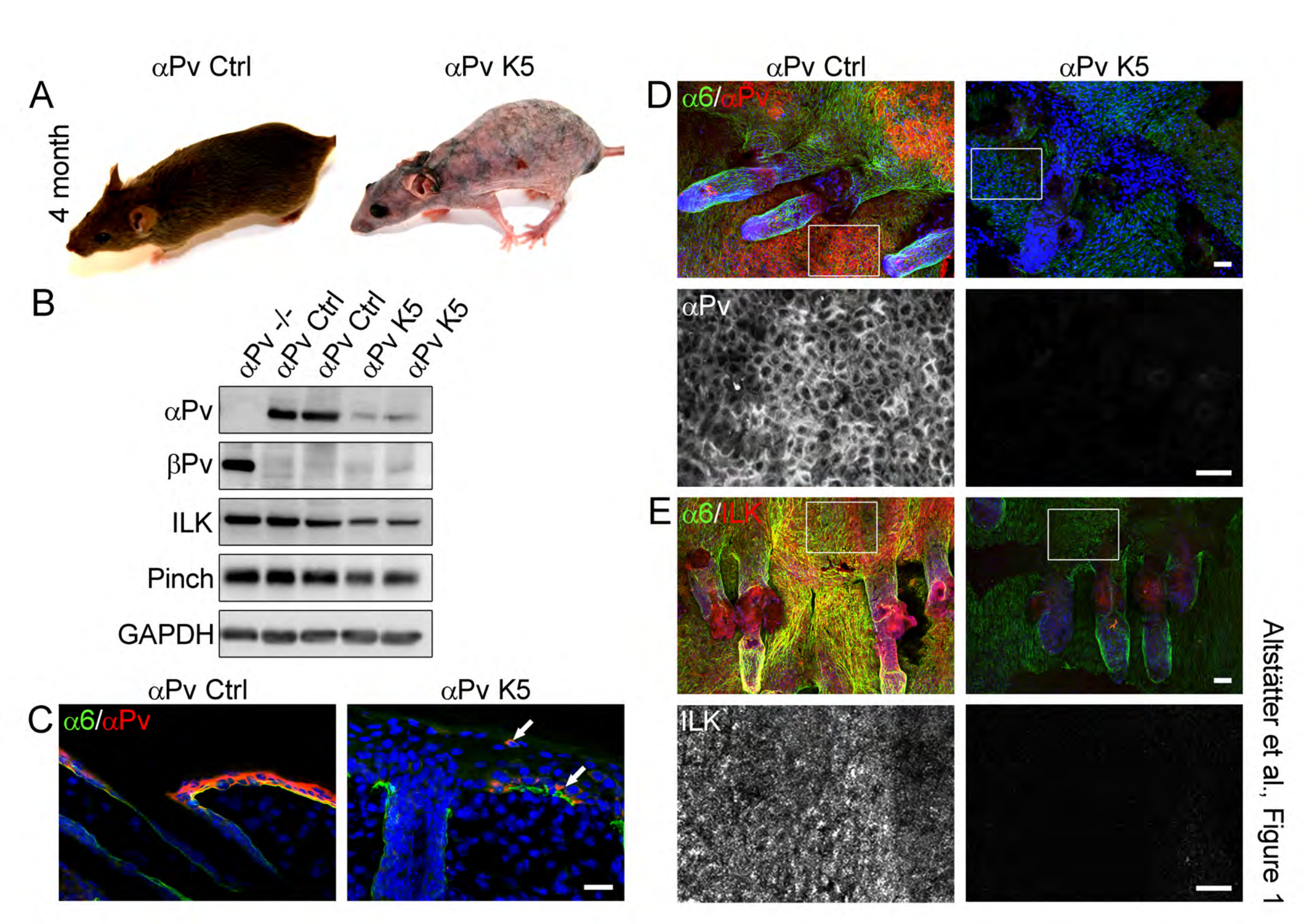
Cryo-section from back-skin of P7 αPv Ctrl and αPv K5 mice stained for: (A) Ln332 (green) and β1 integrin (red). Arrow indicates suprabasal αPv K5 keratinocytes expressing β1 integrin. Asterisk indicates intercellular spaces in αPv K5 epidermis. Note disruption and displacement of the BM in αPv K5 skin. Scale bar: 20µm. (B) Ln332 (green) and β4 integrin (red). Asterisk indicates splitting of the BM in αPv K5 skin. Scale bar: 20µm. (C) Integrin α6 (green) and f-actin (red). Arrow indicates localization of f-actin to the basal side of basal keratinocytes and discontinuous integrin α6 staining in αPv K5 epidermis. Scale bar: 20µm. (D) Ln332 (green) and E-Cadherin (red). Arrow indicates localization of E-Cadherin to the basal side of basal keratinocytes and splitting of the BM in αPv K5 epidermis. Scale bar: 20µm. (E) Ln332 (green) and Gr1 (red). Gr1-positive granulocytes are absent from αPv K5 skin at P7. Scale bar: 20µm.

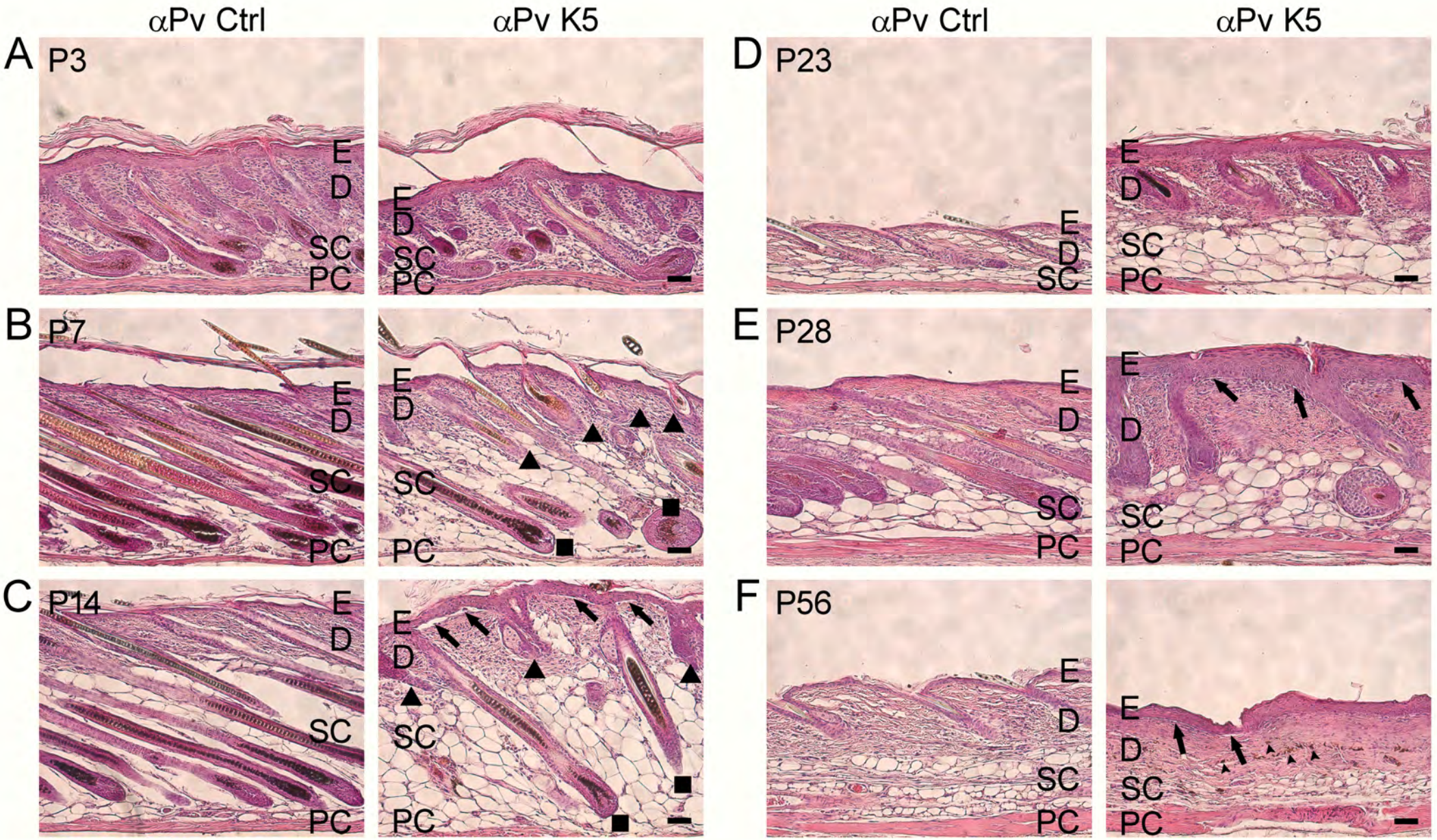
Supplementary figure 4: Compromised FA formation, abnormal organization of the actin cytoskeleton and reduced spreading of αPv-deficient primary keratinocytes

Separate channels from the immunostaining of αPv Ctrl and αPv K5 primary keratinocytes in Fig.6: (A) f-actin (white) and below αPv (white); (B) ILK (white) and below αPv (white); (C) Pinch (white) and below αPv (white); (D) Paxillin (white) and below αPv (white). Scale bars: 20µm. Note the presence of spiky and poorly spread αPv K5 cells in (B) (C) and (D). Paxillin-positive FA are present in spread αPv K5 keratinocytes, however their number and size is reduced compared to αPv Ctrl keratinocytes. FA-recruitment of ILK and Pinch is impaired in αPv K5 keratinocytes.

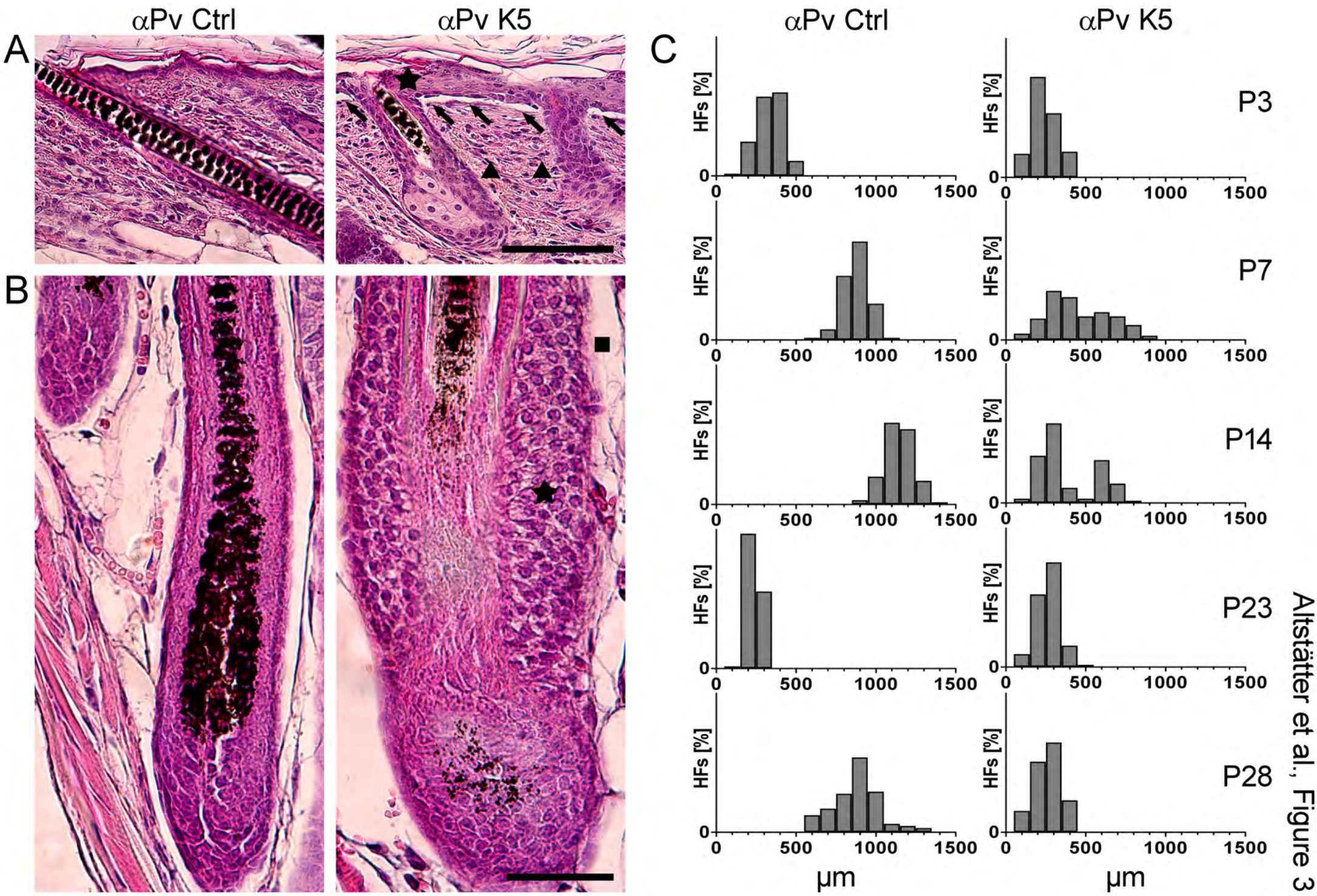
Supplementary figure 5: Edemas and poor interdigitation of keratinocytes upon loss of αPv

Overview of the EM-pictures from back-skin of P14 αPv Ctrl and αPv K5 mice shown in Fig. 4C and D. Arrows indicate edemas in αPv K5 epidermis (asterisk) accompanied with poor interdigitation of αPv-deficient keratinocytes. Scale bar: 5µm. Note the displacement of the BM into the collagen-fibrils of the dermis (cross) in αPv K5 skin as highlighted with a black frame and the local dermal-epidermal detachment. Areas with black or white frame are enlarged in Fig. 4C and D.

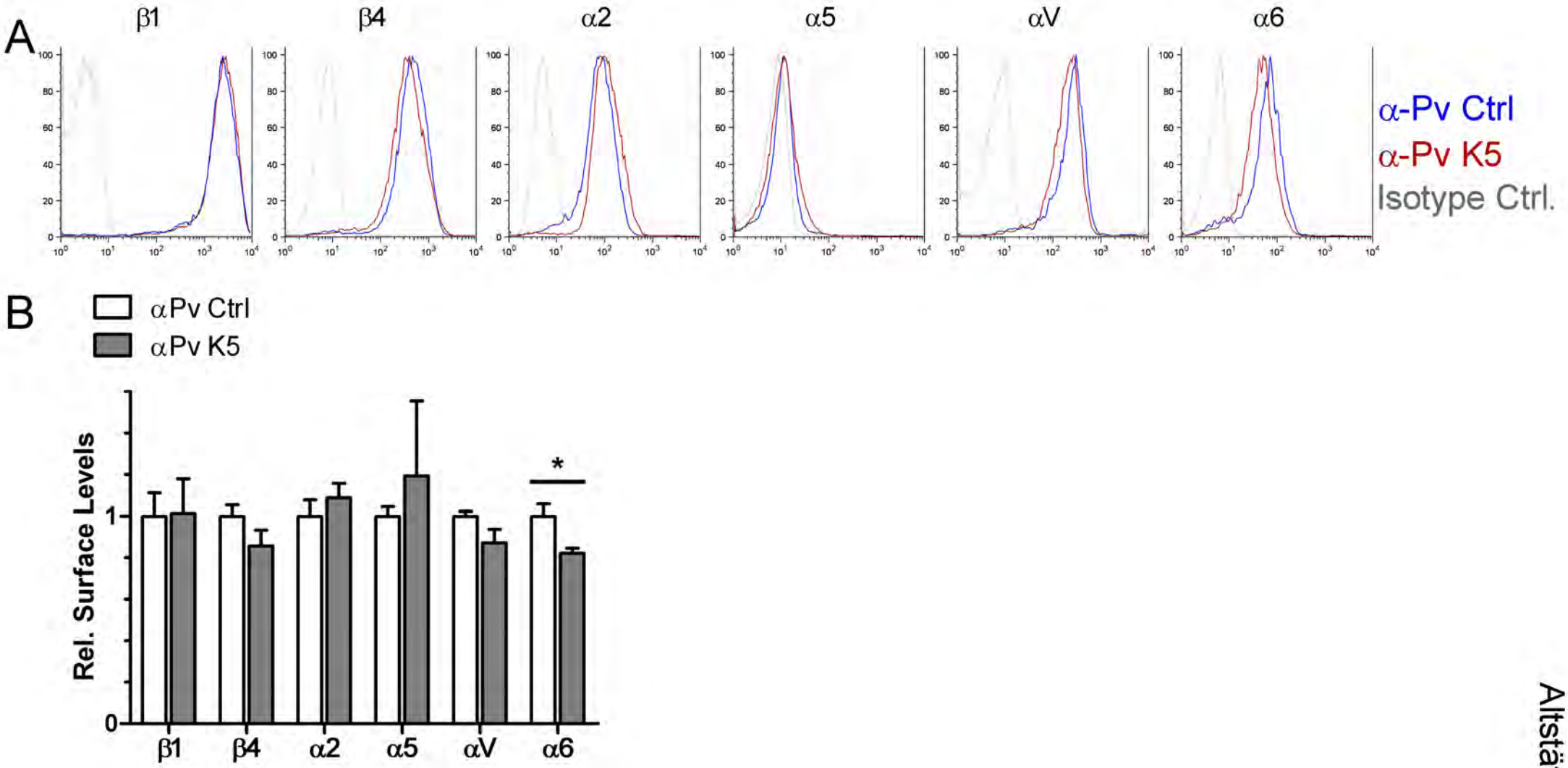

αPv Ctrl
αPv K5
A
4 month
B
αPv -/-
αPv Ctrl
αPv Ctrl
αPv K5
αPv K5
αPv
βPv
ILK
Pinch
GAPDH
αPv Ctrl
αPv K5
C
α6/αPv
D
αPv Ctrl
αPv K5
α6/αPv
αPv
E
α6/ILK
ILK
Altstätter et al., Figure 1



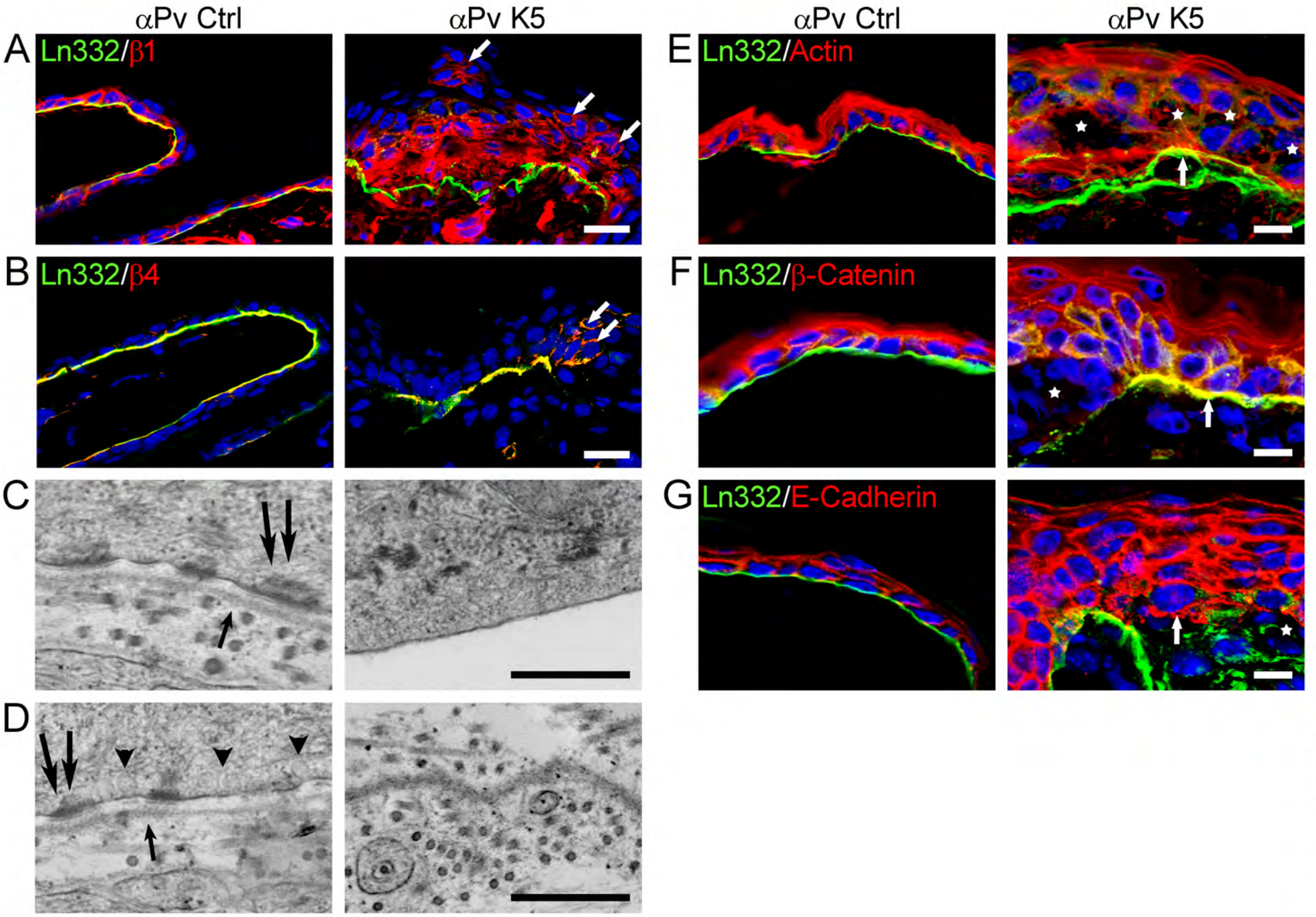
Altstätter et al., Figure 2



Altstätter et al., Figure 3

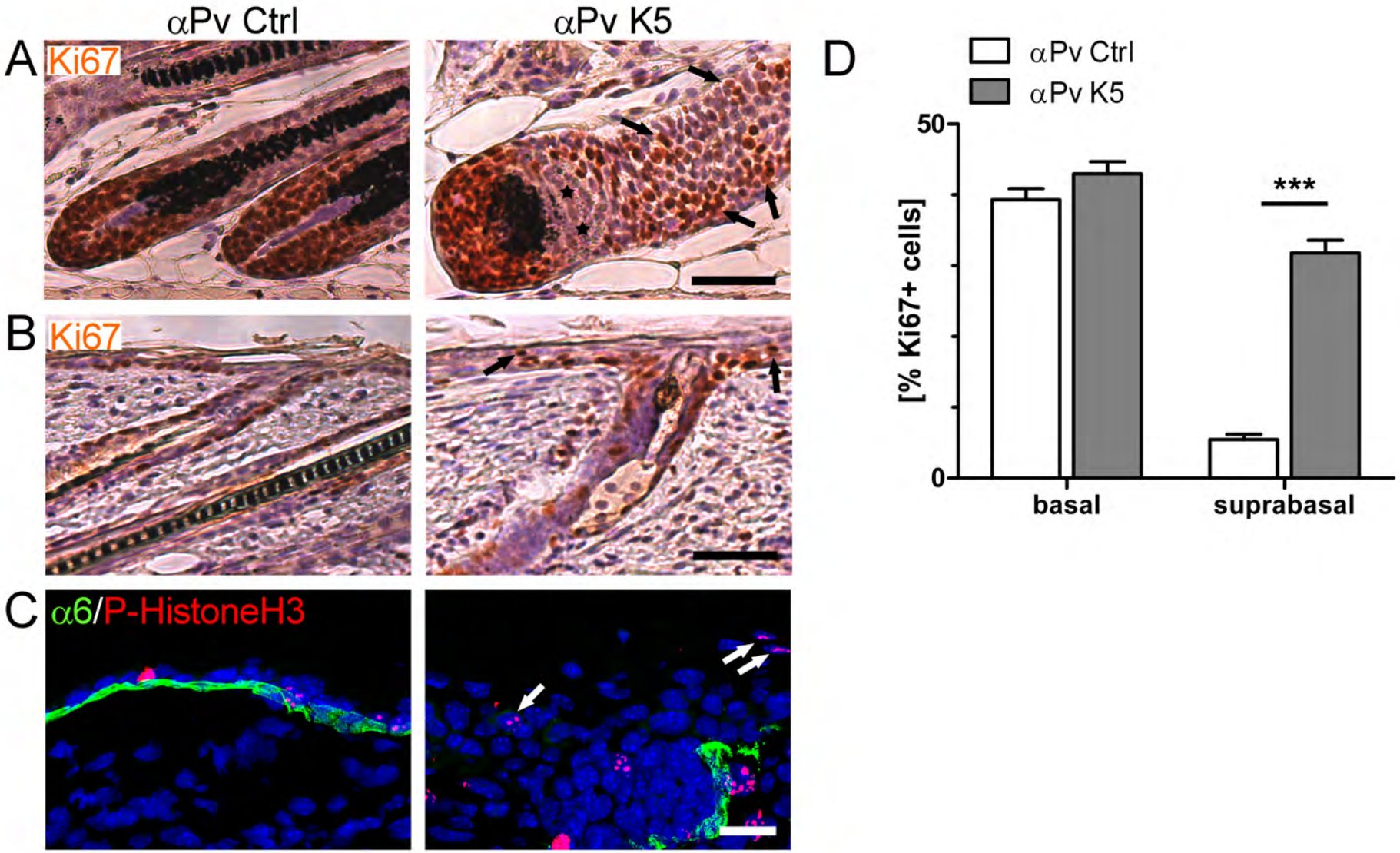


Altstätter et al., Figure 4

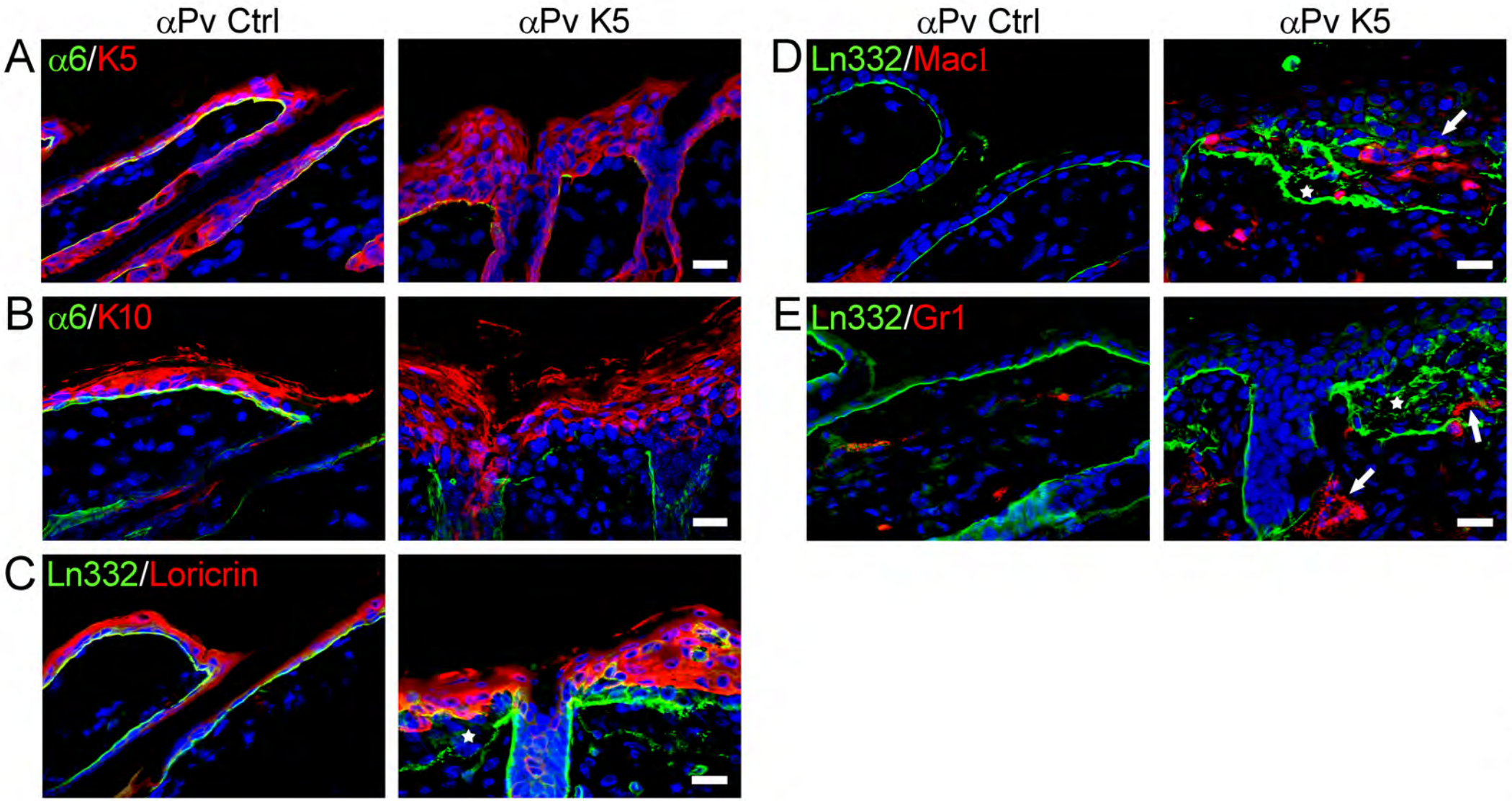


Altstätter et al., Figure 5

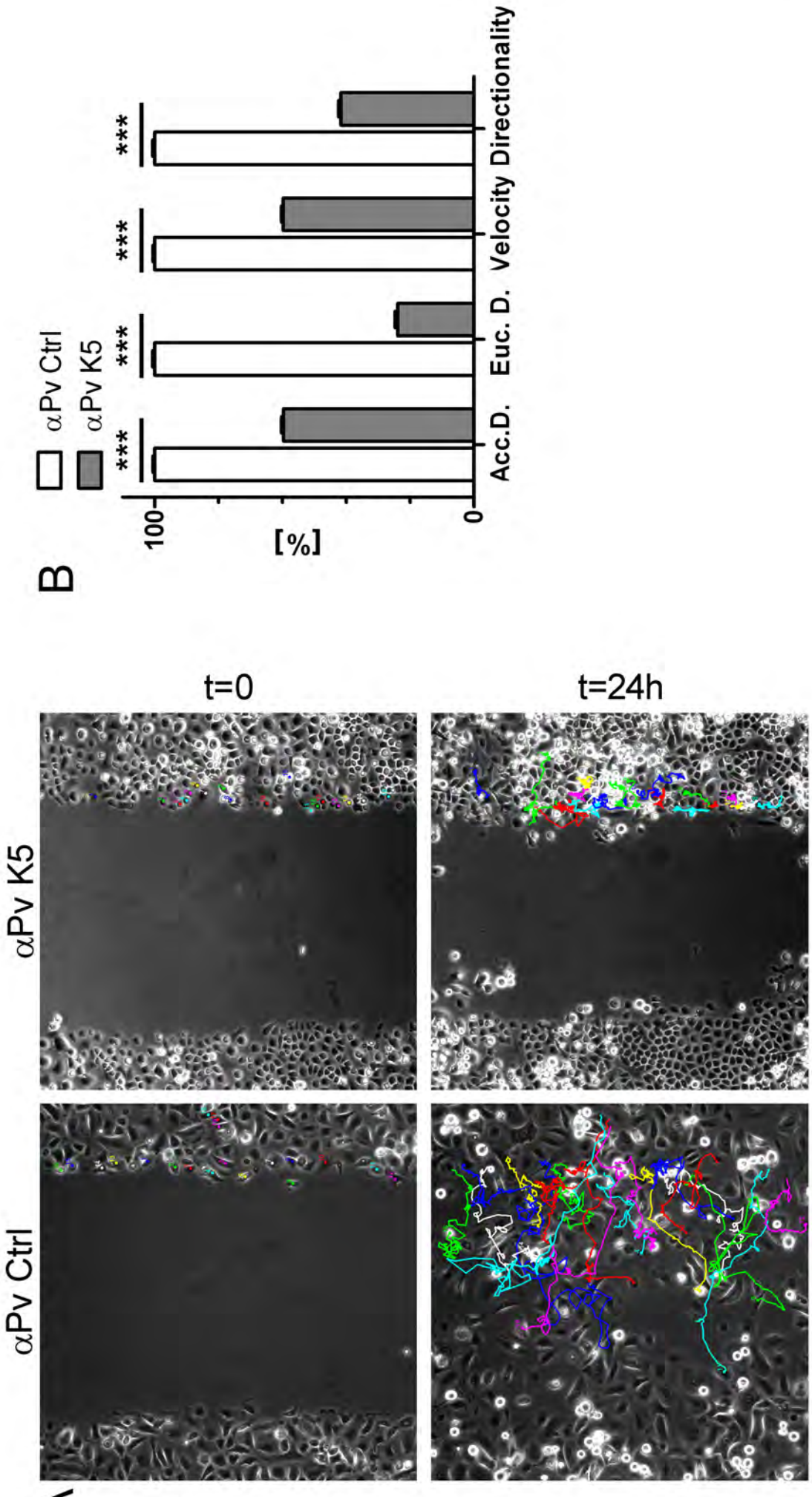
Altstätter et al., Figure 6

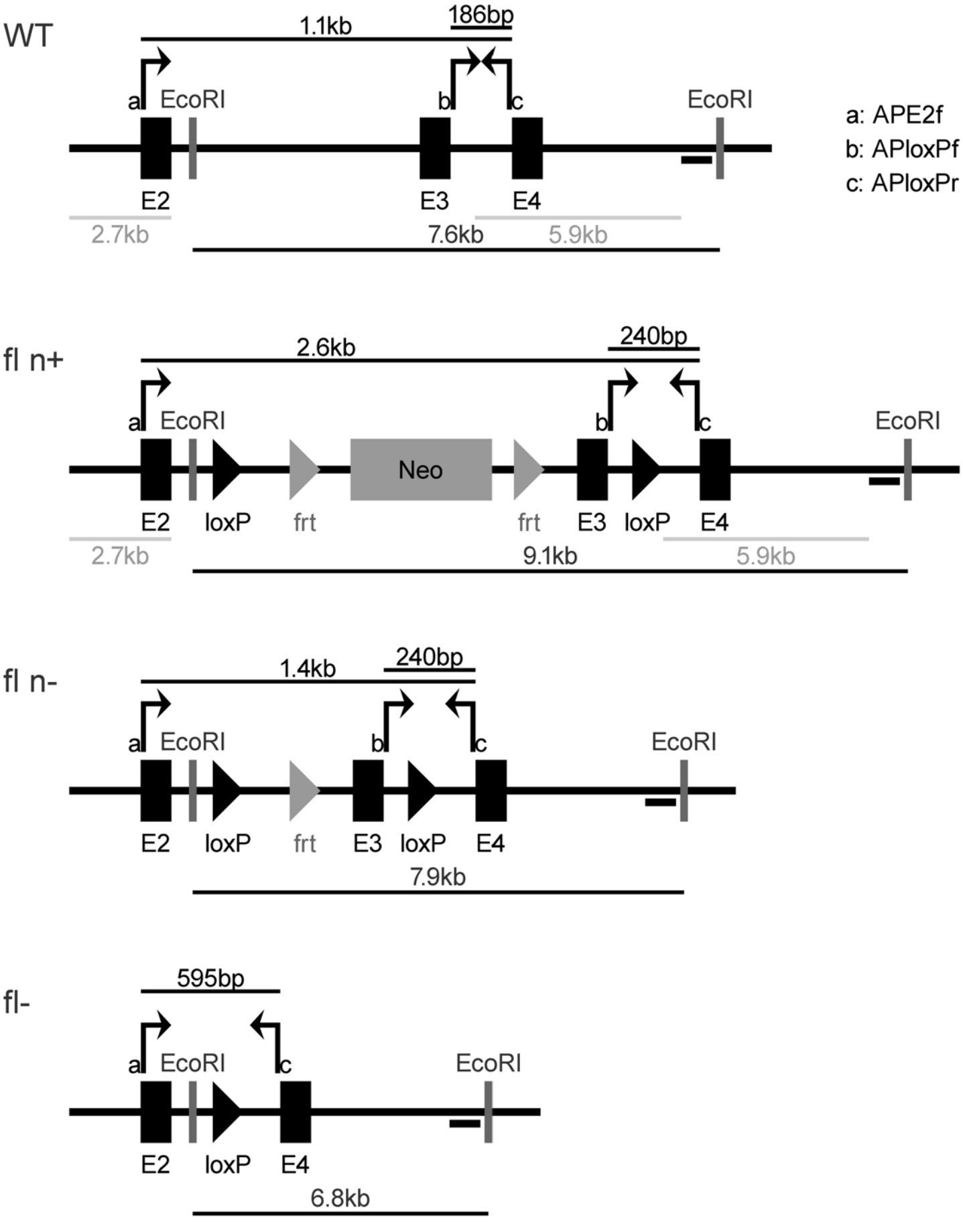


Altstätter et al., Figure 7



Altstätter et al., Figure 8


A
t=0
t=24h
αPv K5
αPv Ctrl
B
αPv Ctrl
αPv K5
***
***
***
***
100
0
[%]
Acc.D.
Euc. D.
Velocity
Directionality


WT
1.1kb
186bp
a
EcoRI
b
c
EcoRI
a: APE2f
b: APloxPf
c: APloxPr
E2
E3
E4
2.7kb
7.6kb
5.9kb
fl n+
2.6kb
240bp
a
EcoRI
b
c
EcoRI
Neo
E2
loxP
frt
frt
E3
loxP
E4
2.7kb
9.1kb
5.9kb
fl n-
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E2
loxP
frt
E3
loxP
E4
7.9kb
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a
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c
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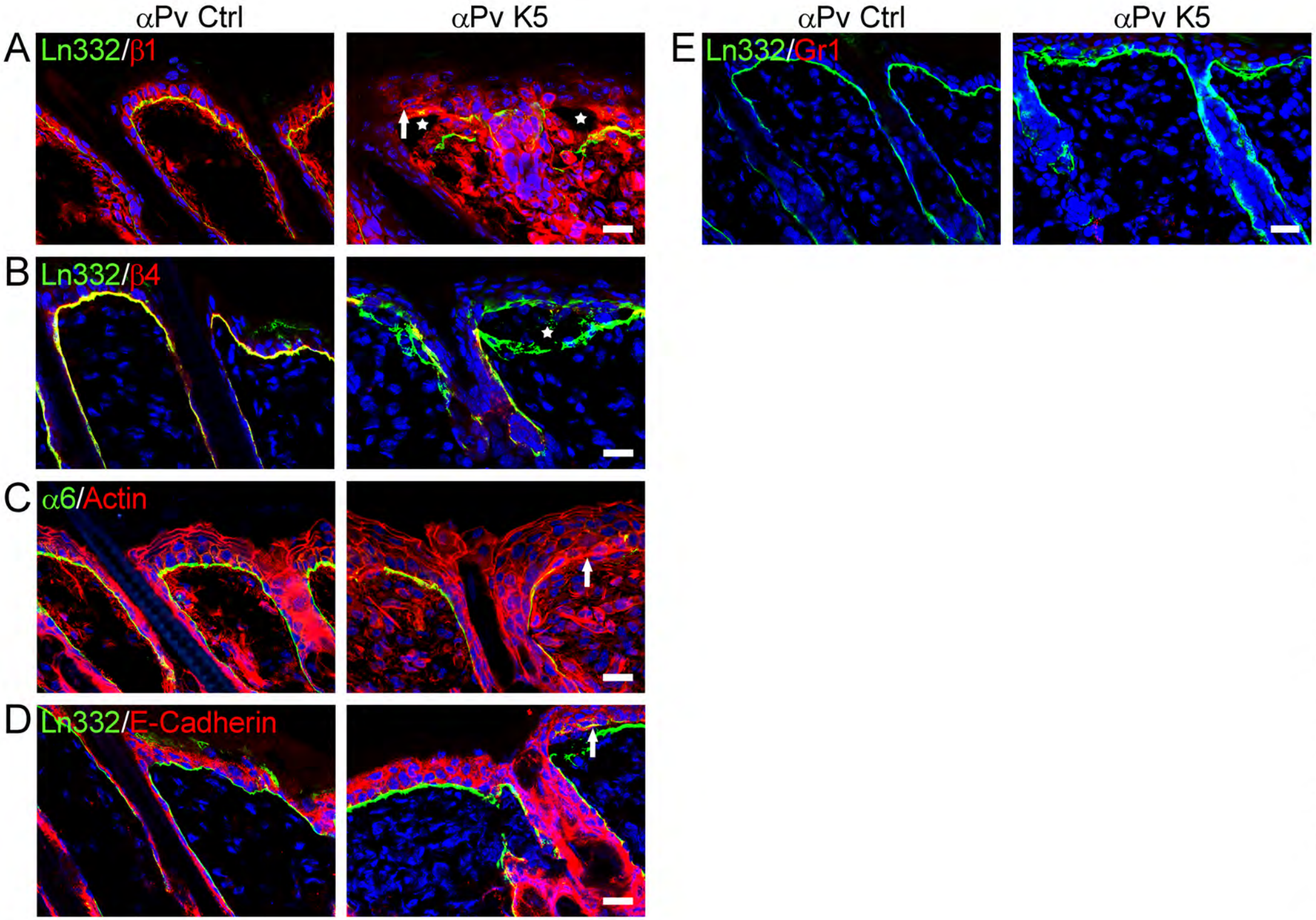
E2
loxP
E4
6.8kb

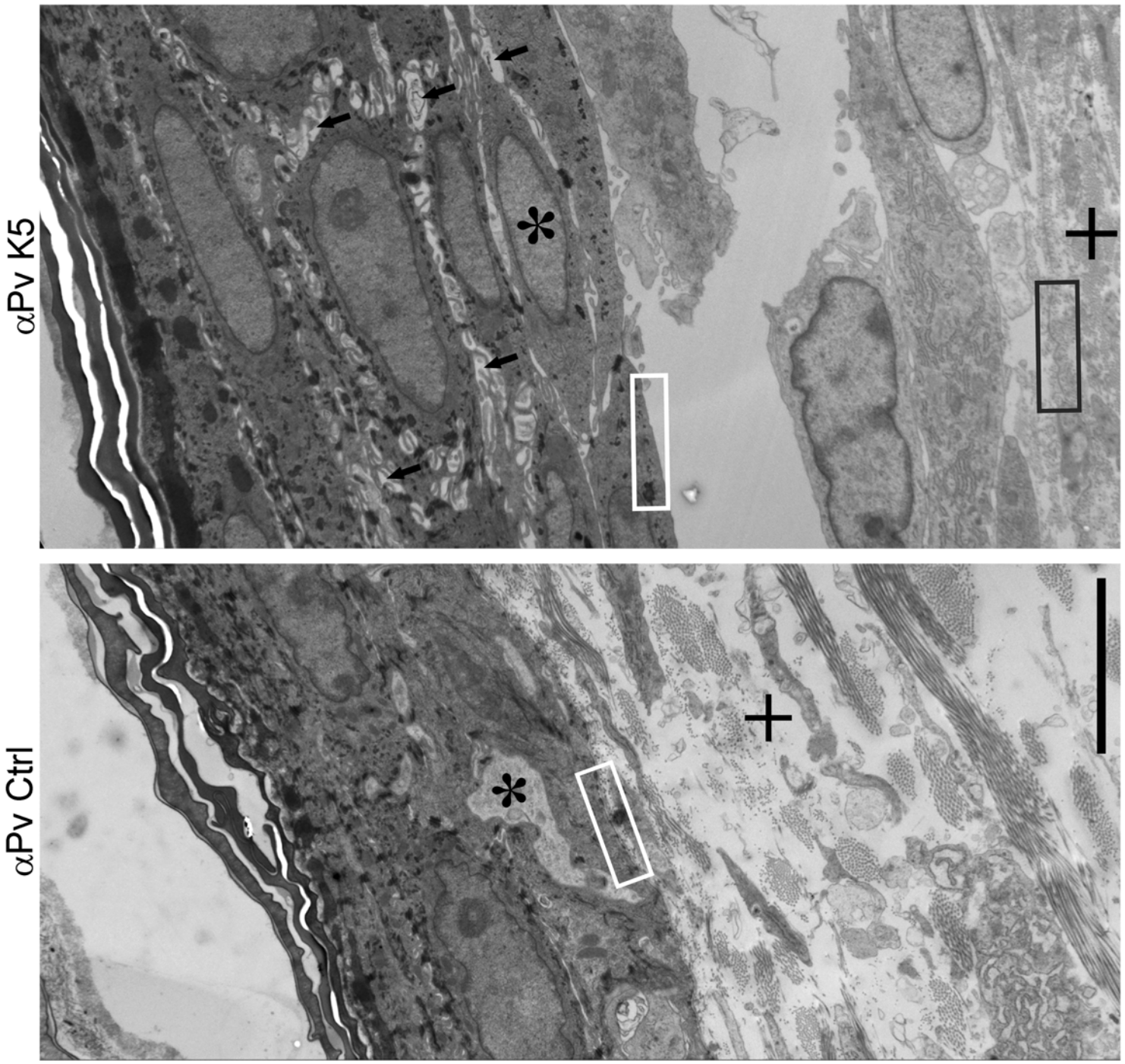
Altstätter et al., Supplementary Figure 2



Altstätter et al., Supplementary Figure 3

Altstätter et al., Supplementary Figure 4

Altstätter et al., Supplementary Figure 5



Curriculum vitae

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Date of birth: xx. xx. xxxx in Munich

School Education:

1986 – 1990	Grundschule
1990 – 1999	Gymnasium

University Education:

1999 – 2001	Studies in chemistry at the LMU, Germany
2001 – 2007	Studies in biology at the LMU, Germany *Main subject:* Cell biology *Minor subjects:* Genetics, biochemistry

University Degree:

03.2007	Diploma in biology Diploma-thesis: Title: „Analyse von ppod4, einem neuen 14-3-3 Bindeprotein aus *Hydra vulgaris*“

Ph. D. thesis:

04.2007– 2011	Doctoral work at the „Max Planck Institute of Biochemistry“ in the department „Molecular Medicine“ Title of the thesis: „ Functional analysis of α-Parvin *in vivo*“