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# Genetic analysis of the catalytic activity of Integrin-linked kinase (ILK) *in vivo*

Anika Lange
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## Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. Dr. Reinhard Fässler betreut.

## Ehrenwörtliche Versicherung

Diese Dissertation wurde selbstständig, ohne unerlaubte Hilfe erarbeitet.

München, am

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Anika Lange

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| 1. Gutachter | Prof. Dr. Reinhard Fässler |
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*Für meine lieben Eltern*

# Table of Contents

## List of Publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-VI):

**I.** Wiesner S, **Lange A** and Fässler R. **Local call: from integrins to actin assembly.** *Trends in Cell Biology* 2006, 16

**II.** Böttcher RT, **Lange A** and Fässler R. **How ILK and kindlins cooperate to orchestrate integrin signaling.** *Current Opinion in Cell Biology* 2009, 21(5): 670-5

**III.** **Lange A**, Wickström SA, Jakobson M, Zent R, Sainio K and Fässler R. **Integrin-linked kinase is an adaptor with essential functions during mouse development.** *Nature* 2009, 461: 1002-1006 .

**IV.** Wickström SA, **Lange A**, Montanez E and Reinhard Fässler. **The ILK/PINCH/parvin complex: the kinase is dead, long live the pseudokinase!** *EMBO J* 2010, 29(2): 281-91.

The following publications were not the focus of my project but I contributed to them:

**V.** Wickström SA, **Lange A**, Hess MW, Krüger M, Pfaller K, Mann M, Bloch W, Huber LA and Fässler R. **Integrin-mediated signals control microtubule dynamics required for plasma membrane targeting of caveolae.** *Submitted manuscript*

**VI.** Kim M, Ogawa M, Fujita Y, Yoshkawa Y, Nagai T, Koyama T, Nagai S, **Lange A**, Fässler R and Sasakawa C. **Bacteria hijack integrin-linked kinase to stabilize focal adhesions and block cell detachment.** *Nature* 2009, 459: 578-583.

## Abbreviations

| | |
|---|---|
| ADAM | a disintegrin and metalloproteinase |
| ADP | adenosine diphosphate |
| Akt | RAC-alpha serine/threonine protein kinase |
| APC | adenomatous polyposis coli |
| Arp2/3 | actin-related protein 2/3 complex |
| ATP | adenosine triphosphate |
| BMP | bone morphogenetic protein |
| Cdc42 | cell division cycle 42 |
| CH | calponin homology |
| Cre | cyclization recombinase |
| DAG | diacylglycerol |
| E | embryonic day |
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| ERK | extracellular signal-regulated kinase |
| FA | focal adhesion |
| F-actin | filamentous actin |
| FAK | focal adhesion kinase |
| FERM | 4.1, ezrin, radixin, moesin |
| FGF | fibroblast growth factor |
| FGFR | fibroblast growth factor receptor |
| FP | foot process |
| GAP | GTPase activating protein |
| GBM | glomerular basement membrane |
| GDI | GDP dissociation inhibitor |
| GDNF | glial-cell-derived neurotrophic factor |
| GDP | guanosine diphosphate |
| GEF | guanine nucleotide exchange factor |
| GFR | growth factor receptor |
| GPCR | G protein coupled receptor |
| Gsk-3β | glycogen synthase kinase-3β |
| HGF | hepatocyte growth factor |
| ICAM | intercellular adhesion molecule |
| ILK | integrin-linked kinase |
| IP3 | inositol triphosphate |
| IPP | ILK/PINCH/parvin |
| JNK | c-jun N-terminal kinase |
| MAPK | mitogen-activated protein kinase |
| MET | mesenchymal-to-epithelial transition |
| MIDAS | metal-ion-dependent adhesion site |
| MLC | myosin light chain |
| MM | metanephric mesenchyme |
| MMP | matrix metalloproteinase |
| Nck2 | noncatalytic region of tyrosine kinase, adaptor protein 2 |
| NS | nephrotic syndrome |

| | |
|---|---|
| PCP | planar cell polarity |
| PH | pleckstrin homology |
| PI3K | phosphatidylinositol 3-kinase |
| PINCH | particularly interesting new Cys-His rich protein |
| PKC | protein kinase C |
| PKD | polycystic kidney disease |
| PLC | phospholipase C |
| PTB | phosphotyrosine binding |
| Ret | rearranged during transformation |
| RGD | arginine, glycine, aspartate |
| RhoA | Ras homology gene family, member A |
| ROCK | Rho-associated protein kinase |
| SD | slit diaphragm |
| SH2 | src homology 2 |
| Src | Rous sarcoma oncogene |
| TGFβ | transforming growth factor β |
| UB | ureteric bud |
| VCAM | vascular cell-adhesion molecule |
| WD | wolffian duct |
| Wnt | wingless-type MMTV integration site family member |
| WT1 | wilms tumour 1 |

## Summary

Integrins are α/β heterodimeric transmembrane receptors that can bind to the extracellular matrix (ECM) and thereby mediate cell adhesion. Integrins are also signal transducing receptors. Since integrins are devoid of enzymatic activity and actin binding capability they depend on the recruitment of adaptor, scaffolding and signalling proteins in order to couple to the cytoskeleton and to propagate signals that regulate a variety of cellular processes including proliferation, survival, differentiation, and migration. One central constituent of this multiprotein complex is integrin-linked kinase (ILK), which is recruited to β1 and β3 integrin-mediated adhesion complexes. ILK consists of an ankyrin, pleckstrin homology (PH) and kinase domain and has been shown to directly bind the cytoplasmic parts of these integrin subunits. At the beginning of my thesis it has also been thought that ILK possesses kinase activity towards substrates such as Akt and Gsk-3β. However, the kinase activity of ILK and its physiological relevance was controversial due to several reasons: (1) ILK lacks well conserved residues that are important for kinase activity, (2) deletion of *ILK* in several cell types such as keratinocytes, fibroblasts or chondrocytes failed to diminsh or ablate phosphorylation of key substrates such as Akt or Gsk-3β, (3) genetic studies in *C.elegans* and *D.melanogaster* failed to confirm a kinase function of ILK *in vivo*.

Due to this controversy, it was important to determine whether the catalytic activity of ILK exists in a mammalian model system. To this end I established knock-in mouse strains with point mutations in ILK that were reported to convert ILK kinase activity *in vitro* either into a constitutive-dead or constitutive-active kinase. Surprisingly, knock-in mice carrying mutations in the putative PH domain (R211A, required for kinase activity) or in the autophosphorylation site (S343A; required for kinase activity, or S343D; renders ILK constitutive-active) do not show any obvious phenotype or changes in Akt or Gsk-3β phosphorylation or actin organization. In contrast, mice carrying point mutations in the potential ATP-binding site (K220A/M; required for catalytic activity) die shortly after birth due to kidney agenesis. This phenotype does not result from impaired kinase activity, as the mutations did not alter the phosphorylation levels of reported ILK substrates *in vivo*. In addition, no evidence of kinase activity was detected *in vitro*.

However, these mutations selectively impair the interaction of ILK and its key binding partner α-parvin. In line with this, similar kidney defects occur also in α-parvin null mice. On the basis of this study, it is now clear that the proposed kinase activity does not exist and thus is neither playing a role in mammalian development or adult life nor the integrin-actin linkage. However, the adaptor function of ILK is crucial for mammalian kidney development. Thus, my studies allow the conclusion that the kinase domain of ILK has been mutated in evolution to provide a novel and essential, non catalytic function to integrins.

# Introduction

## 1. The integrin receptor family

Integrins are α/β heterodimeric type I transmembrane glycoproteins that mediate the attachment of cells to the extracellular matrix (ECM) and to other cells. The receptor family is evolutionarily highly conserved. Integrins have been identified in mammals, chicken and zebrafish, as well as in lower eukaryotes, including sponges, the nematode *C.elegans* (two α and one β subunit, generating two integrins) and the fruitfly *D. melanogaster* (five α and one β subunit, generating five integrins) (Johnson et al., 2009).
The name "integrin" was coined in the 1980ties to denote the importance of these receptors for maintaining the integrity of the cytoskeletal-ECM linkage (Hynes, 2004; Tamkun et al., 1986). The majority of integrins links the ECM to the actin cytoskeleton, while integrin α6β4 connects to the intermediate filament system.
Integrins present a bi-directional conduit for mechanochemical information across the cell membrane, as they provide a major mechanism to connect the inside of the cell with the extracellular environment. Their activation triggers a large variety of signal transduction events that affect cell behaviors such as adhesion, proliferation, survival or apoptosis, shape, polarity, motility, haptotaxis, gene expression, and differentiation, mostly through modulating the cytoskeleton (Takada et al., 2007). Notably, integrins do not possess enzymatic or actin-binding activity of their own.

### 1.1 Structure of integrins

Integrin α- and β-subunits have large extracellular domains (approximately 800 amino acids) that contribute to ligand binding, single transmembrane domains (approximately 20 amino acids) and short cytoplasmic tails (13 to 70 amino acids, with the exception of β4, which has a length of approximately 1.000 amino acids). While there is a striking sequence homology among the different β-subunit cytoplasmic tails, the α-subunit tails are highly divergent apart from a conserved GFFKR motif next to the transmembrane region, which is important for association with the β-tail (Takada et al., 2007). The extracellular domain of the heterodimer consists of a ligand-binding head domain

standing on two long legs (Figure 1). The extracellular domains can also associate laterally with other proteins such as tetraspanins, growth factor receptors, matricellular proteins, and matrix proteases or their receptors at the cell surface (Miranti and Brugge, 2002).

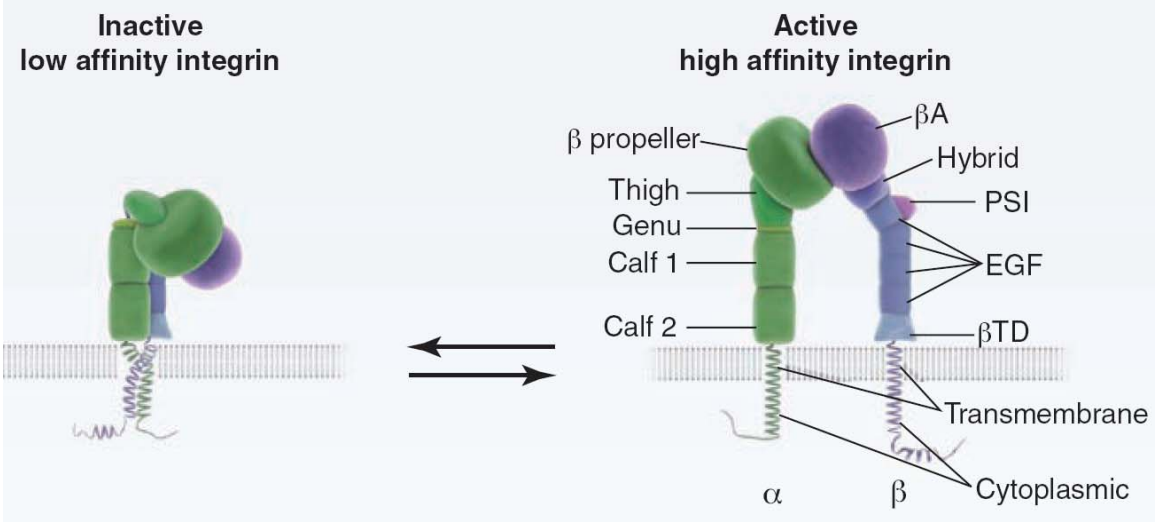



**Figure 1: Integrin architecture and schematic representation of integrin activation (Moser et al., 2009b).**
Specific contacts between the ectodomain, the transmembrane domain and cytoplasmic domains keep the integrin in its bent, inactive conformation. During integrin activation the integrin legs, transmembrane domain and cytoplasmic domains separate, resulting in an extended integrin conformation. βTD, β tail domain; EGF, epidermal growth factor domain; PSI, plexin/semaphorin/integrin domain

The ectodomain of an integrin α-subunit is composed of a seven-bladed β-propeller, which is connected to a thigh, a calf-1 and a calf-2 domain, together forming the leg structure that supports the integrin head. The last blades of the β-propeller contain EF-hand domains that bind $Ca^{2+}$ -ions and thereby affect ligand binding (Humphries et al., 2003). Nine of the integrin α chains contain an additional I domain (Table 1), referred to as a von Willebrand factor A domain that almost always constitutes the ligand binding site. Ligand binding occurs via a coordinating $Mg^{2+}$ -ion in the so-called metal-ion-dependent-adhesion site (MIDAS) motif (Barczyk et al., 2009; Moser et al., 2009b).
The β-subunit is composed of a βA (I) domain, which is analogous to the I domain of the α-subunit, a hybrid domain, a PSI (plexin/semaphorin/integrin) domain, four epidermal

growth factor (EGF) domains and a membrane proximal β tail domain (βTD). In integrins lacking an I domain, ligands bind to a crevice between the αβ-subunit interface, where they interact with a metal ion-occupied MIDAS within the β-subunit and the propeller domain of the α-subunit (Figure 1) (Moser et al., 2009b).

Most integrin β cytoplasmic domains (also called integrin tails) contain one or two motifs that are part of a canonical recognition sequence for phosphotyrosine-binding (PTB) domains: a membrane proximal NPxY motif and a membrane distal NxxY motif. These protein sequences are present in a wide variety of signaling and cytoskeletal proteins, and play a crucial role in integrin activation.

Integrins can exist in low-, intermediate-, and high-affinity states. Based on structural and electron microscopy studies, it is believed that integrins are in a low-affinity state when their extracellular domains are bent and in a high-affinity state when the extracellular domains are extended (Figure 1). Two models have been proposed for the affinity change. In both, the inactive integrin is in a bent conformation, with the headpiece facing the membrane. In the "deadbolt model" the bent conformation is maintained in the activated integrin, but piston-like movements of the transmembrane regions cause sliding of the extracellular stalks of the α- and β-subunits. As a consequence, this sliding disrupts the interaction between the headpiece and the β stalk just beyond the membrane (Arnaout et al., 2005). In the "switchblade model", dissociation of the α and β cytoplasmic and transmembrane regions leads to dislocation of an EGF-like repeat in the β stalk, which causes the head region to extend outwards in a switchblade-like movement (Arnaout et al., 2005). Support for "the switchblade model" came from the crystal structure of integrin αVβ3, which revealed a bent conformation of the head region associated with low-affinity for the ligand. It was therefore proposed that the bent form does not bind to a ligand and that activated integrins have an extended form (switchblade model) (Takada et al., 2007). However, the bent conformation does not always seem to be inactive, especially in the context of binding to small ligands (Askari et al., 2009).

The transmembrane domains have a key role in integrin activation. The transmembrane domains of inactive integrins are engaged in a coiled-coil interaction between canonical GxxxG dimerization motifs in each subunit. Separation of integrin transmembrane domains is a requirement for integrins to adopt the high-affinity state (Moser et al.,

2009b). The role of integrin cytoplasmic tails in regulating integrin affinity has been extensively studied in the rapidly activated leukocyte-specific β2 and platelet-specific aIIbβ3 integrins. High integrin affinity has been shown to be associated with separation of the α and β cytoplasmic tails. The separation is most likely achieved by binding of cytoplasmic proteins to the β-tail and will be discussed later.

### 1.2 Integrins and their ligands

Integrin heterodimers are composed of non-covalently associated α- and β-subunits (Hynes, 2002). In vertebrates, the family is comprised of 18 α-subunits and 8 β-subunits that can assemble into 24 different heterodimers. Some subunits appear only in a single heterodimer, whereas 12 integrins contain the β1 subunit and five contain αV (Figure 2 and Table 1). Integrins assemble in the endoplasmatic reticulum and are transported to the plasma membrane as heterodimers.

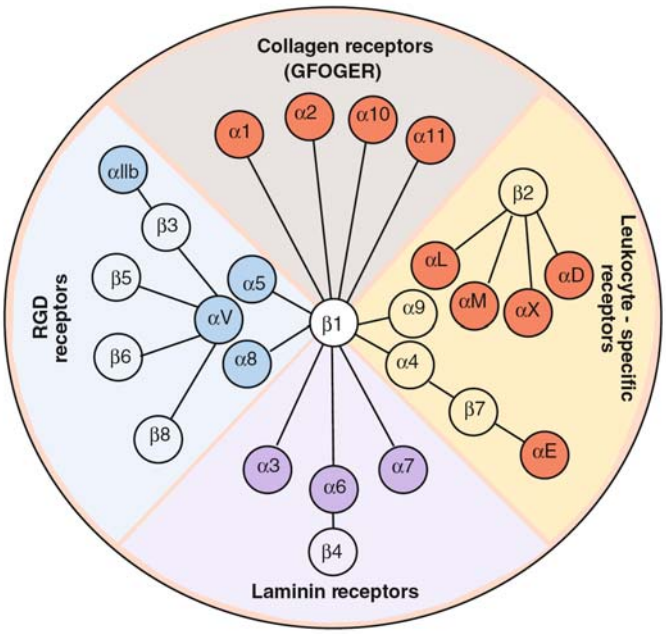



**Figure 2: Representation of the integrin receptor family grouped by their main ligand specificity (Barczyk et al., 2009).**
Vertebrates possess 18 α- and 8 β-subunits, which give rise to 24 heterodimers that can be assembled into four distinct ligand binding classes.

**Table 1: Characteristics of human integrin α-subunits (Barczyk et al., 2009).**

CI, cleavage; αI, αI domain

| Integrin | α chain characteristics | CI | αI | Prototypic ligands | Additional ligands |
|---|---|---|---|---|---|
| α1β1 (CD49a, VLA1) | 1151 aa | - | X | collagens (collagen IV > collagen I; collagen IX) | semaphorin 7A, laminin |
| α2β1 (CD49b, VLA2) | 1181 aa | - | X | collagens (collagen I > collagen IV; collagen IX) | E-cadherin, endorepellin, laminin |
| α3β1 (CD49c, VLA3) | 1051 aa, splice variants α3A and α3B | X | - | laminins (LN-511 > LN-332 > LN-211) | - |
| α4β1 (CD49d, VLA4) | 1038 aa | - | - | fibronectin, VCAM-1 | - |
| α5β1 (CD49c, VLA5) | 1049 aa | X | - | fibronectin (RGD) | endostatin |
| α6β1 (CD49f, VLA6) | 1073 aa, splice variants α6A and α6B | X | - | laminins (LN-511 > LN-332 > LN-211 > LN-411) | - |
| α7β1 | 1137 aa, splice variants X1, X2, α7A and α7B | X | - | α7X1β1: laminins (LN-511 > LN-211 > LN-411 > LN-111) α7X2β1: laminins (LN-111 > LN-211 > LN-511) | - |
| α8β1 | 1025 aa | X | - | fibronectin, vitronectin, nephronectin (RGD) | - |
| α9β1 | 1035 aa | - | - | tenascin-C, VEGF-C, VEGF-D | osteopontin |
| α10β1 | 1167 aa | - | X | collagens (collagen IV > collagen VI > collagen II; collagen IX) | - |
| α11β1 | 1188 aa, serted domain 21 aa | - | X | collagens (collagen I > collagen IV; collagen IX) | - |
| αLβ2 (CD11a) | 1170 aa | - | X | ICAM-1, -2, -3, -5 | - |
| αMβ2 (CD11b) | 1153 aa | - | X | iC3b, fibrinogen + more | - |
| αXβ2 (CD11c) | 1163 aa | - | X | iC3b, fibrinogen + more | - |
| αDβ2 (CD11d) | 1162 aa | - | X | ICAM-3, VCAM-1 | - |
| αIIbβ3 (CD41, GpIIb) | 1039 aa | X | - | fibrinogen, fibronectin (RGD) | - |
| α6β4 | | X | - | laminins (LN-332, LN-511) | - |
| αVβ1 (CD51) | 1048 aa | X | - | fibronectin, vitronectin (RGD) | - |
| αVβ3 | | X | - | vitronectin, fibrinogen, fibronectin (RGD) | tumstatin |
| αVβ5 | | X | - | vitronectin (RGD) | - |
| αVβ6 | | X | - | fibronectin, TGF-β-LAP (RGD) | - |
| αVβ8 | | X | - | vitronectin, TGF-β-LAP (RGD) | - |
| αEβ7 (CD103, HML-1) | 1178 aa | X | X | E-cadherin | - |
| α4β7 | | - | - | MadCAM-1, fibronectin, VCAM-1 | - |

**Table 2: Characteristics of human integrin β-subunits (Barczyk et al., 2009).**

| Integrin β chain | Charateristics | Notes |
|---|---|---|
| β1 (CD29, GpIIa) | 798 aa, splice variants β1A-D, | Splice variants β1B and β1C not present in mice, minor variants with unclear function |
| β2 (CD18) | 769 aa | - |
| β3 (CD61, GpIIIa) | 788 aa, splice variants β3A, β3B and β3C | β3A major form |
| β4 (CD104, TSP-180) | 1875 aa, splice variants β4A-E | β4A and β4B major forms, similar function |
| β5 | 799 aa, splice variants β5A, β5B | Both splice variants have similar functions |
| β6 | 788 aa | - |
| β7 (LPAM-1, βP) | 798 aa | - |
| β8 | 769 aa | - |

Integrins are grouped into subgroups based on ligand-binding properties (Figure 2) or based on their subunit composition (Table 1 and 2). It is possible to cluster integrin-ligand combinations into four main classes, reflecting the structural basis of the molecular interaction: collagen-binding integrins, laminin-binding integrins and RGD (arginine, glycine, aspartate)-binding integrins (Humphries et al., 2006). Leukocyte-specific integrins establish cell-cell contacts with endothelial cells by interacting with cellular counter-receptors such as intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs) (Ley et al., 2007).

All five αV containing integrins and two β1 integrins (α5β1, α8β1) share the ability to recognize ligands containing an RGD tripeptide active site. RGD constitutes the minimal integrin recognition sequence in ligands such as fibronectin, vitronectin, tenascin, osteopontin and fibrinogen. α4β1, α4β7, α9β1, the four members of the β2 subfamiliy and αEβ7 recognize related sequences in their ligands. α4β1, α4β7 and α9β1 bind to an acidic motif, termed “LDV” (lysine, aspartate, valin) that is functionally related to RGD. Fibronectin contains the prototype LDV ligand in its type III connecting segment region; other ligands (such as VCAM-1 and MadCAM-1) employ related sequences. Four α-subunits containing an αA-domain (α1, α2, α10, α11) combine with β1 and form a distinct laminin/collagen-binding subfamily. The fourth group includes three β1 integrins

(α3β1, α6β1 and α7β1) and α6β4 that are highly selective laminin receptors which do not have an αA-domain (Humphries et al., 2006).

Other integrin ligands include milk fat globule-EGF factor 8 (MFGE8) and complement factor iC3b, which facilitate phagocytosis of apoptotic cells and pathogens, respectively; the latency-associated peptide of transforming growth factor β (TGFβ), which regulates the activation of TGFβ and some of the a disintegrin and metalloproteinase (ADAM) family members and matrix metalloproteinase-2 (MMP-2) which participate in ECM remodeling during cell adhesion and migration (Table 1) (Legate and Fassler, 2009).

Integrin ligands can also be generated by proteolysis. Endostatin (derived from collagen XVIII), endorepellin (derived from perlecan) and tumstatin (derived from collagen α3) are the best-known examples (Bix and Iozzo, 2005; Wickstrom et al., 2005). In addition, integrins can bind snake toxins, and certain viruses and bacteria. Some of these interactions occur outside the regular ligand-binding sites in the integrins and display distinct binding characteristics compared with the binding of physiological ligands (Barczyk et al., 2009).

Alternative splicing of mRNA leads to additional complexity of the integrin family. Variants of both the extracellular and cytoplasmic domains have been reported. Alternative extracellular domains may account for different ligand-binding affinities or variations in the state of activation, while variants of the cytoplasmic domain may modulate integrin activity, cytoskeletal associations and/or signaling events (van der Flier and Sonnenberg, 2001). The best studied are the four cytoplasmic variants of the β1 subunit: β1A, β1B, β1C and β1D. Integrin subunit β1A is present in all tissues except mature cardiac and skeletal muscle, which instead express the highly homologous β1D variant. However, β1A and β1D are not functionally equivalent in embryonic development. The replacement of β1A by β1D results in embryonic lethality in mice, whereas replacement of β1D with β1A does not lead to severe abnormalities in striated muscles *in vivo* (Baudoin et al., 1998).

Each of the 24 vertebrate integrins appears to have a specific, non-redundant function. This is in part apparent from their ligand specificities but is best proven by the distinct phenotypes of the knockout mice of single integrin subunits. The phenotypes reflect the different functions of individual integrins and range from a complete block in

preimplantation development (β1) through major developmental defects (α4, α5, αV, β8) to perinatal lethality (α3, α6, α8, αV, β4, β8) and defects in leukocyte function (αL, αM, αE, β2, β7), inflammation (β6), homeostasis (αIIb, β3, α2), bone remodeling (β3), and angiogenesis (α1, β3) as well as others (Bouvard et al., 2001; Hynes, 2002). Generation of tissue-specific integrin knockout mice that gave rise to severe phenotypes during embryonic development provided further insight to the specific function of a given integrin.

### 1.3 Bidirectional regulation of integrin signaling

Integrin receptors possess the rare ability to signal bidirectionally across the plasma membrane. Ligand binding triggers signal transduction into the cell through the recruitment of adaptor and signaling proteins that establish a connection to actin and various signal transduction pathways ("outside-in" signaling) which is important for example in cell spreading and cell migration. Conversely, intracellular non-integrin mediated signals can induce changes in integrin conformation and activation that alters its ligand-binding affinity in a process termed "inside-out" signaling or integrin activation. Integrin clustering follows the engagement of integrins triggered by the naturally multivalent nature of ECM, and it promotes the localized concentration of intracellular signaling molecules.

#### 1.3.1 Inside-out signaling

Inside-out activation of integrins relys on the binding of cytoplasmic ligands to specific sites within the integrin tails. This induces conformational changes that are transmitted to the extracellular ligand-binding domains via the transmembrane domains and stalk regions. The β-integrin interacting proteins talin and kindlin have emerged as important regulators of integrin activation.

Talin orthologs have been identified in all multicellular eukaryotes studied; vertebrates encode two talin isoforms, talin1 and talin2, whereas lower eukaryotes encode only a single talin isoform corresponding to talin1 (Moser et al., 2009b). Talins are ~ 270 kD

proteins, composed of an N-terminal 47 kD head domain and a C-terminal flexible rod domain and form dimers. The talin head consists of a FERM (4.1, ezrin, radixin, moesin) domain composed of three subdomains (F1-F3) and an F0 subdomain with no homology to known domains (Figure 3). The F3 subdomain resembles a phosphotyrosine-binding (PTB) domain and binds to the conserved membrane proximal NPxY motifs in β integrin tails (Calderwood et al., 2002), but also to phosphatidylinositol 4-phosphate 5-kinase γ (PIPK1γ), and the hyaluronan receptor layilin. The talin rod domain is made of a series of domains composed of helical bundles that contain multiple binding sites for the F-actin-binding protein vinculin, and a second integrin binding site (Figure 3) (Critchley and Gingras, 2008). As talin binds to integrin cytoplasmic domains, vinculin and actin filaments it is suggested that it forms an important link between the cytoskeleton and the ECM (Critchley and Gingras, 2008).

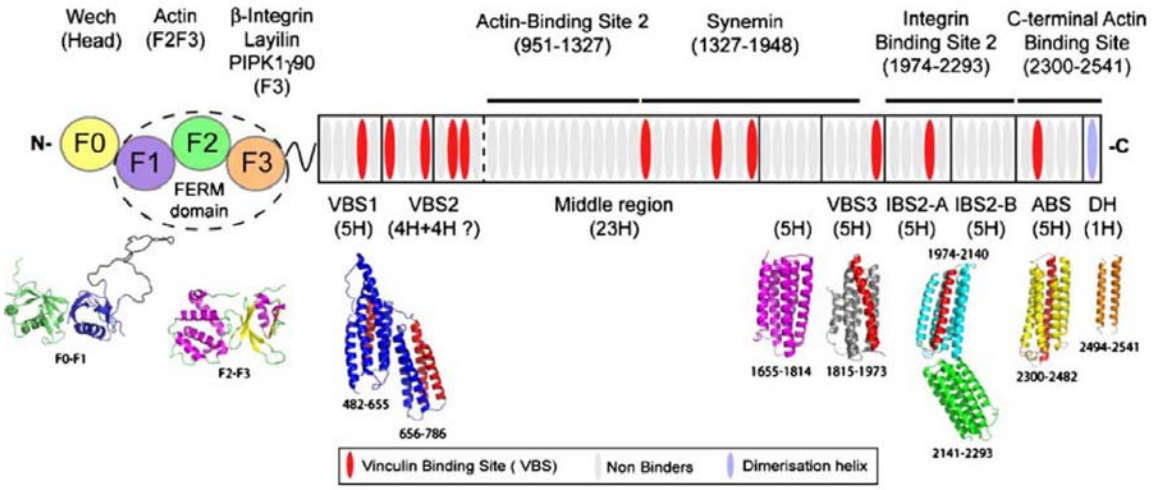



**Figure 3: Schematic representation of the domain structure of talin (Roberts and Critchley, 2009).**
Talin binds to β integrin cytoplasmic tails and regulates integrin activation in cooperation with kindlin proteins. In addition, talin can bind to actin and vinculin and thereby links the ECM to the actin cytoskeleton.

Talin's role in integrin activation was originally demonstrated by the ability of its F3 domain to activate αIIbβ3 integrin when expressed in CHO cells (Calderwood et al., 2002; Calderwood et al., 1999). Knock-out and knock-down experiments subsequently

reinforced the notion that talin is a key component of integrin affinity regulation (Nieswandt et al., 2007; Petrich et al., 2007; Tadokoro et al., 2003).

Inside-out signaling has been extensively studied in circulating blood cells such as platelets, leukocytes and lymphocytes which present integrin αIIbβ3 or β2 integrins on their surfaces. In these cells, integrin activation has to be rapid and tightly controlled as constitutively active αIIbβ3 for example, would trigger pathologic thrombus formation causing strokes, myocardial infarction and other embolic events. This tight regulation is achieved by controlling the binding of integrin-activating proteins such as talin. NMR studies revealed an intramolecular autoinhibitory interaction between the talin C-terminus and its PTB domain that masks the integrin binding pocket. The precise mechanism that disrupts this autoinhibition requires further investigation, although the small GTPase Rap1 and its binding partner RIAM have been shown to play a key role in talin activation (Figure 4). In addition, PIPK1γ90 and PIP2 have also been implicated in this process (Roberts and Critchley, 2009). The talin-integrin interaction might additionally be controlled on the receptor level through phosphorylation of the β integrin tail. The tyrosine residue within the β1 and β3 integrin NPxY motif can be phosphorylated by Src family kinases. When this tyrosine is mutated to phenylalanine it reverses the integrin-dependent spreading and migration defects in v-Src-transformed cells, suggesting that phosphorylation might inhibit talin binding (Moser et al., 2009b; Sakai et al., 2001).

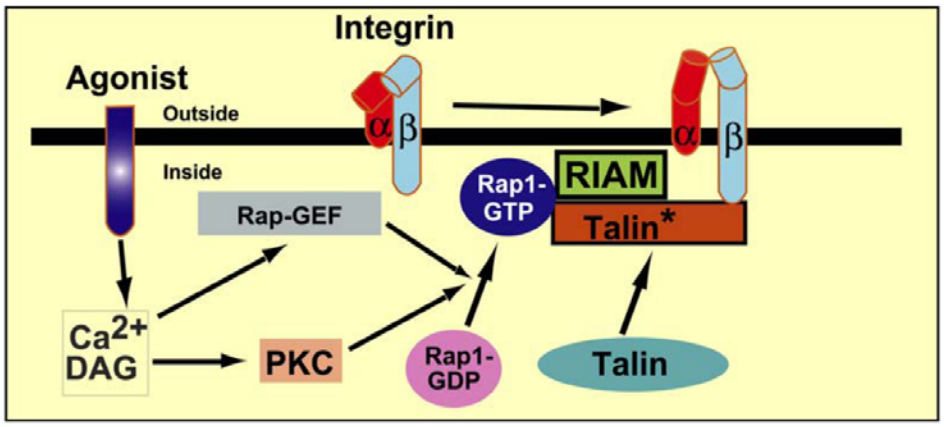



**Figure 4: Agonist stimulation triggers integrin activation (Han et al., 2006).**
Agonist receptors like G-protein coupled receptors or tyrosine-kinase coupled receptors induce the formation of diacylglycerol (DAG) and increased $Ca^{2+}$ leading to the activation and/or translocation of active GTP bound Rap1 to the plasma membrane via activation of protein kinase C (PKC) or a Rap guanine nucleotide exchange factor (Rap-GEF). At the plasma membrane, activated Rap interacts with RIAM, leading to the recruitment of talin to form the integrin activation complex.

Mutations and truncations of the β3 integrin tail C-terminal to the the talin binding site decrease integrin affinity for ligands (Ma et al., 2006), which raised the possibility that additional factors besides talin also affect the affinity states of integrins. Indeed, recent work showed that talin alone is not sufficient for integrin activation and that kindlin proteins are as important in mediating this function (Montanez et al., 2008; Moser et al., 2009a; Moser et al., 2008; Ussar et al., 2008). Kindlins are essential components of the integrin adhesion complex, which bind to the membrane distal NxxY motif of β1, β2 and β3 integrins (Böttcher et al., 2009; Meves et al., 2009). As kindlins and talin bind distinct regions of the β integrin tail, they most likely cooperate to regulate integrin affinity. Although kindlins are not sufficient to shift integrins to a high-affinity state, they facilitate talin function. Conversely, talin depends on kindlins to promote integrin affinity because talin-head overexpression failed to induce activation of αIIbβ3 in kindlin-depleted CHO cells. Thus, kindlins require talin, and talin alone is not sufficient to increase integrin affinity (Moser et al., 2009b).
For more information about the kindlin protein family, the reader is referred to the second publication (review) presented in this PhD thesis.

### 1.3.2 Outside-in signalling

Extracellular ligand binding to integrins is believed to induce conformational changes within the integrin heterodimer, including the outward swing of the hybrid domain, separation of the α and β "leg" domains and separation of the transmembrane and cytoplasmic domains. This leads to the interaction of the cytoplasmic tails with intracellular signaling molecules ("switchblade" model) (Arnaout et al., 2005). Once integrins are activated and clustered they are able to transmit the vast array of intracellular changes collectively referred to as "outside-in" signaling. Interestingly, up to now, around 156 components have been described that build up the so called "integrin adhesome". Theoretically, interactions among those components could give rise to 690 interactions (Zaidel-Bar et al., 2007).

Integrin activation leads to downstream signaling events that can be divided into three temporal stages (Figure 5) (Legate et al., 2009). The immediate effects of integrin activation are the up-regulation of lipid kinase activity that increases the local concentration of the phosphoinositide second messengers PtdIns-4, 5-P2 and PtdIns-3, 4, 5-P3 as well as rapid phosphorylation of specific protein substrates. Within several minutes these changes then lead to the activation of diverse signaling pathways.

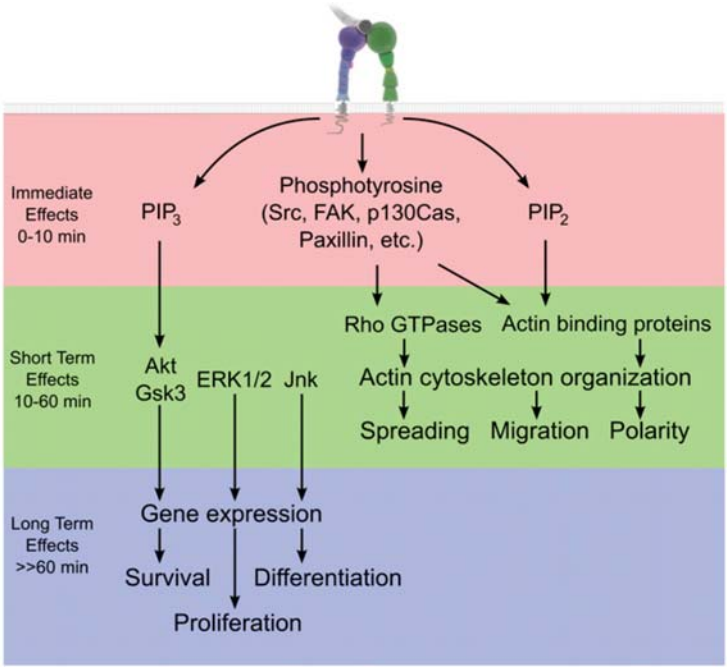



**Figure 5: Integrin activation leads to downstream signaling events that can be divided into three temporal stages (Legate et al., 2009).**
The immediate effects are increased lipid kinase activity and increased tyrosine phosphorylation. Short-term changes consist of cytoskeletal rearrangements and long-term effects are regulation of various signaling pathways and gene expression.

One important event in integrin-mediated signaling is cell adhesion-dependent phosphorylation of key focal adhesion proteins such as FAK and Src. Phosphorylation of FAK at tyrosine-397 creates a docking site for the SH2 domain of Src family kinases. Binding of Src to the FAK phosphotyrosine-397 site releases an autoinhibitory interaction and consequently activates Src. The activated FAK/Src complex in turn phosphorylates components of focal adhesions including FAK, paxillin and p130Cas, resulting in the recruitment of additional signaling intermediates including Grb2 and activation of downstream signaling pathways such as the Ras/MAPK signaling pathway.
Another central event is the activation of Rho family GTPases and other actin regulatory proteins, which drive the reorganization of the actin cytoskeleton allowing cells to adopt their characteristic shape and initiate migration. Long-term consequences of integrin outside-in signaling are the activation of proliferation and survival pathways, leading to the induction of genetic programs to control cell fate (Legate et al., 2009).

Importantly, other signaling pathways such as growth factor signaling interact with integrin-mediated signaling on multiple levels. They can regulate integrin affinity for their ligands, regulate the activity of the integrin-associated signaling proteins, and control the activity of the downstream effectors such as ERK, Akt and JNK and the Rho GTPases.

In addition, key protein complexes such as the integrin-linked kinase (ILK)/PINCH/parvin (IPP) complex are recruited to cell-ECM contacts. The IPP complex connects integrin signaling with growth factor signaling through interaction of PINCH with Nck2 and can connect integrins to the actin cytoskeleton through direct binding of parvin proteins to F-actin or through interaction of ILK with paxillin (Legate et al., 2006). In addition, ILK has initially been identified as a true kinase and later shown to directly phosphorylate substrates such as Akt and Gsk-3β and thereby regulate cell survival and cell proliferation (McDonald et al., 2008). However, ILK lacks well conserved amino acid residues that are required for eukaryotic kinase activity. Therefore its kinase activity and the physiological relevance was hotly debated (Legate et al., 2006).

Interestingly, integrin-dependent processes are strongly influenced by mechanical properties of the matrix such as rigidity and tensile strength. Conversely, endogenous tension (cell contractility) is transmitted through integrins to the ECM to influence matrix rigidity. This occurs through the recruitment of cytoplasmic proteins that induce downstream effectors involved in regulating matrix deposition or remodeling (Berrier and Yamada, 2007).

In summary, the composition of the ECM, its mechanical properties and the growth factor environment regulate the outside-in signaling by integrins in cooperation with growth factor receptors (Figure 6).

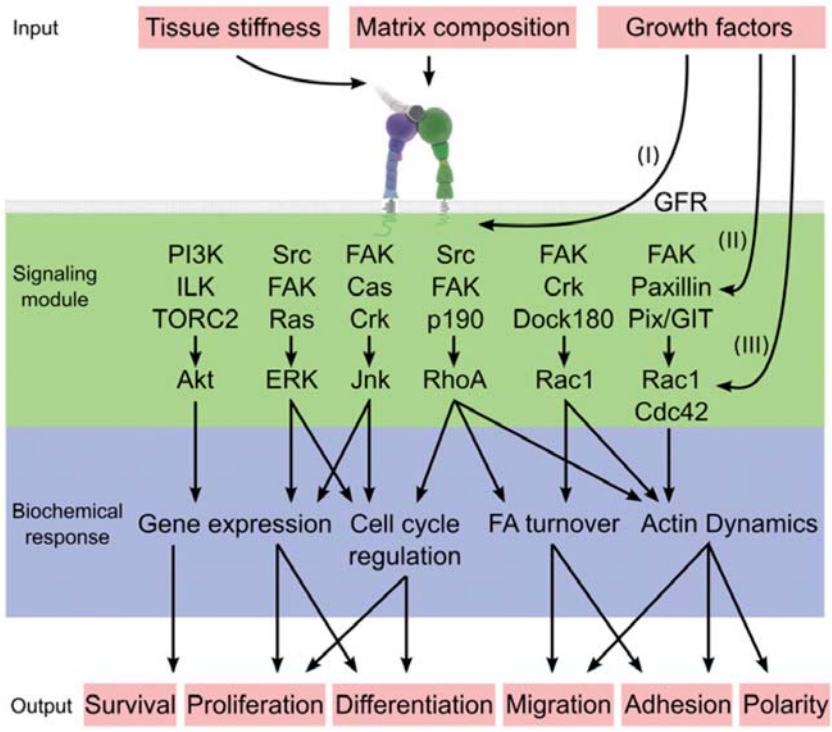



**Figure 6: Examples of signaling pathways located downstream from integrin activation and their possible crosstalk (Legate et al., 2009).**
Growth factor signaling interacts with integrin-mediated signaling on multiple levels: by regulating integrin affinity for ligands (I), by regulating the activity of the integrin-associated signaling proteins such as FAK, Src and PI3K (II), and by regulating the activity of the downstream effectors such as ERK, Akt and JNK and the Rho GTPases (III). The central signaling module downstream of integrins is the Src/FAK complex, which activates ERK and JNK to regulate cell survival, proliferation and differentiation. In addition, through activation of Crk/Dock180 or alternatively PIX/GIT pathways, the Src/FAK complex regulates Rho GTPase activity, resulting in cytoskeletal reorganization and regulation of cell migration, adhesion and polarity. Integrins also activate PI3K, which in collaboration with ILK and mTOR is thought to regulate cell survival through Akt.
Growth factor receptor (GFR), PI-3-kinase (PI3K), Integrin-linked kinase (ILK), mammalian target of rapamycin complex (TORC), Focal Adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), Crk-associated substrate (Cas), Janus kinase (JNK), dedicator of cytokinesis 1 (DOCK180), PAK interactive exchange factor (PIX), G-protein-coupled receptor kinase-interacting protein (GIT).

### 1.4 Assembly of integrin-dependent adhesion structures

Matrix adhesions are highly dynamic structures that organize around activated integrin clusters. All subtypes of matrix adhesions are areas of very close contact between the plasma membrane and the substrate. The number of adhesions, their morphology and molecular composition vary widely between cell types.

The current nomenclature differentiates between five different types of integrin-containing cell-substrate adhesion structures: focal complexes, focal adhesions (FAs), fibrillar adhesions, podosomes and 3D-matrix adhesions. Focal complexes are small (~100 nm in diameter), dot-like transient matrix contact structures that provide early cell attachment at the leading edge. If stabilized, they will subsequently mature and form FAs (size around 1-5 μm). The molecular nature of this transition is still enigmatic, even though differences in protein composition, phosphorylation status and dynamics were detected. The LIM-domain protein zyxin, for example, constitutes a distinctive protein marker that localizes to FAs but not to the nascent focal complexes (Zaidel-Bar et al., 2003).

Focal adhesions are structures that are predominantly found in resting cells or in areas of cells with low motiliy and display much slower turnover than focal complexes. Structurally, mature FAs are elongated and localized at the termini of stress fibers. Stress fibers consist of actin filament bundles that contain a multitude of accessory proteins, including actin filament crosslinkers (such as α-actinin and filamin) and myosin II. Myosin II possesses both actin-bundling activity (motor-independent) and contractile activity. The presence of myosin II is responsible for the contractile nature of the stress fibers such that FAs experience continuous pulling forces, which they in turn transmit to the ECM through the associated integrins (Geiger et al., 2009). Interestingly, it has recently been shown that the formation of focal complexes does not require myosin II activity whereas both functions seem to be essential for adhesion maturation (Choi et al., 2008).

FAs can subsequently transform into streak-like fibrillar adhesions which differ from FAs in their characteristic morphology, consisting of elongated fibrils or array of dots, and their distribution in more central areas under the cells. Certain integrin receptors are

preferentially concentrated at different cell-matrix adhesion structures. For example, fibroblasts adhering to a 2D fibronectin matrix will form focal complexes and focal adhesions that are rich in integrin αVβ3 (Figure 7). In the same cells integrin α5β1 is often excluded from the focal adhesion core, but localizes to fibrillar adhesions (Berrier and Yamada, 2007).

Podosomes are found naturally in osteoclasts or cells of hematopoietic origin. They compare with focal complexes in both size and half-life, but are composed of a ring-like assembly of matrix adhesion components surrounding an F-actin core (Gimona et al., 2008).

The biological relevance of FAs was initially questioned, since equivalent structures to these prominent 2D adhesion structures were not observed in tissues. However, FAs have been found in cells at points of high fluid shear stress in blood vessels (Romer et al., 2006).

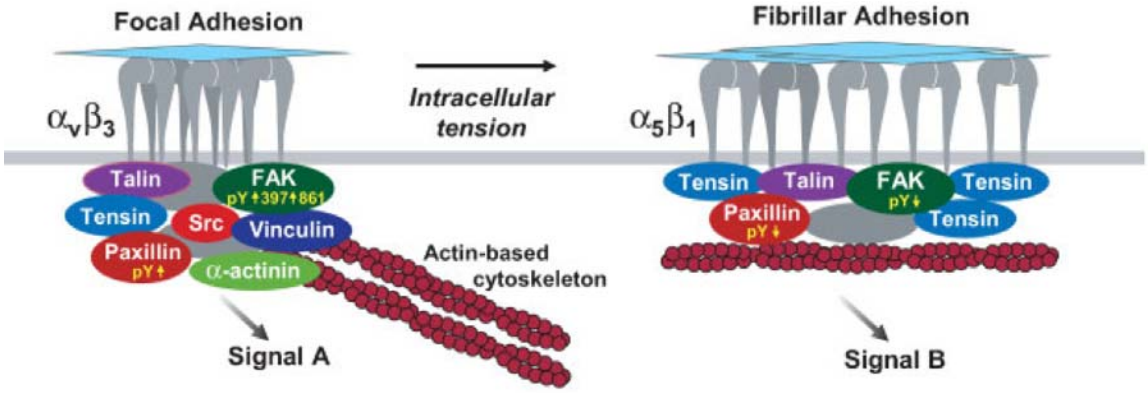



**Figure 7: Comparison of focal adhesions with fibrillar adhesions (Berrier and Yamada, 2007).**
Subsets of proteins are recruited to different adhesion structures suggesting that adhesions may have signaling specificity. For instance, focal adhesions contain vinculin and numerous tyrosine-phosphorylated proteins including FAK and paxillin. In contrast, fibrillar adhesions contain high levels of tensin, low levels of protein tyrosine phosphorylation, and integrin α5β1 instead of integrin αVβ3.

### 1.4.1 The integrin-actin connection

A central consequence of integrin outside-in signaling is the establishment of the integrin-actin connection allowing cells to change their cell shape and to initiate migration. This linkage is a network of transient, highly dynamic interactions between FA proteins and F-actin. FA proteins that are involved in establishing and maintaining the integrin-cytoskeleton linkage can roughly be divided into four classes: (1) integrin-bound proteins that directly bind actin, such as talin, α-actinin and filamin; (2) integrin-bound proteins that indirectly associate with/regulate the cytoskeleton, such as kindlin, ILK, paxillin and FAK; (3) non-integrin-bound actin-binding proteins, such as vinculin; and (4) adaptor and signaling molecules that regulate the interactions of the proteins from the above mentioned groups (Legate et al., 2009).

Experiments utilizing novel imaging technologies such as total internal reflection fluorescence microscopy and fluorescent speckle microscopy, in combination with structural, biochemical, and *in vivo* data, point to talin, vinculin, α-actinin, and ILK as the crucial structural elements of the integrin-actin linkage, as well as the main components regulating FA growth.

The initial integrin-cytoskeleton linkage following fibronectin binding involves the recruitment of talin to β integrins and the establishment of a 2-pN slip bond, which provides the initial force applied by the cytoskeleton to the extracellular ligand (Jiang et al., 2003). The importance of talin in connecting integrins to the actin cytoskeleton is underlined by *in vivo* studies in mice. Mice lacking talin1 die during gastrulation due to a defect in cytoskeletal organization and cell migration (Monkley et al., 2000). Ablation of both talin1 and talin2 in skeletal muscle causes defects in myoblast fusion, sarcomere assembly and maintenance of myotendinous junctions. Interestingly, talin1/2-deficient myoblasts express functionally active β1 integrins suggesting that the defects are caused by disruptions of the interaction of integrins with the actin cytoskeleton (Conti et al., 2009).

Talin binding is rapidly followed by the recruitment of proteins such as vinculin to the nascent adhesion. Vinculin binds to several sites in the talin rod that are normally buried in helical bundles but may become exposed upon mechanical stretch. Expressing the talin

head domain, which does not bind vinculin, in talin-null cells, activates integrins but fails to form detectable focal contacts (Zhang et al., 2008). This study suggests that talin makes the initial contacts between integrins and actin, but it is not sufficient to maintain this connection on its own. Vinculin is required to strengthen the linkage by acting as a crosslinker and by stabilizing the talin-actin interaction by binding directly to both proteins (Legate et al., 2009).

Recent studies have highlighted the importance of α-actinin, a binding partner of both talin and vinculin, in linking integrins to the actin cytoskeleton. It has been shown that the force-dependent strengthening of integrin-cytoskeleton linkages correlates with the incorporation of α-actinin into integrin adhesion sites (Laukaitis et al., 2001).

ILK as a core scaffold protein connecting integrins with the actin cytoskeleton and with growth factor signaling will be discussed in more detail below.

Most proteins that mediate the integrin-cytoskeleton linkage act by some means or other on Rho GTPases. Mammalian Rho GTPases are a family of 20 signaling proteins, which cycle between an active GTP-bound state and an inactive GDP-bound state. Three types of proteins can regulate the cycling and thereby the activation of Rho GTPases: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). The most important regulators of actin dynamics downstream of integrins are RhoA, Rac and Cdc42. The activation of RhoA, Rac or Cdc42 leads to the assembly of contractile actin-myosin filaments, protrusive lamellipodia and protrusive actin-rich filopodia, respectively (Etienne-Manneville and Hall, 2002). Although Rac and Cdc42 lead to morphologically distinct protrusions at the plasma membrane (lamellipodia and filopodia), they both initiate peripheral actin polymerization through the Arp2/3 complex whereas RhoA stimulates actin polymerization through formins (Jaffe and Hall, 2005).

Regulation of actomyosin-based contraction by RhoA, Rac and Cdc42 is antagonistic. RhoA activates Rho-kinase (ROCK), which in turn phosphorylates and inactivates the phosphatase that dephosphorylates myosin light chain (MLC), resulting in increased contractility. Conversely, Rac activates PAK, which phosphorylates and inactivates MLC kinase, leading to decreased contractility, which promotes cell spreading (Vicente-Manzanares et al., 2005).

Interestingly, besides the remote actin nucleation upon integrin activation by Rho-family GTPases, Butler *et al*. have shown that purified adhesion complexes possess the entire machinery to actively assemble F-actin suggesting that Arp2/3 and formins might also be recruited directly to matrix adhesion complexes through integrin-associated adaptor proteins (Butler et al., 2006). For more details, the reader is referred to the first publication (comment) presented in this PhD thesis.

In addition, mechanotransduction is an essential function of FAs that requires an intact integrin-actin connection. Conversion of physical signals into chemical signals is critical for many biological and pathological processes including morphogenesis, wound healing, cancer, atherosclerosis and osteoporosis. In principle, mechanotransduction can be achieved through force-induced protein conformational changes, modifications and/or positional changes (Bershadsky et al., 2006; Orr et al., 2006). The ECM protein fibronectin is so far the best-studied example. Friedland *et al.* provide evidence that the major fibronectin-binding integrin, α5β1, undergoes a force-dependent conformational transition. The emerging picture from these results is that initial low-tension binding of integrin α5β1 to fibronectin involves association of the integrin with the RGD sequence, which under force converts to a higher-strength, more readily cross-linked bond that involves the synergy site. Only this second conformation can activate FAK and transmit downstream signals (Friedland et al., 2009; Schwartz, 2009). Focal adhesion proteins that serve as force sensors in cells include talin and p130Cas (Sawada et al., 2006). Interestingly, also ILK has been recently shown to act as a cardiac stretch sensor, fulfilling a structural role as a mechanical integration site that links membrane-bound β integrins via β-parvin and α-actinin to the sarcomeric Z-disc to exert a functional role in the regulation of cardiomyocyte contractility via Akt/VEGF signaling (Bendig et al., 2006).

### 1.5 The ILK/PINCH/parvin (IPP) complex

The response of the cell to integrin ligation depends not only on the type of integrin heterodimer but also on the molecular composition of the adhesion complex. The

ILK/PINCH/parvin (IPP) complex is a central constituent of at least β1 and β3 integrin containing adhesion sites, from where it regulates multiple cellular processes.

### 1.5.1 The molecular composition of the IPP complex

The assembly of the IPP complex precedes cell adhesion, which indicates that these complexes first form in the cytosol (Zhang et al., 2002). Interestingly, the stability of the individual IPP components is dependent on complex formation (Fukuda et al., 2003).
ILK, which is ubiquitously expressed in mammalian tissues, is composed of three structurally distinct domains. The N-terminus consists of four (five) ankyrin repeats followed by a PH-like domain and a C-terminal kinase-like domain (Figure 8). Interestingly, almost all adaptor proteins that bind either directly or indirectly to ILK regulate the actin cytoskeleton and hence could be responsible for the shape change and FA dysfunction associated with altered ILK expression.
The ankyrin repeats mediate the interaction between ILK and PINCH, a family of LIM domain only containing proteins consisting of two members, PINCH-1 and PINCH-2. Both PINCH proteins contain five LIM domains, the first of which is responsible for their interaction with ILK (Chiswell et al., 2008; Tu et al., 2001; Tu et al., 1999). PINCH can signal to receptor tyrosine kinases (RTKs) through the SH2-SH3 adaptor Nck2, thereby PINCH couples growth factor signaling to integrin signaling (Vaynberg et al., 2005). The PH domain of ILK has been shown to bind phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) (Delcommenne et al., 1998; Pasquali et al., 2007). The C-terminal kinase-like domain binds several adaptor proteins including the parvins that consist of three members; the ubiquitously expressed α-parvin (also known as actopaxin or CH-ILKBP), β-parvin (also known as affixin), which is primarily expressed in heart and skeletal muscle, and γ-parvin, which is expressed exclusively in the haematopoietic system (Chu et al., 2006; Nikolopoulos and Turner, 2000; Olski et al., 2001; Tu et al., 2001; Yamaji et al., 2001). Parvins are characterized by an N-terminal polypeptide stretch followed by two calponin homology (CH) domains arranged in tandem, of which the second has been shown to mediate its interaction with ILK (Tu et al., 2001).

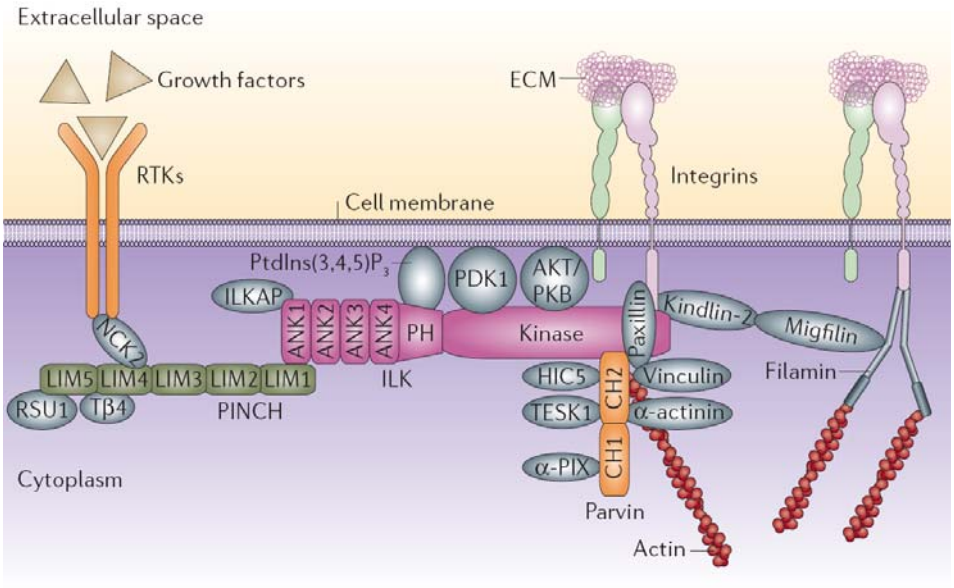



**Figure 8: Anatomy of the IPP complex and its main binding partners (Legate et al., 2006).**
ILK consists of three structurally distinct domains, N-terminal ankyrin repeats (ANK), a pleckstrin homology domain (PH) and a C-terminal kinase-like domain. Via ANK1, ILK binds to the PINCH isoforms as well as to ILK associated phosphatase (ILKAP). The PH domain is believed to bind phosphatidylinositol-3, 4, 5-trisphosphate (PtdIns(3, 4, 5)P3. The kinase domain of ILK binds parvins, paxillin, kindlin-2, the cytoplasmic tails of β integrins, and maybe the kinase substrate Akt/PKB and PDK1.

### 1.5.2 The biological functions of the IPP complex

The biological functions of the IPP complex proteins have been extensively studied in several organisms and cell types. Genetic ablation of ILK or PINCH-1 in mice results in embryonic lethality (Li et al., 2005; Sakai et al., 2003). Mice lacking ILK expression die during peri-implantation due to a failure in epiblast polarisation, which is associated with severe defects in F-actin organization at adhesion sites (Sakai et al., 2003). ILK-deficient fibroblasts display defects in cell adhesion, spreading and migration due to a delay in the formation of FAs, which also fail to mature and are poorly linked to a disorganized actin cytoskeleton (Sakai et al., 2003; Stanchi et al., 2009). The defective maturation of ILK-deficient FAs into fibrillar adhesions leads to defects in deposition of the fibronectin matrix (Stanchi et al., 2009). Interestingly, this function requires the interaction of ILK with α-parvin but not with PINCH-1 (Stanchi et al., 2009). The essential role of ILK in

linking integrins to the actin cytoskeleton has been further confirmed in several tissue and cell types (McDonald et al., 2008). Recent studies suggest that ILK does not only regulate the actin cytoskeleton but can also modulate the microtubule network and influence mitotic spindle orientation (Dobreva et al., 2008; Fielding et al., 2008). However, as loss of ILK can lead to both increased or decreased proliferation rates *in vivo*, depending on the cellular context (Gkretsi et al., 2008; Grashoff et al., 2003; Lorenz et al., 2007; Sakai et al., 2003), the relevance of these functions needs to be established.

PINCH-1 is ubiquitously expressed throughout mammalian development and adult life, whereas PINCH-2 expression starts during the second half of embryonic development and has a slightly more restricted expression pattern (Braun et al., 2003). Ablation of PINCH-2 does not affect mouse development, but loss of PINCH-1 results in abnormal epiblast polarity, impaired cavitation, and detachment of endoderm and epiblast from basement membranes (Li et al., 2005). However, the functions of PINCH-1 are not restricted to the regulation of cell-matrix adhesions as PINCH-1 has been shown to regulate cell-cell adhesion of the endoderm and epiblast as well as cell survival in the endoderm layer (Li et al., 2005). As ILK has not been shown to play a role in these processes, it suggests that several functions of PINCH are independent of the IPP complex.

Like ILK and PINCH, parvins play a role in modulating cell spreading and actin organization downstream of integrins. However, the role of parvins in these processes is more complex and the precise functions of the different isoforms *in vivo* are not clear. Mice lacking β- or γ-parvin show no obvious phenotypes, whereas α-parvin null mice die between E11.5 and E14.5, suggesting that the parvin isoforms can functionally substitute for each other during development (Chu et al., 2006; Montanez et al., 2009). All parvins contain two CH-domains and bind F-actin *in vitro*, but the functional significance of this interaction is unknown (Olski et al., 2001; Yamaji et al., 2004; Yamaji et al., 2001). The primary sequences of both CH domains of α-parvin are highly diverged from the typical CH-domains found in actin binding domains (Gimona et al., 2002), and it has been shown that α-parvin uses these domains to interact with paxillin (Lorenz et al., 2008; Nikolopoulos and Turner, 2000; Wang et al., 2008). Since the C-terminal region containing the CH-domains is highly conserved throughout the parvin family, it is likely

that all parvin paralogues may be able to bind paxillin and its homologue Hic-5 (Lorenz et al., 2008).
ILK is an important scaffold protein due to its interaction with several actin regulatory proteins whereas the importance of its signaling function is unclear.

### 1.5.3 The putative kinase activity of ILK

As integrins themselves lack enzymatic activity, they propagate intracellular signals by recruiting signalling proteins such as tyrosine and serine/threonine kinases to their cytoplasmic tails. In the study that identified ILK, it was proposed that ILK acts as a direct binding partner of β1 integrin. Furthermore, it was shown that bacterially expressed recombinant ILK possesses kinase activity and phosphorylates serine and threonine residues in the cytoplasmic tail of β1 integrin (Hannigan et al., 1996). Since then, about 200 studies confirmed the putative kinase activity of ILK towards a diverse set of substrates ranging from Akt, a kinase regulating key cellular functions such as cell cycle progression, survival, differentiation and energy homeostasis, to myosin light chain (MLC) whose phosphorylation regulates actomyosin contractility and vascular tone.
As ILK lacks well conserved motifs required for eukaryotic protein kinase activity (Hanks et al., 1988), the putative kinase activity and its physiological relevance has remained a subject of debate and controversy. Although ILK contains the lysine residue in subdomain II required for phosphotransfer and the A/SPE motif in subdomain VIII involved in substrate recognition, the GxGxxG consensus sequence of the kinase subdomain I required for covering and anchoring the non-transferable phosphates of ATP is not conserved in ILK from different species. This suggests that if ILK is indeed a kinase, this function would have evolved late during evolution. It is, however, even more difficult to reconcile that ILK lacks the catalytic base in subdomain VIb, which accepts the proton from the hydroxyl group of the substrate during the phosphotransfer reaction, as well as the DFG motif in subdomain VII required to align the γ-phosphate of ATP. A conserved lysine, which neutralizes the charge on the γ-phosphate of ATP and a conserved asparagine which chelates the secondary magnesium ions, both in subdomain VIb, are also missing. Due to these characteristics, ILK has also been classified as a

pseudokinase, a catalytically inactive remnant of an active kinase that uses its substrate recognition motif to interact with other proteins (Boudeau et al., 2006).

However, several lines of biochemical and cell biological evidence pointed to the possibility that ILK might be an active kinase (Hannigan et al., 1996). It has been shown that recombinant ILK expressed in bacteria can phosphorylate the cytoplasmic tail of β1 integrin as well as the model substrate myelin basic protein (Delcommenne et al., 1998; Hannigan et al., 1996). Furthermore, purified ILK from mammalian cell extracts was shown to co-immunoprecipitate and phosphorylate Akt (Persad et al., 2001). Mutational analysis has been used to gain further insight to the catalytic activity of ILK, and several mutations have been described to abrogate the kinase activity *in vitro*. A serine (S) to alanine (A) substitution in the potential autophosphorylation site (S343A), an arginine (R) to A substitution in the potential PtdIns(3,4,5)P3 binding site of the PH-domain (R211A), or a lysine (K) to A or to methionine (M) substitution in the putative ATP-binding site (K220A/M) have all been shown to result in a catalytically inactive ILK (Filipenko et al., 2005; Persad et al., 2001), whereas a S to aspartate (D) substitution in the autophosphorylation site (S343D) was shown to generate a hyperactive kinase (Persad et al., 2001). Importantly, however, these mutations have also been shown to disrupt the interaction of ILK with essential binding partners. The inactivating R211A mutation apparently disrupts the interaction with α-parvin and impairs the recruitment of ILK to FAs (Attwell et al., 2003), whereas the K220A mutation reduces β-parvin binding (Yamaji et al., 2001). These findings together with the observation that a combination of two inactivating mutations (S343D and K220M) can reverse the kinase dead phenotype despite abolishing the ability to bind ATP (Lynch et al., 1999), suggest that these mutations might affect the activation status of downstream substrates such as Akt phosphorylation by an indirect mechanism.

For more details and information the reader is referred to the third and fourth publication presented in this PhD thesis.

## 2. Kidney physiology

Kidneys are paired, mesoderm-derived organs that belong to the excretory/urinary system. In mammals, the urinary system includes two kidneys, two ureters, urinary bladder and urethra. In producing urine, the kidneys excrete wastes such as urea and ammonium and reabsorb glucose and amino acids. However, the kidneys' role is not merely excretion. As part of the urinary system they are also regulatory organs, maintaining homeostasis along with the respiratory (lungs remove water and $CO_2$) and the integumentary system (blood vessels dilate to allow more heat to escape; sweat glands produce sweat). The urinary system controls the composition of the blood, eliminates waste through filtration, reabsorption, and secretion and regulates the water balance through retention and excretion of acids, bases, ions and salts. In addition, it influences the blood pressure through production of the renin hormone and it helps to maintain a constant calcium level in the blood through conversion of cholecalciferol into vitamin D, which stimulates calcium absorption from the kidney.

Anatomically the kidney consists of an outer cortex and an inner region, which is divided into the medulla and the renal pelvis (Figure 9). Nephrons are the functional units of the kidney which reside in the cortex and extend partly into the medulla. The initial filtering portion of a nephron is the renal corpuscle which is formed from a tuft of capillaries at the head of each nephron known as the glomerulus which is enclosed in the Bowman's capsule. The glomerular capillaries originate from an afferent arteriole and recombine to form an efferent arteriole. Between the glomerular capillaries are clusters of phagocytes called mesangeal cells. The proximal tubule arises directly from the Bowman's capsule. The epithelial cells of the proximal tubule are closely fused with one another via tight junctions near their apical surfaces, which are densely covered by microvilli giving rise to a prominent brush border. The proximale tubule connects with the intermediate tubule also known as the descending loop of Henle. The distale tubule arises from the ascending loop of Henle and merges via connecting tubules to form collecting ducts which pass through the cortex and medulla to the renal pelvis.

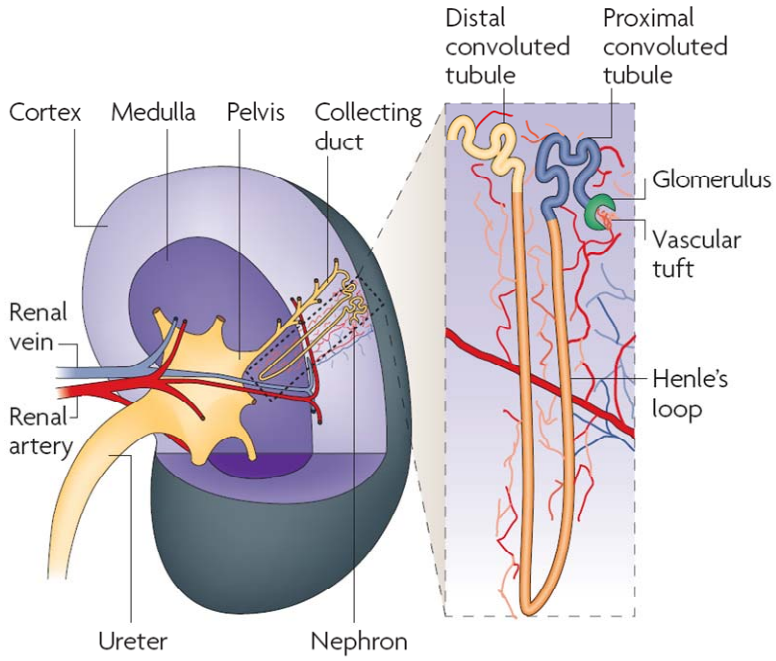



**Figure 9: Anatomy of the adult kidney and the position and arrangement of a nephron (Schedl, 2007).**
The kidney consists of an outer cortex and an inner region, which is divided into the medulla and the renal pelvis. Nephrons are the functional unit of the kidney and contain the glomerulus where blood ultrafiltration takes place.

The nephrons (about a million in each human kidney) form urine by three precisely regulated processes: filtration, reabsorption, and secretion. The first step of urine formation is glomerular filtration (ultrafiltration). The glomerular blood pressure provides the driving force for water and solutes to be filtered out of the blood into the space made by Bowman's capsule. The glomerular capillary wall consists of three layers, which together combine to an effective filtration system: the fenestrated capillary endothelial layer, the basement membrane, rich in collagen IV, laminin and nidogen and the podocyte (specialized epithelial cell) cell layer. The most selective filtration is believed to take place at the diaphragms of the slit pores formed by foot-like projections of podocytes onto the basement membrane. The glomerular filter is freely permeable to water, mineral ions ($Na^+$, $Ka^+$, $Ca^{2+}$, $Cl^-$) and to small organic molecules such as glucose. Plasma proteins with molecular weights above 40.000 daltons are passing only in small amounts, so urine is normally almost protein-free.

Pathological malfunction of podocytes causes, however, large quantities of protein in the urine (proteinuria). But also the loss of many of the plasma constituents (sodium, potassium, and water) that are freely filtered across the glomerular capillary walls must be avoided. This is achieved in a second step in the proximal tubule in which these substances are actively reabsorbed regardless of the ionic balance of the body. The energy for active reabsorption is provided by a $Na^+$-concentration gradient generated by $Na^+$-$K^+$ pumps located in the basolateral membrane of proximal tubular cells. In addition to its role in reabsorption, the proximal tubule is the site at which secretion of numerous substances such as metabolic products (hydroxybenzoates, neurotransmitters, bile pigments, uric acid) or drugs and toxins occurs. In contrast, the uptake and secretion of mineral ions in the distal tubule and collecting ducts is regulated by the renin-angiotensin system. This is achieved by two specialized cell types, the P and I cells in the epithelium of the distal tubule and the collecting duct. The P cells absorb sodium and water from the tubular fluid and secrete potassium into the tubular fluid while I cells secrete hydrogen ions and reabsorb bicarbonate. The efficiency of sodium uptake and potassium secretion is mainly regulated by the hormone aldosterone which activity is in turn regulated by the enzyme renin. The osmolality of the plasma is regulated by adjusting the amount of water reabsorbed by the collecting ducts. Finally, renal calyxes, ureters, urinary bladder, and urethra comprise the urinary tract. Their function is to collect and store the urine formed by the kidneys (Richards, 2006).

All animals must excrete waste products of their metabolism in order to maintain a constant body composition despite changes in the external environment. The excretory organs in higher species range from a single excretory cell in the nematode C.*elegans* to malphigian tubules in insects; nephridia in annelids; rectal glands in sharks as well as kidneys in amphibians, birds and mammals (Table 3). Despite the heterologous appearance of the different excretory systems many genes that are involved in the development of such organs are evolutionarily conserved.

**Table 3: Selected characteristics of excretory systems of different species. Modified from (Igarashi, 2005).**

| Organism | Excretory organ | Composition and function | Differences from human |
|---|---|---|---|
| *C.elegans* | excretory cell | four cell types make up the excretory system: pore cell, duct cell, canal cell, and a fused pair of gland cells; excretes saline fluid via the duct and pore to maintain the salt balance and to remove metabolites | unicellular excretory cell |
| *D.melanogaster* | malphigian tubules | four blind-ended epithelial tubes connected to the hindgut, mainly elimination of excess potassium | absence of filtration |
| *Zebrafish* | pronephros (larvae) | single midline glomerulus which drains into two pronephric tubules; tubules process the blood filtrate produced from glomeruli | single glomerulus, no urinary concentration |
| *Mus musculus* | kidneys (metanephros) | paired-organ, ultrafiltration in glomeruli; excretion and reabsorption in tubules; hormone production | - |

## 2.1 Kidney development in mice

A variety of developmental processes play a role in kidney organogenesis such as inductive tissue interactions, branching morphogenesis, differentiation, cell polarization, mesenchymal-to-epithelial transformation (MET), and pattern formation.
Branching morphogenesis is fundamental to the development of a number of organ systems that share similar tissue architecture, such as the kidney, lung, mammary gland and salivary gland.

### 2.1.1 Kidney morphogenesis – pronephros, mesonephros and metanephros

In mammals and birds, the kidney develops in three stages: pronephros, mesonephros and metanephros of which only the metanephros differentiates into the permanent kidney whereas the other structures form transiently (Figure 10a). Renal differentiation in mice starts at E8.0 with the formation of the pronephros from the intermediate mesoderm. It consists of the nephric duct and pronephric tubules which lack glomeruli. While in amphibians and fish the pronephros constitutes the functional excretory organ in their larval stages, a functional pronephros does not develop in mammals (Table 3). However, the pronephros is essential for the development of the more complex later kidneys. In mammals, the cranial part of the pronephric duct degenerates but the caudal portion, termed wolffian duct (WD) elongates and becomes part of the excretory system.
Organogenesis of the mesonephros is initiated when the nephric duct reaches the presumptive mesonephric mesenchyme and induces adjacent mesenchymal cells to condensate and to form nephrons (Dressler, 2006). Mammalian mesonephric nephrons consist of a glomeruli-like structure and a proximal and distal tubule. The number, size and functional properties differ between species. In pigs and humans mesonephric nephrons are functional excretory organs during embryogenesis whereas the murine mesonephros is more primitive and non-secretory (Kuure et al., 2000; Sainio et al., 1997a). Two distinct sets of mesonephric tubules can be observed in mice: cranial mesonephric tubules which are connected to the WD and caudal mesonephric tubules, which do not fuse with the WD. Eventually, murine mesonephric tubules degenerate by

apoptosis. The regression in mice starts from the caudal mesonephric tubules, progresses cranially and is completed by E15. In males this degradation is incomplete. The remaining cranial tubules form the epididymal ducts and the WD becomes the vas deferens (Sainio et al., 1997a). The permanent metanephric kidney starts to develop at E10.5-E11 after the WD has further extended caudally along the body axis and has reached a region close to the cloaca. This region is specified by a distinct intermediate mesoderm derived structure, termed metanephric mesenchyme (MM) (Figure 10a). The cloaca, an endodermal sac, becomes partitioned into the hindgut and the urogenital sinus, the primordium of the bladder and the urethra. Due to inductive signals arising from the MM, the ureteric bud (UB) is formed as an epithelial outgrowth from the WD, which then invades the MM (Saxen, 1987). Reciprocal induction between the UB and the MM leads to a number of iterative, dichotomous branching events of the UB epithelium to form the collecting duct system while the MM is induced to condense around the tip of the UB. Eventually mesenchymal pre-tubular aggregates undergo MET to form comma-shaped and S-shaped bodies, which subsequently give rise to the components of the nephron: renal tubules (proximal and distal) and the epithelial component of the glomeruli (Figure 10b-d). In addition, mesenchymal–derived endothelial cells are attracted to the glomeruli and thereby contribute to the formation of the functional nephron (Dressler, 2006). Finally, at ~E13, the ureters dissociate from the WDs and merge with the bladder epithelium in the trigone, the muscular region located at the base of the bladder. It has been suggested that ureter insertion into the bladder depends on distinct events, starting with apoptosis between E11 and E12 that enables separation of the ureters from the WD, followed by fusion with the bladder epithelium that occurs on E13 and finally growth of the bladder, which expands enormously between E12 and E14 (Mendelsohn, 2009).

With the ureter fusion to the bladder the development of the functional metanephros is completed. Over the next weeks the metanephros continues to mature into the adult kidney.

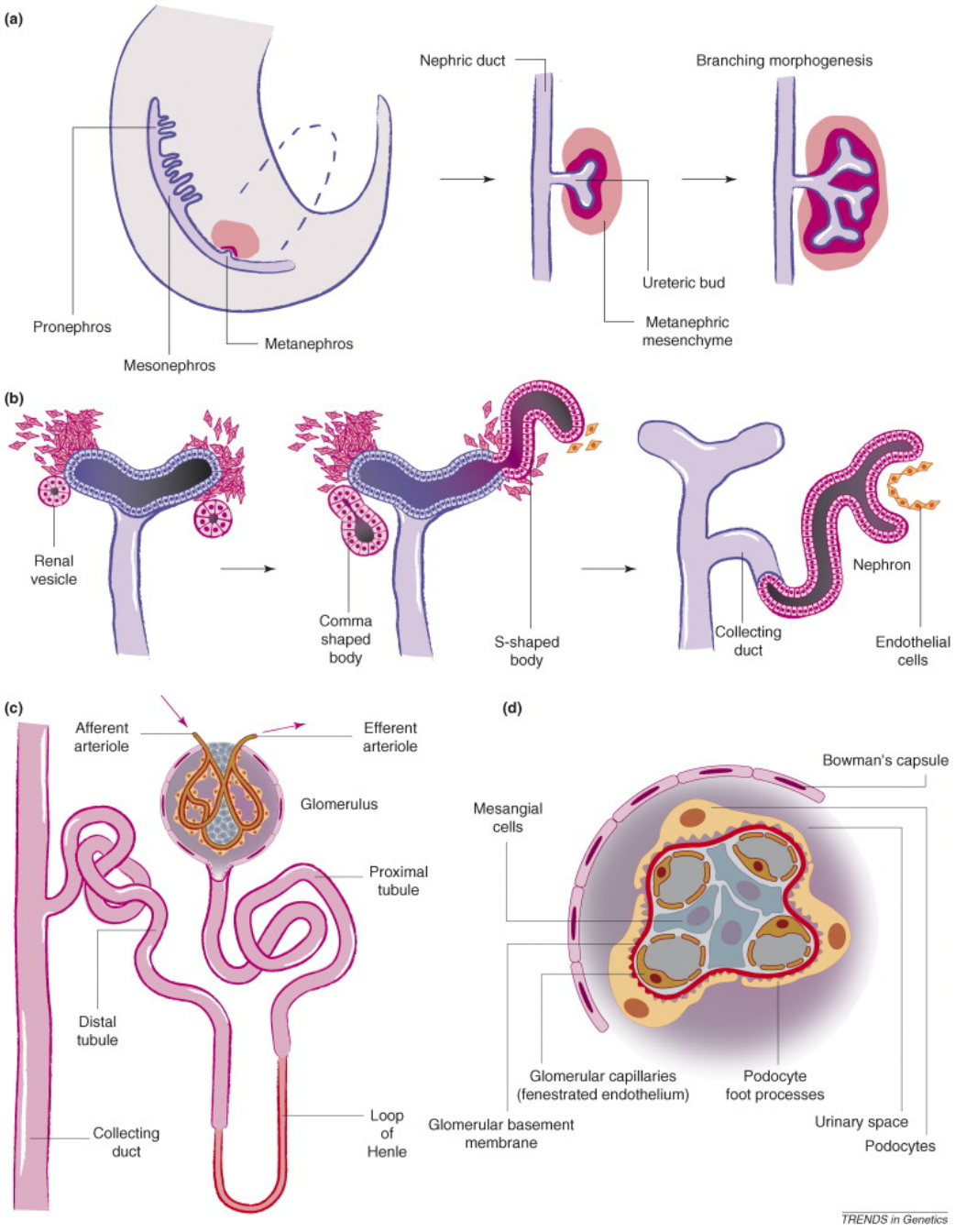



**Figure 10: Schematic overview of mammalian kidney development and morphology (Uhlenhaut and Treier, 2008).**
a) Kidney development proceeds through three stages: pronephros, mesonephros and metanephros. The metanephros develops into the permanent kidney and is formed by the outgrowth of the UB from the WD and invasion and branching of the UB in the MM. b) Ureteric branches give rise to the collecting duct system and induce MET of the surrounding mesenchymal cells resulting in nephron formation. c) The nephron represents the functional unit of the kidney. d) The initial blood filtration takes place inside the glomerulus.

### 2.1.2 Mesenchymal signals initiate kidney development

Over the last decades the understanding of the molecular pathways underlying the different processes during metanephros formation has increased considerably. Such molecular players include genes that specify and pattern the intermediate mesoderm and thereby control the formation and survival of its derivatives WD and MM such as *Lim1*, *Odd1* and *Pax2/8* (Bouchard et al., 2002; Dressler, 2006; Pedersen et al., 2005; Wang et al., 2005). The specification of the MM as a unique region within the intermediate mesoderm is at least in part controlled by genes that provide positional information along the anterior-posterior axis such as mammalian *homeobox* (*Hox*) genes. *Hoxb-7* expression, for example, can already be detected in the mesonephros and later in the WD, ureter and collecting duct. Due to its early expression, the promoter of the *Hoxb-7* gene is used to drive reporter gene expression in the urogenital system as well as to enable conditional deletion of a gene of interest specifically in the UB/collecting system using the cre/loxP system (Igarashi, 2004). In mice, *Hox11* paralogous genes (*Hoxa11*, *Hoxc11*, and *Hoxd11*) are essential for early patterning of the MM, as their loss results in a complete failure of UB outgrowth (Wellik et al., 2002).

Prior to UB outgrowth, the MM expresses a unique combination of markers including *Hox11* paralogs, *Osr1*, *Pax-2*, *Eya1*, *WT-1*, *Six1*, *Six2* and *GDNF* (glial cell derived neurotrophic factor) (Dressler, 2006). The early transcriptional regulation of key genes such as *GDNF* is still not fully understood. However, it has been suggested that a *Hox11-Eya1-Pax-2* positive regulatory network is necessary for early *GDNF* and *Six2* expression in the uninduced MM (Gong et al., 2007).

A recent study also illustrated the importance of ECM signals to maintain GDNF expression in the MM. Linton *et al*. show that the ECM protein nephronectin, which is expressed by epithelial cells binds to integrin α8β1 on the surface of mesenchymal cells. Mice lacking either integrin α8 or nephronectin fail to maintain *GDNF* expression at E11, the critical time point for induction of UB outgrowth (Linton et al., 2007; Muller et al., 1997). Interestingly, *Hoxa11* is required for integrin α8 expression in mesenchymal cells suggesting that the lack of GDNF expression in *Hoxa11* knockout mice is caused by a failure of integrin α8 expression rather than through direct *GDNF* gene regulation (Valerius et al., 2002).

Infact, the growth factor GDNF has a critical role in induction of UB outgrowth from the WD. Initially, GDNF has been identified as a factor maintaining dopaminergic, noradrenergic and motor neurons of the central nervous system (Airaksinen and Saarma, 2002). The GDNF family members GDNF, neuturin, artemin and persephin are distant members of the transforming growth factor-β (TGF-β) superfamily. Unlike other members of the TGF-β superfamily, which signal through receptor serine/threonine kinases, GDNF signals via the receptor tyrosine kinase c-Ret (rearranged during transformation, Ret9 isoform in the kidney,) and the GPI-linked cell surface co-receptor GFRα1 (Costantini and Shakya, 2006; Vega et al., 1996). Like many other growth factors, GDNF also requires heparan sulphate proteoglycans for signaling (Barnett et al., 2002). Accordingly, mice lacking either GDNF, c-Ret or GFRα1 show similar excretory system defects, ranging from renal agenesis to blind ending ureters with no renal tissue, and tiny disorganized kidney rudiments (Cacalano et al., 1998; Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994).

As indicated above, a tight spatiotemporal control of GDNF expression is crucial for proper kidney development (Figure 11). GDNF is expressed broadly throughout the nephrogenic cord at E9.5 but becomes restricted to the region of the MM by E10.5. The control mechanisms either directly regulate GDNF expression or modulate the signal transduction downstream of c-Ret. Deletion of negative regulators such as the transcription factor FOXC1 that is expressed in the MM and ROBO2/SLIT2 that are expressed in the nephrogenic mesenchyme and WD, respectively, cause an anterior expansion of the GDNF expression domain leading to the formation of multiple ureteric buds and ectotopic ureters that remain connected to the WD instead of the bladder (Grieshammer et al., 2004; Kume et al., 2000). In contrast, Sprouty1, a negative regulator of receptor tyrosine kinase signaling expressed by the WD and UB, modulates the c-Ret/GDNF signaling intensity in the WD and thereby prevents the formation of multiple UBs (Basson et al., 2005). In addition, BMP-4 mediated signaling has been shown to prevent the formation of supernumerary buds. Spatially restricted expression of the BMP antagonist gremlin1 in the MM close to the budding site enables UB outgrowth, its invasion into the MM and the establishment of an autoregulatory GDNF/WNT-11

feedback loop that is crucial for branching morphogenesis (Majumdar et al., 2003; Michos et al., 2007).

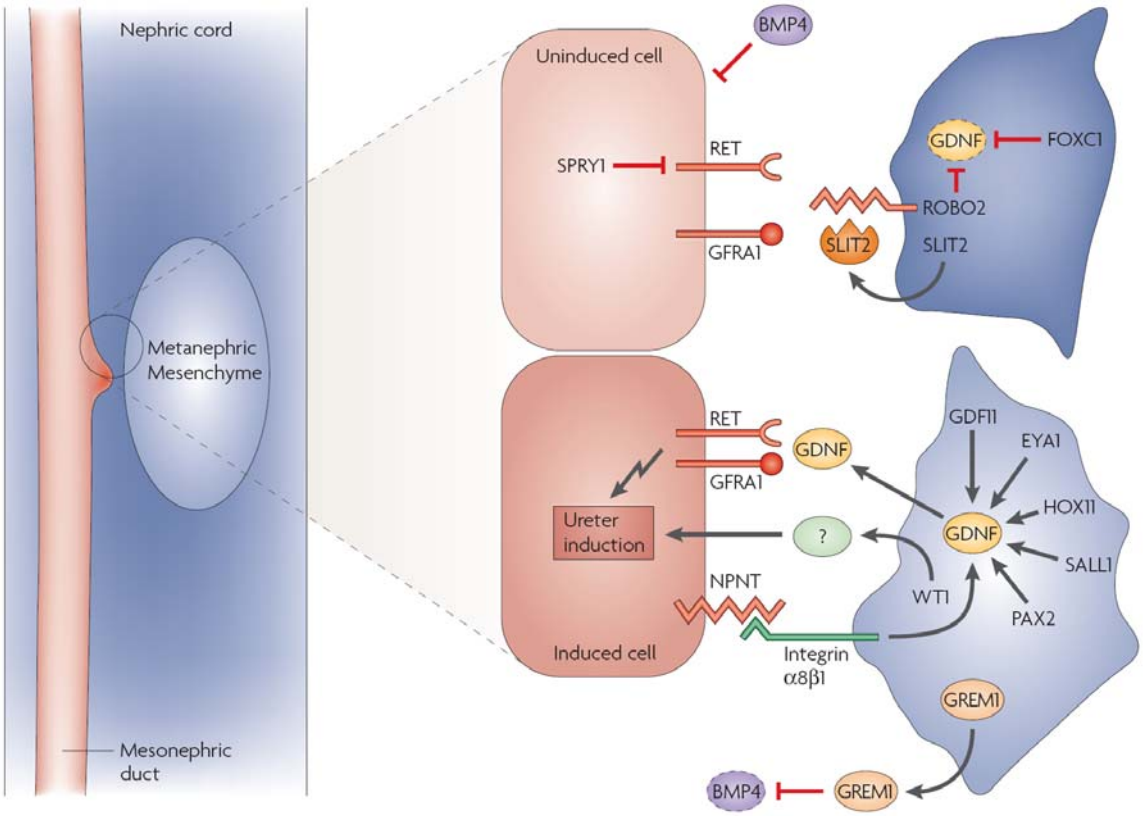



**Figure 11: Molecular pathways that control kidney induction (Schedl, 2007).**
GDNF expression is tightly controlled. GDNF expression is spatially restricted through expression of the forkhead box protein C1 (FOXC1) transcription factor, Slit homologue 2 (SLIT2) and its receptor Roundabout homologue 2 (ROBO2). GDNF binds and signals through c-RET (rearranged during transformation) and GDNF-family receptor α1 (GFRα1) receptors that are expressed by the UB epithelium (mesonephric duct). The tyrosine kinase inhibitor sprouty 1 (Spry1) modulates Ret signaling. Bone morphogenic factor 4 (BMP-4) inhibits ureter outgrowth.
GREM1, gremlin1; NPNT, nephronectin; EYA1, Eyes absent homologue 1; GDF11, growth factor differentiation factor 11; HOX11, homeobox protein 11; WT-1, Wilms tumour transcription factor.

### 2.1.3 Ureteric bud outgrowth and branching

The region of the WD, which gives rise to the UB, is a site of intensive cell proliferation (Bridgewater and Rosenblum, 2009; Michael and Davies, 2004). The newly formed UB segregates into two functional domains, the ureteric stalk and the ureteric tip. Although the entire UB is derived from the WD, tip and stalk express a distinct set of genes. The tip, which is the site of branching and cell proliferation, expresses *Ret*, *WNT-11*, *Sox9* while expression of *WNT-7b, aquaporin 3*, *collagen XVIII*, and binding of the lectin Dolichos biflorus agglutinin (DBA) is restricted to the stalk epithelium. Eventually the tip cells differentiate into stalk cells and thereby cause elongation of the stalk. Finally, the stalk will give rise to the collecting duct system (Figure 12).

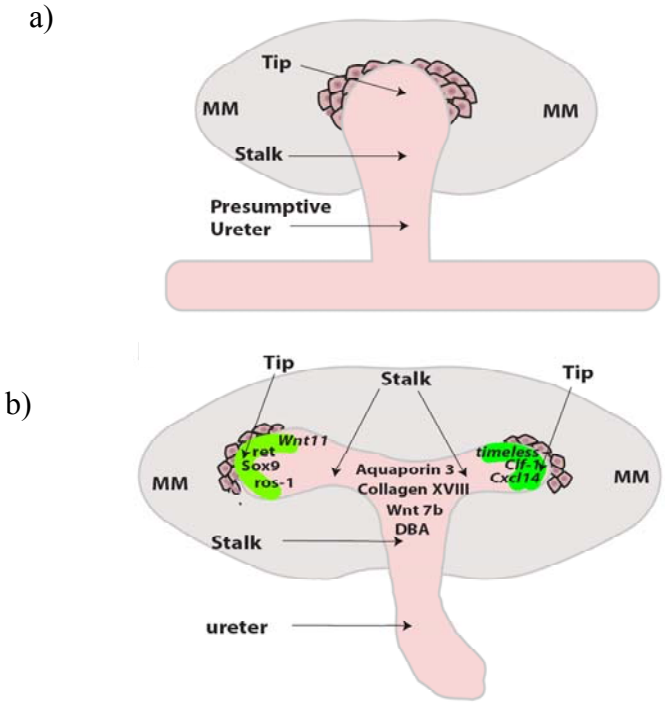



**Figure 12: Cell fate determination in the developing UB (Bridgewater and Rosenblum, 2009).**
a) The UB grows out from the WD and migrates into the MM. b) Further segmentation of the UB into tip and stalk (T-bud stage, in mice at E11.5). The tips will give rise to the ampulla and form new branches, while the stalks will differentiate into the collecting system. The tip segment (shown in green) expresses a unique set of genes, including *WNT-11*, *Ret*, *Sox9*, *Ros1*, *Clfl*, *Cxcl14* and *timeless*. The stalk portion of the UB binds the lectin Dolichos biflorus agglutinin (DBA) and expresses stalk specific genes, such as *aquaporin 3*, *collagen XVIII*, and *WNT-7b*.

As mentioned above, proper UB induction and outgrowth requires GDNF expression in the MM adjacent to the caudal portion of the WD as well as c-Ret expression along the WD. The co-receptor GFRα1 is expressed in the WD and MM. Wnt/β-catenin signaling has been reported to be a critical regulator of c-Ret expression in the WD/UB and appears to maintain the epithelium in an undifferentiated state allowing progression of morphogenesis (Bridgewater et al., 2008; Marose et al., 2008). Another important regulator of c-Ret expression in the WD is the Zn-finger transcription factor Gata3. *Gata3*-deficient embryos show an aberrant elongation of the WD and loss of c-*Ret* expression in the WD (Grote et al., 2006). Interestingly, Gata-3 expression is downregulated in β-catenin mutant WD epithelial cells, while β-catenin expression is retained in the WD epithelium of *Gata3*-deficient mouse embryos indicating that Gata3 acts downstream of β-catenin to activate c-Ret expression. Once the UB forms and begins to branch, c-Ret expression is downregulated in the WD and UB trunks and becomes restricted to the distal tips of the branches.

What are the signaling pathways downstream of GDNF/c-Ret and what cellular processes are stimulated by GDNF? So far, three signaling pathways have been shown to be important in GDNF/c-Ret mediated UB outgrowth and branching: Ras/ERK MAP kinase pathway, PI3-Kinase/Akt, and PLC-γ/calcium pathway (Costantini, 2006). Recently, it has been shown that the ETS transcription factors Etv4 and Etv5 are positively regulated by Ret signaling in the ureteric bud tips. Several genes have been identified whose expression in the ureteric bud depends on Etv4 and Etv5, including *Cxcr4*, *Myb*, *Met*, and *MMP14* (Lu et al., 2009).

Among the cellular processes, GDNF-mediated signaling has been implicated in the regulation of cell proliferation and migration/chemoattraction, but both functions are still controversial. *In vitro*, GDNF stimulates proliferation of cultured primary UB cells (Towers et al., 1998) and collecting ducts of whole kidney explants (Pepicelli et al., 1997). However, it was also demonstrated that the primary response to GDNF is not mitogenic but rather a combination of decreased apoptosis, increased adhesiveness, secretion of basal lamina, and maintenance of the polarization of the ureteric cells in a hanging drop culture (Sainio et al., 1997b). In addition, time-lapse imaging of chimeric organ cultures has recently revealed that WD cells expressing the GDNF receptor c-Ret

undergo extensive movements to generate a specialized epithelial domain that gives rise to the first UB tip, while cells lacking c-Ret are excluded from this domain, suggesting that directed cell migration and not localized cell proliferation initiate UB formation (Figure 13) (Chi et al., 2009).

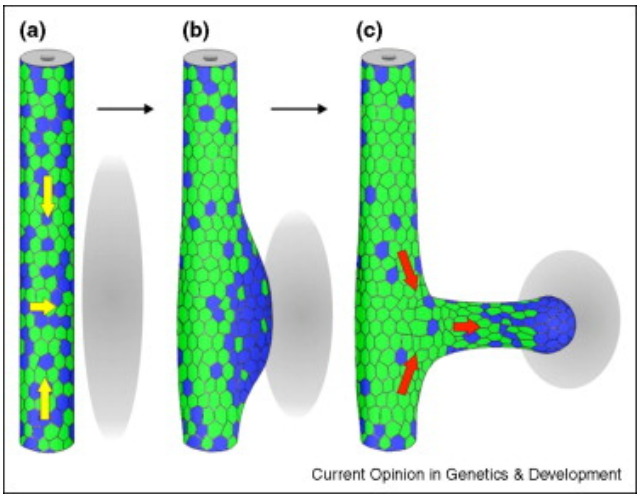



**Figure 13: c-Ret-dependent cell movements initiate UB formation (Chi et al., 2009; Michos, 2009).**
a) Ret-expressing cells (in blue) are initially dispersed along the WD and eventually start moving (yellow arrows) to caudal position next to the MM to form the primary UB tip domain. b) WD cells compete for inclusion in the forming primary UB based on their Ret activity levels. c) The tip of the UB elongates toward the source of GDNF secreted by the MM (in grey).

The observations further imply that rearrangements result from competition among cells based on the level of signaling, a mechanism which is similar to that described for fly trachea branching where tracheal epithelial cells express Btl/FGFR and compete for the ligand Bnl/FGF to become a tip cell (Chi et al., 2009; Lu and Werb, 2008). Thus, receptor tyrosine kinase signaling-based cell competition might be an evolutionarily conserved mechanism of epithelial branching. However, the role of GDNF as a chemoattractant in this process is not completely clear. *In vitro*, GDNF can act as a chemotactic guidance cue for c-Ret-expressing epithelial cells, and this is mediated at least in part by the PI-3 kinase (Tang et al., 2002; Tang et al., 1998). However, misexpression of GDNF in WD and UB results in the formation of multiple ectotopic buds that branch independently of the MM, indicating that GDNF can signal in an

autocrine manner and mesenchymal GDNF is not required as a chemoattractive factor for the attraction of c-Ret-expressing UB cells *in vivo* (Shakya et al., 2005).

Taken together, the experimental data point to the existence of multiple signaling pathways downstream of activated c-Ret, which coordinate proliferation and cell migration of UB epithelial cells.

Upon invasion of the MM, the UB secretes survival factors such as TGF-α, TIMP-2, EGF and FGF2 to prevent apoptosis in the MM (Davies and Fisher, 2002; Koseki et al., 1992) and induces the MM to establish two cell fates - the stromal progenitor cells and the nephrogenic mesenchyme that undergoes MET.

Many of the factors that regulate UB outgrowth are also essential for subsequent branching morphogenesis. Branching morphogenesis is characterized by the following repetitive sequence: 1) expansion of the UB branch at the leading tip (called ampulla); 2) division of the ampulla causing the formation of two new ureteric bud branches; and 3) elongation of newly formed branches (Figure 14).

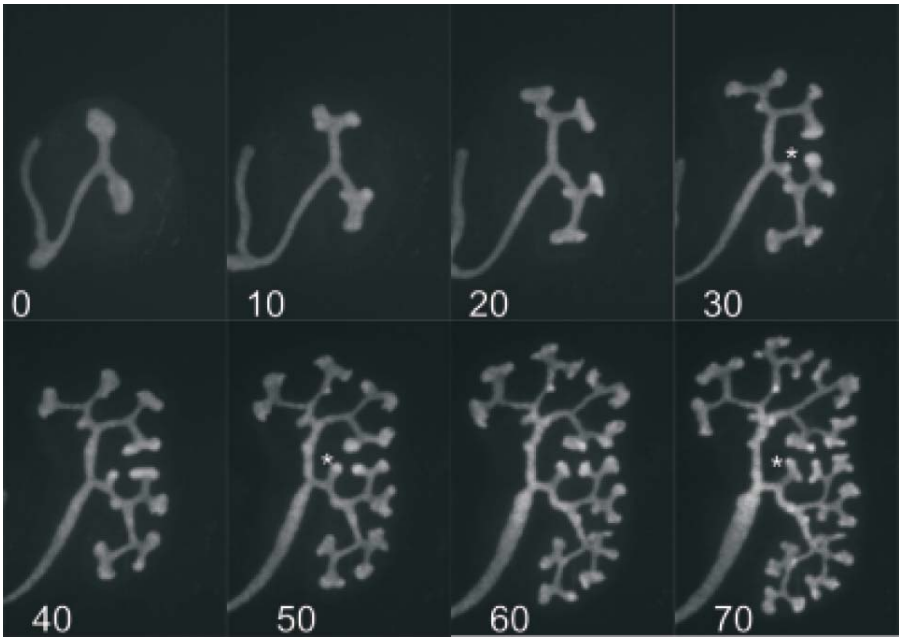



**Figure 14: UB branching in organ culture (Costantini, 2006).**
Kidneys, isolated from E11.5 Hoxb-7/GFP transgenic embryos were cultured and photographed at 10 hours intervals. The UB can branch in a variety of complex patterns, including terminal bifid, terminal trifid and lateral branching (indicated with asterisks). The most UB branching events are terminal bifurcations.

The formation of new branches requires radical cellular changes. The bud epithelium gradually changes shape without losing its integrity or polarity. Such epithelial shape changes can be accomplished by several mechanisms such as localized cell proliferation or cell death, cell migration, changes in cell shape or cell adhesion or through forces exerted on the epithelium by the ECM or by surrounding cells or tissues (Lecuit and Lenne, 2007). Indeed, it seems that branching morphogenesis requires several of these processes. Localized proliferation appears to contribute to the outgrowth of the UB from the WD, as well as to the formation of ampullae at the UB tips (Michael and Davies, 2004). In contrast, the contribution of apoptosis to branching morphogenesis is not clear as the number of apoptotic cells in normally growing UBs is low (Coles et al., 1993). However, Bcl-2, a proto-oncogene that inhibits apoptosis does not only act as a survival factor during kidney development but in addition promotes UB branching by modulating cell adhesion and migration, most probably through its interaction with paxillin (Sheibani et al., 2008; Sorenson, 2004). Branching morphogenesis could also be driven by the directed migration of epithelial cells towards the ampullae as has been shown for *Drosophila* air sac morphogenesis (Cabernard and Affolter, 2005). Extensive c-Ret-dependent cell movements have been described for UB outgrowth from the WD (Figure 13) (Chi et al., 2009). Apical constriction, which changes the cell shape from cuboidal to a wedge shape has been observed in UB cells of the ampullae and in forming outpouches. This could cause localized folding of the epithelial sheet and eventually lead to initiation of branching (Lecuit and Lenne, 2007; Meyer et al., 2004). This so-called “purse-string” model is further supported by accumulation of actin and myosin along the apical surface of the wedge-shaped cells, suggesting localized contraction of the actin-myosin cytoskeleton (Meyer et al., 2004). Acto-myosin contractility is mediated through activation of the small GTPase RhoA and its downstream target ROCK, a serine/threonine protein kinase. ROCK in turn can either directly activate MLC by phosphorylation or indirectly via inactivation of myosin light chain phosphatase (Amano et al., 1996; Kimura et al., 1996). Indeed, treatment of isolated UBs with a ROCK inhibitor increases budding and/or tip formation, while stalk formation is severely reduced, leading to the generation of a “stubby” UB with numerous stunted ampullae (Meyer et al., 2006).

Interestingly, the opposite phenotype was observed after interference of the MAPK pathway by MEK inhibition, which reduced UB branching whereas tubule elongation was not affected, giving rise to UBs with long tubules and just a few branches (Fisher et al., 2001). In an *in vivo* model for tumor angiogenesis it has been shown that MAPK/ERK inhibition leads to increased MLC2 phosphorylation indicating a crosstalk between the two pathways (Mavria et al., 2006). Therefore, in the developing kidney, the balance between MAPK/ERK signaling and RhoA/ROCK signaling could play a critical role in modulating the responsiveness of the epithelial cells to inductive factors and to eventually create branching tips with elongating stalks (Figure 15) (Meyer et al., 2006).

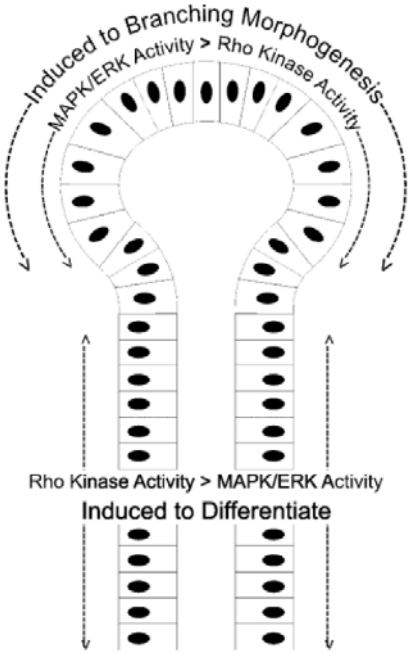



**Figure 15: Proposed model of balance of MAPK/ERK signaling to Rho-kinase signaling in the branching ureteric bud (Meyer et al., 2006).**
Stalk elongation requires higher ROCK activity whereas tip generation requires high MAPK activity that is thought to inhibit ROCK activity.

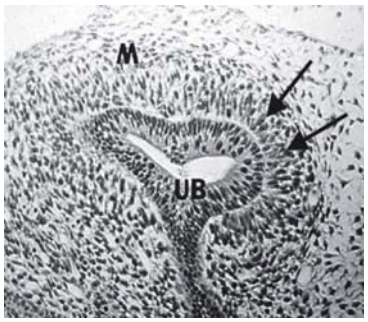
Finally, the cellular crosstalk between UB tip cell (expressing *Ret*, *WNT-11* and *Emx2*), stromal cells (expressing RARs and FoxD1/BF-2) and condensed mesenchyme (high expression of *Pax-2*) promotes branching. This crosstalk is in part mediated by BMP-7, which is expressed by the UB tip and the condensed mesenchyme and maintains

proliferation and survival. Interestingly, it has been shown that ILK is an effector of BMP-7-dependent epithelial cell morphogenesis through activation of p38 MAPK phosphorylation (Leung-Hagesteijn et al., 2005).
Cortico-medullary patterning of collecting ducts and formation of the pelicalyceal system also requires the function of *p57*$^{KIP2}$, BMP-4, BMP-5, and components of the renin-angiotensin pathway (Piscione and Rosenblum, 2002).

### 2.1.4 Tubulogenesis – MET

Once the UB has reached the MM, mesenchymal cells adjacent to the UB organize in such a way that their long axis is perpendicular to the surface of the tubule (called "condensing mesenchyme"), unlike more distant layers of mesenchyme and stroma, which show a horizontal orientation (Figure 16a). Mesenchymal cells that adopt this cell shape change start to undergo MET.
MET is characterized by a stepwise change in gene expression pattern (Figure 16b). Cells that undergo MET start to express R-cadherin, cadherin-6 and subsequently E-cadherin whereas the expression of the mesenchymal specific cadherin-11 is suppressed. This cadherin switch is followed by the translocation of ZO-1 and β-catenin to the lateral cell surfaces, and finally the deposition of a laminin-containing basement membrane. The renal vesicle remains associated with the UB epithelia. By the comma-shaped-body stage, the distal end of the growing nephron fuses to the epithelium of the duct to form a continuous lumen (Figure 10b) (Dressler, 2006). Several UB-derived factors have been identified to induce epithelial conversion in the MM. It has been shown that leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) are among the first UB-derived inductive molecules (Barasch et al., 1999).

a)




b)

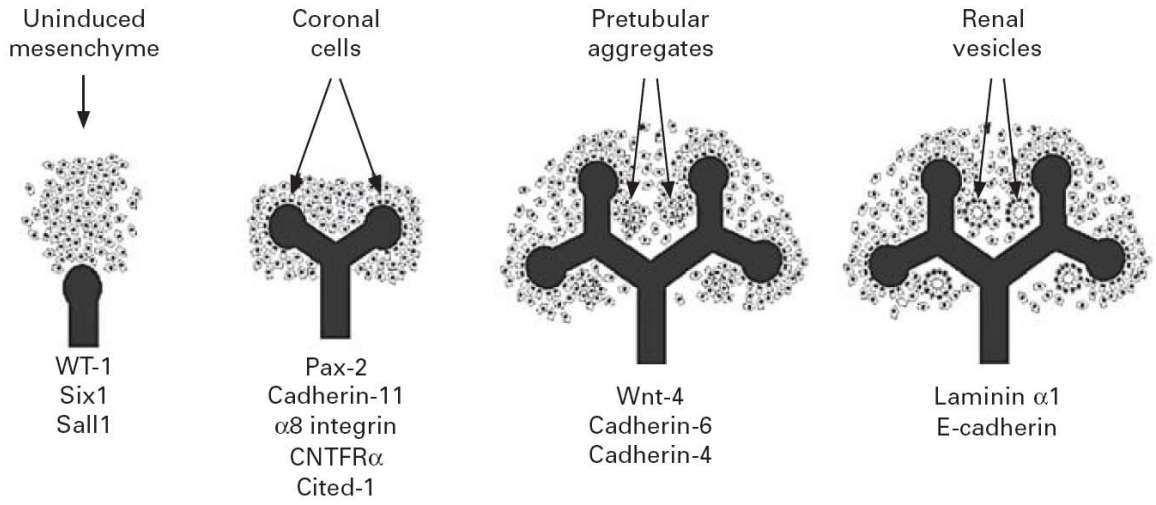



**Figure 16: Mesenchymal to epithelial transformation (MET) during kidney development (Schmidt-Ott et al., 2006).**
a) Early signs of polarity can already be observed at the coronal cell stage. Mesenchymal cells at the tip of the ureteric bud reveal a columnar shape (arrows). b) MET in the developing kidney leads to the formation of renal vesicles and is characterized by discret morphological stages and the expression of distinct marker genes.

Other factors that are associated with tubulogenesis are *WNT-9b*, *WNT-4*, *Notch-2* and *BMP-7*. Genetic deletion of *WNT-9b* leads to an arrest of epithelialization in the MM and loss of WNT-4 expression (Carroll et al., 2005). In *WNT-4*$^{-/-}$ kidneys, the mesenchyme initially condenses, but MET fails, and tubules do not form (Stark et al., 1994). It has been shown that stabilization of β-catenin through the inhibition of Gsk-3β, with for instance lithium, is sufficient to induce nephron differentiation in isolated mouse kidney mesenchymes suggesting that canonical WNT signaling induces nephrogenesis (Kuure et

al., 2007). Notch-2 expression can be detected in the early renal vesicle. It is required for the differentiation of proximal nephron structures such as podocytes and proximal tubules (Cheng et al., 2007). BMP-7, which is expressed in the UB seems to be important for early induction of nephrogenesis as in the absence of BMP-7 only a few and/or incomplete mesenchymal condensations are observed. This implies that BMP-7 regulates target genes in metanephric mesenchymal cells that are critical for nephrogenesis such as genes whose products are important for proliferation, differentiation and survival (Luo et al., 1995).

The mature nephron is subdivided into segments that are dedicated to specific tasks (see 2.1). This specialization, which is acquired during terminal differentiation, is reflected in protein expression profiles of various segments. However, the factors that drive segmental nephron identity are still poorly understood. LIM1 is required to induce the initial stages of patterning in the renal vesicle, by controlling the expression of the POU-domain transcription factor BRN1 and the Notch ligand DLL1. DLL1 itself contributes via Notch-2 activation to the specification of the proximal tubule fate. Under the control of the transcription factors BRN1 and Iroquois-class homeodomain proteins IRX1-3, distal segments further extend and differentiate towards distal tubule and the Henle's loop. Finally, to terminally differentiate cells need to withdrawal from the cell cycle and repress genes involved in cell cycle control. Proteins of the p53 family perform a dual function by inducing cell cycle arrest genes (e.g., $p21^{Cip1}$) and repressing proliferation genes (e.g., *PCNA* and *CDC2*) on one hand and by activating terminal differentiation markers, including the bradykinin B2 receptor, aquaporin-2 and the $Na^+$-$K^+$-ATPase a1 (El-Dahr et al., 2008) on the other hand.

### 2.1.5 Role of stroma in kidney development

After induction of the MM by the UB at least two different sets of cells are found: epithelial cells contributing to the nephron and stromal cells. Renal stromal cells have been identified as an important source of metanephric regulatory signals. So far three different factors secreted or expressed by stromal cells have been shown to influence kidney development. The first hints to the importance of the stromal compartment came

from forkhead box D1 (*Foxd1*) knockout mice which display defects in the collecting duct system and nephrons, suggesting that it regulates the expression of a stromal-cell-derived signal that promotes branching and tubulogenesis (Hatini et al., 1996). Vitamin A has also been shown to play a role in branching morphogenesis of the UB epithelium. A double knockout of both vitamin A receptors, the nuclear retinoic acid receptor (rar)-α und –β, leads to reduced growth of the UB which is accompanied by reduced expression of *c-Ret* and *WNT-11* (Mendelsohn et al., 1999). The third factor is FGF-7 which is specifically secreted from stromal cells that surround the UB and the developing collecting duct. Its receptor FGFR2 is expressed in the UB itself. *FGF-7*-deficient mice have reduced growth of the UB and collecting ducts and ~30% fewer nephrons (Qiao et al., 1999). In isolated WDs treated with a combination of FGF-7 and blockade of the TGF-β family member activin A with Follistatin, outgrowth of extra-UBs was induced (Maeshima et al., 2007). Therefore, it has been suggested that the FGF-7/activin A pathway might also modulate UB outgrowth and branching *in vivo* and thus explain why 30 to 50% of knockout mice that lack either *GDNF*, *c-Ret* or *GFRα* have normal ureters.

### 2.2 Renal abnormalities in humans

Developmental abnormalities of the kidney are diverse and include renal agenesis (the absence of a kidney), multiple ureters, renal hypoplasia (reduced kidney size, reduced number of intact nephrons) and dysplasia (a kidney containing abnormal structures). Each defect corresponds to irregularities at a particular stage of development (Figure 17).

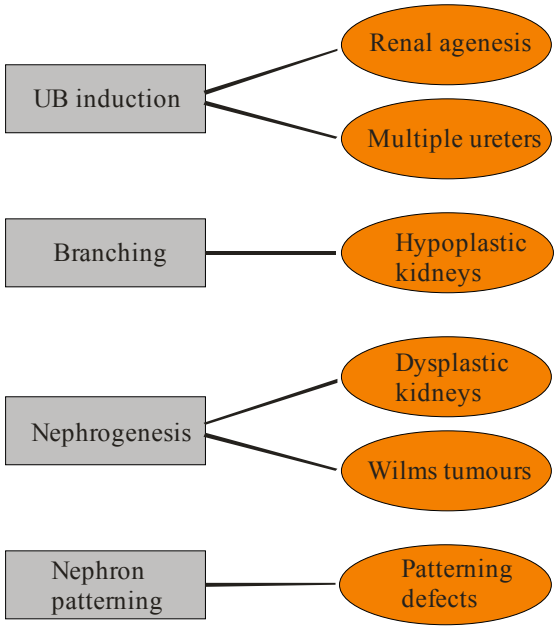



**Figure 17: Developmental abnormalities of the kidney. Adapted from (Schedl, 2007).**
Development of the kidney can be subdivided into several distinct stages. Abnormalities in these stages usually lead to specific developmental defects that in turn can be related to a human disease.

Human urinary tract abnormalities are phenotypically variable and can affect several segments simultaneously. As a consequence the renal and urologic malformations are grouped together into "Congenital Anomalies of the Kidney and the Urinary Tract" (CAKUT).

Renal agenesis is a relatively frequent congenital defect in humans. An estimate of a congenital absence of the kidney is 0.48 to 0.58 per 1000 live births. Unilateral agenesis occurs with a frequency of 1 in 200 births and lethal bilateral agenesis with a frequency of 1 in 5.000-10.000 births. In addition, major malformations, including those involving

the kidneys or lower urinary tract, are often lethal *in utero* or shortly after birth (Pohl et al., 2002).

In humans, renal agenesis seems to arise mainly from mutations in genes known to affect *GDNF* expression or signaling. These include diseases such as Townes-Brock syndrome (*SALL1*), Renal-coloboma syndrome (*Pax-2*) or Brancho-Oto-Renal syndrome (*Eya1*) (Shah et al., 2004). In addition, in 37% of stillborn fetuses with congenital renal agenesis mutations in *Ret* have been found suggesting that also in humans mutations in *Ret* may contribute significantly to abnormal kidney development (Skinner et al., 2008). Mutations in ROBO2 which restricts GDNF expression to the caudal part of the WD have been identified in patients with vesicoureteral junction defects and vesicoureteral reflux (Table 4).

Kidney size is primarily determined by the total number of nephrons that are formed during development. The average nephron number varies between individuals, ranging from 300.000 to 1 million in each kidney. Recent studies suggest that there is a strong correlation between the number of nephrons and the risk to develop primary hypertension (high blood pressure for which no particular cause is known). The number of ureteric branches also determines the nephron number. As the GDNF/c-Ret pathway is important for UB outgrowth and branching, mutations that affect the expression of transcriptional regulators of these genes will also result in reduced ureter branching. Indeed, heterozygous mutations in *Pax-2* cause Renal-coloboma syndrome in humans, a congenital disease that is characterized by optic nerve coloboma and renal hypoplasia (Sanyanusin et al., 1995).

Fraser syndrome is an autosomal recessive disorder occurring in 11/100.000 stillbirths and 0.4/100.000 live births and is characterized by cryptophthalmos (eyeball covered by skin), syndactyly (fused digits) and kidney malformations. Some Fraser syndrome individuals have mutations in either *FRAS1* or *FRAS1-related ECM gene 2* (*FREM2*), that encode for basement membrane related transmembrane proteins. FRAS1 is found to be expressed around the UB and is upregulated as the MM differentiates into nephrons, particularly in nascent glomeruli. FRAS1 deficiency is associated with failed UB growth into the MM causing kidney agenesis and glomeruli defects (Pitera et al., 2008).

**Table 4: Human congenital abnormalities and gene defects linked to the diseases (Schedl, 2007).**

| Renal congenital defects | Symptoms | Syndromes | Gene defects |
|---|---|---|---|
| Renal agenesis | Absence of kidneys; usually unilateral, but can occur bilaterally | Brancho-Oto-Renal syndrome (BOR) | *Eya1*, *Six1*, *Six4*, *Six5* |
| Duplex (multiple) ureter | Formation of several ureters resulting from defective ureter induction | Usually no symptoms; can however be associated with hydroureter | |
| Renal hypoplasia | Reduction of kidney size without abnormal development, probably caused by a reduced number of nephrons | Renal-coloboma<br>Townes-Brocks<br>Pallister-Hall | *Pax-2*<br>*Sall1*<br>*Gli3* |
| Renal dysplasia | Kidney contain abnormally developed structures; often associated with hypoplasia | Fraser<br>Campomelic dysplasia | *Fras1*, *Frem1*<br>*Sox9* |
| Tubular dysgenesis | Defective proximal tubules formation | Renal tubular dysgenesis | *Ren*, Agt, *ACE*, *AGTR1* |
| Nephrotic syndrome (NS) | Proteinuria resulting from a failure of blood filtration; usually caused by glomerular defects | Frasier, Denys-Drash<br>Nail-Patella-syndrome | *WT-1*<br>*LMX1B* |
| Polycystic kidneys | Formation of cysts affecting either tubules, collecting ducts or both | Renal cysts and diabetes<br>Polycystic kidney disease | *HNF1B*<br>*PKD1*, *PKD2*<br>*AP2beta* |

Wilms tumour is a pediatric kidney cancer that affects 1 in 10.000 children. Wilms tumours seem to develop from so called nephrogenic rests, an abnormal structure in the kidney that is formed as a result of a failure of the mesenchymal tissue to differentiate into nephrons. *WT-1* is required for kidney induction but it has also an important role in nephron formation and podocyte differentiation. Hence, loss of *WT-1* during kidney development is likely to arrest nephron precursors in a multipotent state. Usually, a second mutation in the *β-catenin* gene preventing its degradation is associated with *WT-1* mutations promoting cell growth (Schedl, 2007).

### 2.2.1 The glomerulus and proteinuria

One primary function of the mature kidney is the filtration of high-molecular-weight proteins present in the blood which takes place in the glomeruli. This highly specialized function resides in the glomerular filtration unit, composed of (1) fenestrated endothelial cells of the capillary tuft; (2) an intervening glomerular basement membrane (GBM), rich in specialized collagens, laminins, and heparan sulphate proteoglycans; and (3) the podocytes, terminally differentiated epithelial cells with specialized major cell processes that extend to interdigitate with adjacent foot processes, forming an intervening slit diaphragm (Figure 18). The epithelial foot processes serve as a molecular sieve that selectively restricts the filtration of different molecules on the basis of their size, shape and charge (Tryggvason and Wartiovaara, 2001).

The GBM has a thickness of 300 to 350 nm and it derives initially from fusion of two independent basement membranes, that of endothelial cells with that of the glomerular epithelial cell. As the glomerular epithelial cell differentiates into the mature podocyte, it continues to synthesize GBM, whereas the contribution of the endothelial cell is thought to cease. The main components of the GBM are type IV collagen, proteoglycans, laminin and nidogen. Interestingly, a developmental switch in the composition of the GBM matrix expression occurs during glomerulogenesis. During embryogenesis and postnatal life, heterotrimeric type IV collagen containing α1 and α2 chains are replaced with heterotrimeric type IV collagen containing tissue-specific α3, α4 and α5 chains that are primarily present in the glomeruli (Miner and Sanes, 1994). Parallel to this, heterotrimeric laminin 511, containing α5/β1/γ1 chains switch to heterotrimeric laminin 521, containing α5/β2/γ1 (Miner et al., 1997). Ablation of the *laminin β2* gene in mice causes a lack of laminin 521 resulting in proteinuria and neonatal death (Noakes et al., 1995). Mutations in the *laminin β2* gene cause Pierson's syndrome, an early lethal form of congenital nephrotic syndrome in humans (Zenker et al., 2004). Hence, laminin 521 seems to be important for macromolecular filtration whereas mutations in adult collagen IV cause only mild proteinuria (Tryggvason et al., 2006).

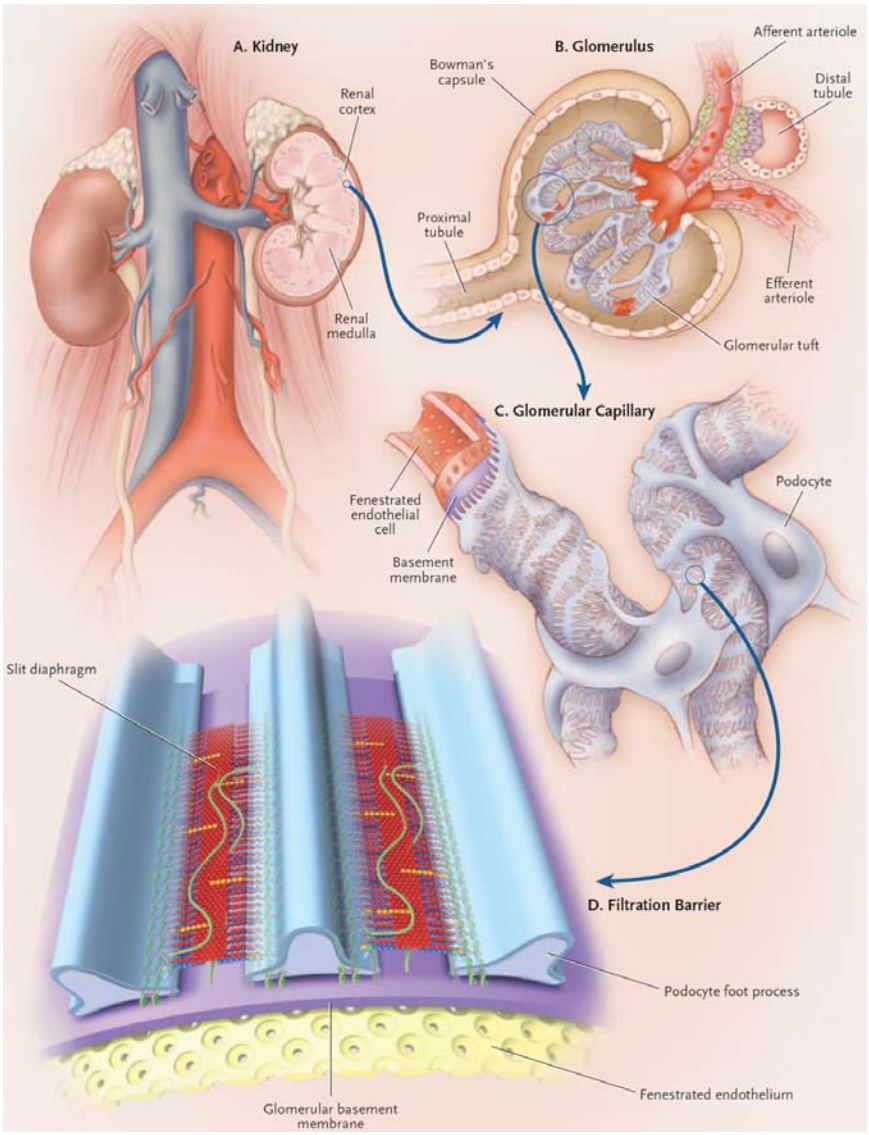



**Figure 18: The glomerular filtration system of human kidneys (Tryggvason et al., 2006).**
a) Each kidney contains about 1 million glomeruli in the renal cortex. b) An afferent arteriole enters the Bowman's capsule and branches into several capillaries that form the glomerular tuft. c) The filtration barrier of the capillary wall contains a fenestrated endothelium, the glomerular basement membrane and a layer of interdigitating podocyte foot processes. d) Cross section through the glomerular capillary. An ultra thin slit diaphragm spans the filtration slit between the foot processes.

Podocytes are highly specialized, pericyte-like cells, with large arborization of cell processes that make up the unique foot processes covering the GBM. Their function is to control the turnover of the GBM and to regulate ultrafiltration of urine. An electron-dense

slit diaphragm (SD) approximately 40 nm in size extends from foot processes (FP) of adjacent cells to form specialized cell-cell junctions, thereby establishing the final barrier to urinary protein loss (Figure 10d). The SD shares certain features with other intercellular junctions. Similarly to tight junctions, SDs contain zona occludens-1 (ZO-1) protein, whereas the presence of FAT and P-cadherin resembles the composition of desmosomes and adherens junctions.

Nephrin, podocin and CD2AP are considered as the main structural elements of the SD. Nephrin is a single-pass transmembrane protein that homodimerizes and forms heterodimers with its homolog NEPH1, thus connecting adjacent foot-processes to each other and transducing signals that control glomerular permeability (Liu et al., 2003).

Nephrin interacts through its C-terminal part with podocin and CD2AP. The nephrin/NEPH1 complex transduces phosphorylation mediated signals that assemble an actin polymerization complex at the podocyte intercellular junction and recruits Grb2 and Nck1/2 adaptor proteins, which mediate downstream activation of the cytoskeletal regulators N-WASP and Pak. In addition, nephrin phosphorylation by Fyn kinase increases its interaction with PI3K and the subsequent PI3K-dependent activation of Akt and Rac modifies the actin cytoskeleton, confirming the determinant role of nephrin signaling on podocyte morphology. Similarly, CD2AP has been implicated in the PI3K/Akt survival pathway and in dynamic actin remodeling. Another function of this complex is the regulation of podocyte polarity, which occurs via its interactions with Par3, Par6 and aPKC complex (Figure 19) (Machuca et al., 2009).

FPs are further characterized by a podosome-like, cortical network of short branched actin filaments and the presence of highly ordered parallel, contractile actin filament bundles, which are thought to modulate the permeability of the filtration barrier through changes in FP morphology. FPs are functionally defined by three membrane domains: the apical membrane domain (AMD), the SD and the basal membrane domain, which is associated with the GBM. All three domains are physically and functionally linked to the FP actin cytoskeleton. Interference with any of the three FP domains changes the actin cytoskeleton from parallel contractile bundles into a dense network resulting in FP effacement and proteinuria. Thus, proteins regulating the plasticity of the podocyte actin

cytoskeleton are of crucial importance for the maintenance of the glomerular filter function (Faul et al., 2007) and their loss of function results in proteinuria (Table 5).

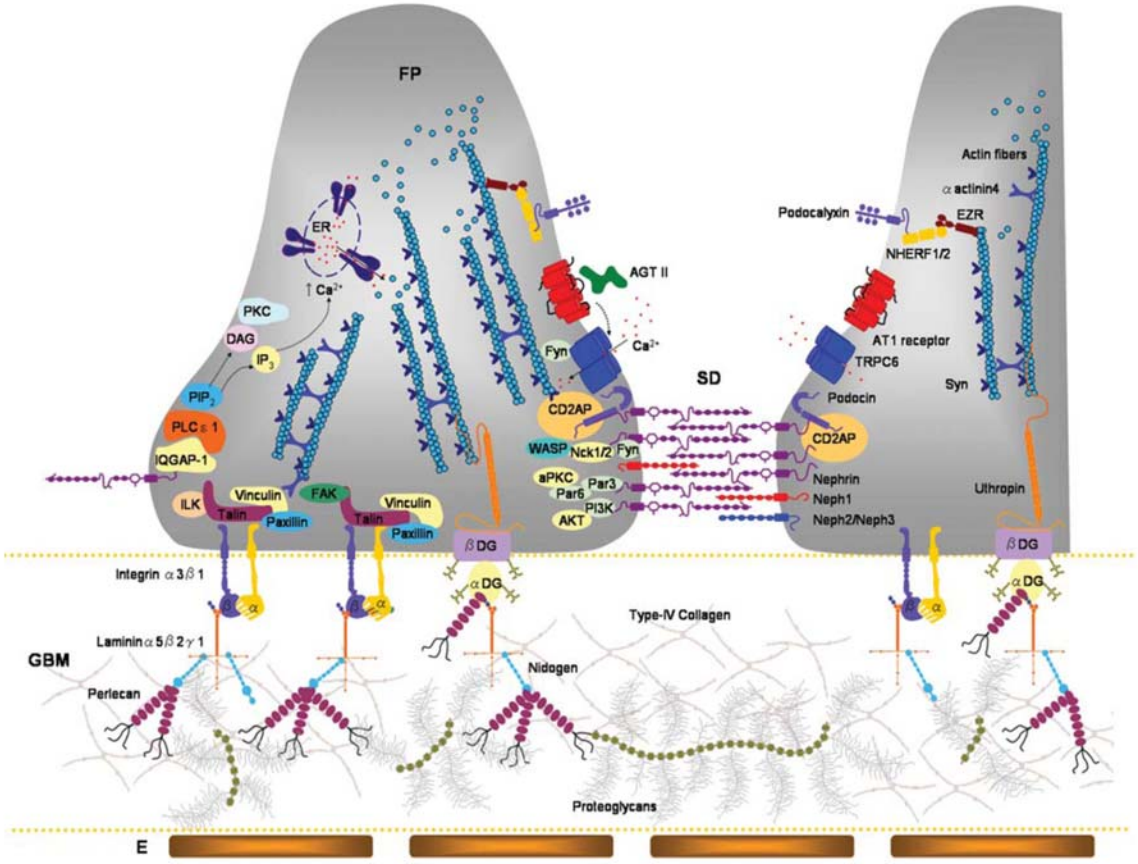



**Figure 19: Molecular overview of the slit diaphragm and podocyte cell-matrix interactions (Machuca et al., 2009).**
At the slit diaphragm, nephrin mediates signals that control actin cytoskeleton remodeling (Nck1/2, WASP), cell polarity (Par3/6, aPKC) and survival (PI3K, Akt). TRPC6-podocin interactions modulate mechanosensation, whereas angiotensin II type 1 receptor (AT1) may increase TRPC6 mediated calcium influx upon stimuli by angiotensin II (AGT II). The main component of the podocyte-matrix interaction structure is the integrin α3β1-laminin 521 and dystroglycan-uthropin complex that connects GBM components (proteoglycans, nidogen, perlecan and type IV collagen) to the actin cytoskeleton.
FP, foot-process; SD, slit diaphragm; GBM, glomerular basement membrane

**Table 5: Hereditary forms of Nephrotic syndrome (Machuca et al., 2009).**
Nephrotic syndrome (NS) is a group of disorders characterized by heavy proteinuria with hypoalbuminemia, edema and dyslipidema.
AR, autosomal-recessive; AD, autosomal-dominant; SRNS, steroid-resistant NS; SSNS, steroid-sensitive NS; FSGS, focal segmental glomerulosclerosis; ESKD, end stage kidney disease; CNS, congenital kidney disease

| Gene | Locus | Inheritance | Protein | Function | Phenotype or Syndrome |
|---|---|---|---|---|---|
| **Actin cytoskeleton components** | | | | | |
| *ACTN4* | 19q13 | AD | α -actinin-4 | F-actin cross-linking protein | Late-onset SRNS with imcomplete penetrance and slow progression to ESKD |
| *MYH9* | 22q12.3 | complex | NMMHC-A | Cellular myosin: cytokinesis and cell shape | High risk haplotypes associated with increased risk of FSGS and ESKD in African-Americans |
| **Glomerular basement membrane proteins** | | | | | |
| *LAMB2* | 3p21 | AR | Laminin-β2 | GBM component, scaffold for type IV collagen assembly | Pierson syndrome |
| *ITGB4* | 17q25.1 | AR | Integrin-β4 | Cell-matrix adhesion, structural role in the hemidesmosome of epithelial cells | Epidermolysis bullosa. Anecdotic cases presenting with NS and FSGS |
| **Slit diaphragm protein complex** | | | | | |
| *NPHS1* | 19q13.1 | AR | Nephrin | Main component of the SD. Anchors the SD to the actin cytoskeleton. Modulates actin cytoskeleton | CNS of the Finnish type. Early-onset SRNS in cases carrying at least one mild mutation |
| *NPHS2* | 1q25–31 | AR | Podocin | Scaffold protein linking plasma membrane to the actin cytoskeleton | CNS. Early and late onset AR SRNS. Juvenile and adult SRNS in cases bearing the R229Q variant in compound heterozygous state with a pathogenic mutation |
| *PLCE1* | 10q23 | AR | Phospho-lipase C ε1 | Involved in cell junction signaling and glomerular development | Early-onset SRNS with DMS and FSGS |
| *CD2AP* | 6p12.3 | AR (?) | CD2 associated protein | Adaptor protein, may anchor the SD to the actin cytoskeleton | Not precisely defined in humans, may cause early-onset SRNS and FSGS. Mice model exhibits a severe phenotype resembling CNS in humans |

### 2.2.2 Cystic kidney diseases

Renal cystic diseases are a major clinical concern as they are the most common genetic cause of end-stage kidney disease. During the course of the disorders clusters of cysts (noncancerous round sacs containing water-like fluid) develop primarily in the kidney leading to an increase in kidney size while its function decreases. Among other symptoms, patients suffer from a high risk of high blood pressure and kidney failure. Cyst formation might be primarily caused by defective planar cell polarity (PCP) and/or ciliary defects. Healthy mammalian nephrons are characterized by a striking structural organization. A series of morphogenic remodeling events characterized by limited cellular proliferation progressively shape vesicles into slightly elongated and folded tubular structures called “comma” and “S-shaped” bodies. Subsequently, extensive cell proliferation at the corticomedullary junction gives rise to the final elongated structure. Normally, this proliferation does not give rise to a huge increase of tubular diameter. Instead, this intense proliferation specifically produces tubular elongation, so that at the end of the maturation process, tubules are several hundred folds longer than their width. Polycystic kidney disease (PKD) represents an example of a drastic dysfunction of this morphogenetic process (Figure 20a) (Fischer and Pontoglio, 2009). Autosomal-dominant polycystic kidney disease (ADPKD) is caused by mutations in the PKD1 and PKD2 loci, which code for polycystin-1 (PC-1) and 2 (PC-2). PC-1 is a large transmembrane protein while PC-2 is an L-type calcium channel that modulates calcium signaling in response to mechanical deformation. PC-1 and PC-2 form a complex and assemble in cilia of renal epithelial cells. The primary cilium is a microtubule-based, antenna-like extension that projects from the surface of most cells. These structures are non-motile and composed of an axoneme comprised of nine microtubule doublets surrounded by the ciliary membrane. The primary cilium is anchored in the cell by the basal body, a structure that also functions as one of the centrioles during cell division (Figure 20b).

a) b)

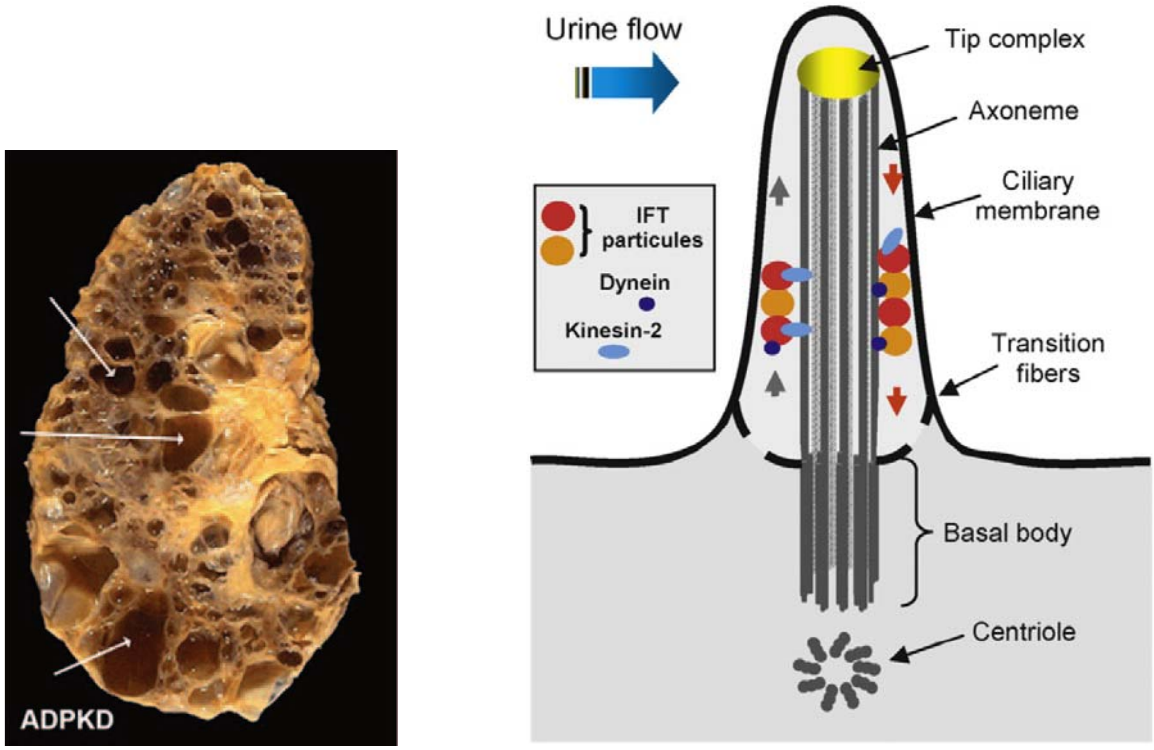



**Figure 20: Role of cilia in polycystic kidney disease.**
a) Kidney from a patient having ADPKD (Bacallao and McNeill, 2009). Large cysts are marked with arrows. b) Schematic presentation of the structure of a primary cilium (Fischer et al., 2006). Primary cilia are microtubular structures surrounded by a ciliary membrane and separated from the cytoplasm by transition fibers. The basal body contains nine microtubules triplets and is oriented perpendicular to the daughter centriole, represented here in cross section.

In the kidney, a single primary cilium is found on the apical surface of most tubular epithelial cells. Primary cilia have been implicated in cell cycle regulation, hedgehog signaling, Wnt signaling and PCP signaling (Bacallao and McNeill, 2009). During normal kidney function, urine flows over kidney epithelial cells, bending their primary cilia. This bending results in a PC-1- and PC-2-dependent increase in intracellular $Ca^{2+}$ ion concentration and the inhibition of the regulated intramembrane proteolysis of PC-1. Disruption of urine production or flow allows the cilium to straighten, blocks $Ca^{2+}$ ion flux and activates the proteolysis of PC-1. PC-1 proteolysis releases a portion of its cytoplasmic tail which translocates to the nucleus in a complex with Stat6 and P100. Once in the nucleus, the complex activates transcription. In ADPKD, mutations in *PKD1*

and *PKD2* lead to constitutive activation of this pathway which results in uncontrolled cell proliferation and cyst formation (Singla and Reiter, 2006). Another form of human PKD is caused by mutations in the gene *inversin* which leads to constitutive activation of the canonical Wnt pathway and aberrant proliferation. Finally, a defective PCP pathway may also contribute to the pathogenesis of PKD. During kidney tubule elongation, the mitotic apparatus of cells is precisely oriented to direct cell division parallel to the axis of the tubule. Decreased expression of the ciliary protein Pkhd1 results in both PKD and disoriented kidney cell mitosis (Fischer et al., 2006).

## 2.3 Integrins and kidney

Formation, growth and branching morphogenesis of the collecting system of the kidney requires interaction between UB and MM. As mentioned above, this complex developmental process is regulated by a set of growth factors including GDNF and FGF-7 and is dependent on interactions between cells and ECM components mediated by integrin receptors.

### 2.3.1 ECM and its receptors in mammalian nephrogenesis

ECM glycoproteins influence intracellular events via their receptors, e.g. integrins, and thereby regulate cell differentiation, migration and polarization. On the other hand, transcription, translation and posttranslational modification of such ECM components are regulated by various growth factors or hormones and their receptors indicating an interdependence of growth factors and ECM proteins. This interdependence is in addition reflected by the fact that the ECM can act as a storage depot for certain growth factors such as GDNF or FGFs.

A large number of ECM proteins are expressed during renal development (Table 6) in a spatiotemporal manner. ECM proteins expressed in the MM include interstitial collagens, tenascin, nidogen, fibrillins, osteopontin, and fibronectin while type IV collagen, laminin, proteoglycan, and nephronectin are associated with the basement membrane (Kanwar et al., 2004).

**Table 6: Spatiotemporal expression of ECM proteins during metanephric development (Kanwar et al., 2004).**
BL, basal lamina; GBM, glomerular basement membrane; CS-PG, chondroitin sulphate-proteoglycan; TIN-Ag, tubulointerstitial nephritis antigen; BMP-7, bone morphogenetic protein-7

| ECM protein | Ureteric Bud | Stage of Vesicle | Comma/S-Shaped | Precapillary Stage | Glomerular Capillary, (GBM) | Glomerular Mesangium | Proximal Tubule | Distal Tubule | Metanephric Mesenchmye |
|---|---|---|---|---|---|---|---|---|---|
| Collagen IV (BL) | +, α 1, α 2 | +, α 1, α 2 | +, α 1, α 2 | +, α 1, α 5 | +, α 3, α 5 | +, α 1, α 2 | +, α 1, α 2 | +, α 1, α 2 | - |
| Laminins (BL) | +, α 1, α 5 | +, α 1, α 4, β1 | +, α 1, α 4, α 5, β1 | +, α 1, α 4, α 5, β1, β2 | +, α 1, β2 | +, α 1, α 2 | +, α 1, α 5 | +, α 5 | - |
| Perlecan (BL) | + | + | + | + | + | - | + | + | - |
| CS-PG (BL) | - | - | + | - | - | + | - | - | - |
| Nidogen (BL) | + | ± | + | ± | + | - | + | + | ± |
| TIN-Ag (BL) | + | + | + | - | - | - | + | + | - |
| Collagen I/III | - | - | - | - | - | + | - | - | + |
| Fibronectin | - | - | - | - | - | + | - | - | + |
| Tenascin-C | - | - | - | - | - | - | - | - | + |
| Fibrillin-1 | - | - | - | - | - | + | - | - | + |
| Nephronectin | + | ± | + | + | + | - | + | + | - |
| Osteopontin | + | - | +, cleft | - | - | + | - | + | ± |
| BMP-7 | + | + | + | + | - | + | - | - | + |

**Table 7: ECM proteins and their binding proteins/integrin receptors expressed during metanephric development (Kanwar et al., 2004).**

| ECM protein | Integrin receptor(s), binding proteins |
|---|---|
| Collagen IV (BL) | α 1β1, α 2β1, α 3β1, α Vβ3 |
| Laminins (BL) | α 1β1- α 3β1, α 6β1, α 6β4, α 7β1, α Vβ3, Dystroglycan |
| Perlecan (BL) | Dystroglycan |
| Nidogen (BL) | α 3β1 |
| TIN-Ag (BL) | α 3β1, α Vβ3 |
| Collagen I/III | α 1β1, α 2β1, α Vβ3 |
| Fibronectin | α 1β1, α 5β1, α 8β1, α Vβ3 |
| Tenascin-C | α 8β1, α Vβ3, α9β1 |
| Fibrillin-1 | α Vβ3 |
| Nephronectin | α 8β1 |
| Osteopontin | α 8β1, α Vβ3, α9β1 |

Integrins serve as receptors for a variety of ECM molecules, including laminins, collagens, osteopontin, nephronectin, vitronectin, and tenascin (Table 7 and chapter 1.2). The expression pattern of distinct integrin heterodimers in the developing and adult kidney has been analysed in recent years.

The α1β1-integrin, a receptor for collagen and laminin, is expressed in S-shaped tubules mainly by cells invading the glomerular cleft. In more mature glomeruli, integrin α1β1 is restricted to the mesangial area within glomeruli.

Another laminin and collagen binding integrin, α2β1-integrin, is expressed in the part of the S-shaped tubule that will contribute to distal tubules as well as in endothelial cells within the capillary loops of immature glomeruli. In more mature kidneys, α2β1-integrin is expressed in collecting ducts and glomerular endothelial cells.

The α3β1-integrin was originally characterized as a promiscuous receptor that can bind to collagen, fibronectin, laminin, and nidogen and with higher affinity to α5 chain containing laminin isoforms. α3β1 is expressed weakly by the UB and most highly in those cells of the early tubule that represent the presumptive podocytes. In more mature

kidneys expression is observed in distal tubules and collecting ducts. In maturing glomeruli, α3β1 is highly expressed by glomerular podocytes in a polarized pattern along the glomerular basement membrane.
The laminin receptor α6β1, is expressed along both proximal and distal tubular basement membranes, as well as by collecting ducts (Kreidberg and Symons, 2000).

### 2.3.2 Role of integrins and their binding partners in the development of the collecting system

To address the role of integrins during kidney development different mouse strains have been generated that lack expression of specific integrin subunits. Among the different integrin α-null mice generated, only integrin α3 and α8 knockout mice show a severe collecting system phenotype whereas integrin α6-deficient mice do not display any obvious kidney phenotype (Georges-Labouesse et al., 1996; Kreidberg et al., 1996; Muller et al., 1997). Mice lacking the α3 subunit show decreased branching of the medullary collecting ducts suggesting impaired branching morphogensis of the UB. In addition, glomerular development is markedly affected. The glomerular basement membrane is disorganized and glomerular podocytes are unable to form mature foot processes (Kreidberg et al., 1996). Specific deletion of the integrin α3-subunit in the UB leads to either absent or abnormal kidney papillae, while the rest of the collecting system of the kidney is unaffected (Liu et al., 2009). Interestingly, integrin α3β1 and the HGF receptor c-Met, signal in concert to regulate the expression of WNT-7b, which is required for the establishment of the cortico-medullary axis through regulation of the cell cleavage plane (Liu et al., 2009; Yu et al., 2009).
Integrin α8 expression is induced in mesenchymal cells upon contact with the ureter. In integrin α8 knockout mice, growth and branching of the UB as well as recruitment of mesenchymal cells into epithelial structures are defective (Muller et al., 1997). Nephronectin has been identified as the ligand for integrin α8β1 in the kidney (Brandenberger et al., 2001). Mice lacking nephronectin also frequently display kidney agenesis, similar to integrin α8-null mice (Linton et al., 2007). Interestingly, GDNF expression in the MM at the time of UB invasion is transiently reduced in both

nephronectin and integrin α8-null mice suggesting that nephronectin/integrin α8β1 are part of a signaling pathway that regulates GDNF expression (Linton et al., 2007). However, the mechanism of GDNF expression regulation by nephronectin/integrin α8β1 is not understood as integrin α8β1 is not expressed in the uninduced MM when the GDNF signal is required for UB outgrowth.

Recently, the role of β1 integrins in the development of the collecting duct system has been addressed. The deletion of β1 integrin at E10.5, the time where UB outgrowth/branching is initiated leads to a severe branching phenotype and decreased nephron formation followed by the death of mice by 4 to 6 weeks of age. Integrin β1 null collecting duct cells are impaired in FGF and GDNF mediated signaling, growth factors known to be important for UB outgrowth and branching morphogenesis. Interestingly, the abnormality in branching morphogenesis is significantly worse in mice in which β1 integrin rather than α3 was specifically deleted in the UB (Liu et al., 2003; Zhang et al., 2009), suggesting that other αβ1 integrin heterodimers play a role in this process.

In contrast, when β1 integrin was deleted in collecting ducts at E18.5, kidney development preceded normally. However, severe collecting system injury is observed in adult animals following ureteric obstruction (Zhang et al., 2009). Thus β1 integrins are required to maintain structural integrity when the collecting system is subjected to the increased hydrostatic pressure induced by tying the ureter.

### 2.3.3 Role of integrins and their binding partners in the development and function of the glomerulus

β1 integrins are also highly expressed in the glomerulus of the kidney. Recently, the integrin α3-subunit was selectively deleted in podocytes, which resulted in mice that developed proteinuria within the first week after birth and a nephrotic syndrome (NS) by 5-6 weeks of age (Sachs et al., 2006). Newborn mice had podocyte foot process effacement and the glomeruli of the 6-weeks-old mice were severely sclerosed, had a disorganized GBM and protein casts in dilated proximal tubules. Deletion of all β1 containing integrins using the podocin-cre that is active at the S-shaped body stage results in normal morphogenesis of the glomerulus, despite podocyte abnormalities, a defective

glomerular filtration barrier present at birth and podocytes loss over time. Three weeks old mice develop severe end-stage renal failure characterized by both tubulointerstitial and glomerular pathology (Pozzi et al., 2008). The overall phenotype is similar to that found in mice where the α3 integrin subunit is selectively deleted in podocytes suggesting that integrin α3β1 is the main integrin required to maintain the structural integrity of the glomerulus. In a parallel study, β1 integrin has also been deleted in podocytes. Here, the phenotype is more severe as the mutant mice have proteinuria on day 1 and die within one week after birth (Kanasaki et al., 2008). Interestingly, the podocyte/slit diaphragm defects are accompanied by structural defects in the GBM and matrix assembly which has not been observed in a study by Pozzi *et al*. (Pozzi et al., 2008).

The glomerular endothelial cells and also the podocytes contribute to the GBM that initially develops as two separate layers. These two layers fuse to form the mature GBM. In the podocin-cre integrin β1-fl/fl mice, the GBM remains as two layers and in many areas it is further disrupted (Kanasaki et al., 2008). The role of integrin β1 for proper formation of basement membranes is well established (Li and Yurchenco, 2006; Miner and Yurchenco, 2004). Integrin β1 has been shown to facilitate the recruitment of laminin followed by type IV collagen to organize the basement membrane structure at the basolateral site of the cell. Podocytes express integrin α3β1 and bind to laminin 511 and laminin 521 in the GBM suggesting that an impaired laminin recruitment could be causal for the observed GBM defects (Li and Yurchenco, 2006; Miner and Yurchenco, 2004).

ILK, PINCH and parvin function as a signaling platform for integrins by regulating the actin cytoskeleton and diverse signaling pathways (Legate et al., 2006). As mentioned above, podocytes are anchored to the GBM through the α3β1-integrin complex that is present in the sole of the foot processes. However, whether the integrin and SD signals are connected and how this occurs is still not clear. Dysregulation of ILK expression is implicated in the pathogenesis of a wide variety of chronic kidney diseases, including nephrotic syndrome and diabetic and obstructive nephropathy (Guo et al., 2001; Kretzler et al., 2001; Li et al., 2003; Teixeira Vde et al., 2005). Overexpression of ILK can be observed in patients with congenital nephrotic syndrome and in glomerular podocytes of murine models of proteinuria (Kretzler et al., 2001). Selective ablation of ILK in podocytes causes aberrant distribution of the SD protein nephrin and α-actinin-4 as well

as early foot process effacement. This results in heavy albuminuria, glomerulosclerosis, and kidney failure, which leads to lethality at 10 weeks of age. In addition, ILK is in a complex with nephrin together with α-actinin suggesting that cell-matrix integrin signaling and the cell-cell adhesion SD signaling are intrinsically coupled through an ILK-dependent mechanism (Figure 21) (Dai et al., 2006).

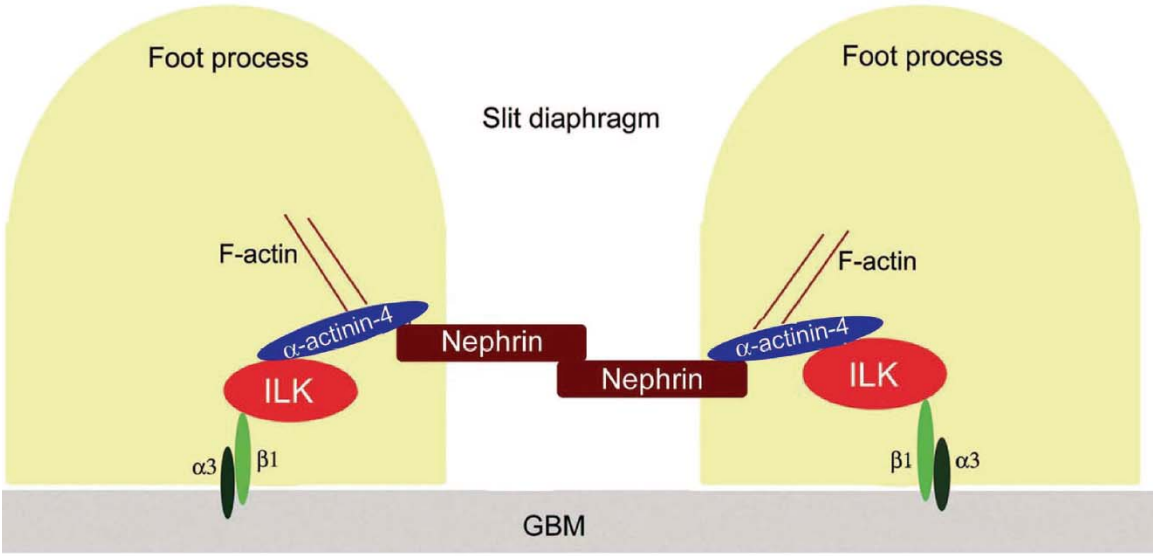



**Figure 21: Schematic model that illustrates how ILK bridges the integrin and slit diaphragm signaling (Dai et al., 2006).**
ILK functions as an adaptor protein that physically and functionally associates with the slit diaphragm protein nephrin. α-Actinin-4 participates in the ILK/nephrin complex formation.

The redistribution of the actin cross-linking protein α-actinin-4 could change the actin cytoskeleton dynamics and may cause the collapse of the actin meshwork underneath or near the SD of the foot processes. As the actin cytoskeleton defines cell shape and morphology, altered cytoskeletal structure of the foot processes as observed in the absence of ILK could lead to disappearance of the SD structures and development of an "effaced" phenotype. Interestingly, mutations or deletion of α-actinin-4 cause proteinuria and focal segmental glomerulosclerosis in animal models and patients (Kaplan et al., 2000; Kos et al., 2003; Weins et al., 2005).

## Aim of the Thesis

Although ILK was discovered 13 years ago, it is still a puzzling integrin adaptor protein with respect to its function. The essential role of ILK in strengthening the integrin-actin linkage as well as in actin reorganization is well established through numerous studies. However, it is still largely unknown how ILK exerts its effects on the actin cytoskeleton. In addition, a large amount of studies suggested that ILK regulates phosphorylation of several substrates such as Akt, Gsk-3β or MLC downstream of integrin engagement, yet the potential kinase activity remained controversial since ILK's discovery.

The aim of the thesis was to analyse the function of the ILK kinase domain, and the relevance of the putative kinase activity for mouse development. The tasks were defined as follows:

(1) Generation of mice carrying point mutations that have been shown to affect the ILK kinase activity *in vitro*.

| Mutation | Motif | Reported altered kinase function |
|---|---|---|
| **K220A** | ATP-binding site | kinase dead |
| **K220M** | ATP-binding site | kinase dead |
| **E359K** | conserved APE-motif of subdomain VIII | kinase dead |
| **S343A** | (auto)phosphorylation site | kinase dead |
| **S343D** | (auto)phosphorylation site | kinase active |
| **R211A** | PH-domain | reduced phosphorylation of Akt |

(2) Analysis of the phenotypes of these mice with a special focus on the role of ILK as a serine/threonine kinase.

## Short Summaries of Publications

### Publication I: Local call: from integrins to actin assembly

The focus of this commentary is an article published by Butler *et al*.

Integrin receptors link the extracellular matrix to the actin cytoskeleton. As integrins lack actin binding capacity they depend on the recruitment of other proteins to fulfill this function. Because many of those proteins bind to integrin cytoplasmic tails and F-actin simultaneously, actin binding to matrix adhesions was considered as recruitment of pre-assembled filaments.

The activity of actin nucleation factors such as the Arp2/3 complex and the formin protein family, which both have been shown to localize to adhesion sites, is controlled by small GTPases of the Rho family, which are in turn activated downstream of integrin engagement. However, this suggests that actin polymerization occurs at a certain distance to the core adhesion site.

Butler *et al*. show here that isolated αVβ3 adhesion complexes from hematopoietic cells can induce the polymerization of actin filaments suggesting that local actin polymerization through the direct recruitment of Arp2/3 and formins to matrix adhesion sites could be possible. A few proteins were found to be involved in the regulation of the actin nucleation activity. Among them were PI3K, Vav1, Pyk2 and Src.

**Publication II: How ILK and kindlins cooperate to orchestrate integrin signaling**

This review article summarizes the current knowledge about the role of ILK and kindlins, a family of three integrin-binding proteins, in the regulation of integrin function. It also includes a discussion of cell-matrix adhesion-independent functions of both proteins.

Integrin receptors possess the rare ability to signal bi-directionally across the membrane and appear in different conformations characterized by their affinity to the ligand but do not possess enzymatic activity or actin binding capability by their own. Ligand binding triggers the recruitment of a multiprotein complex to the cytoplasmic tails of integrins which establishes a connection from the ECM to the actin cytoskeleton and to various signal transduction pathways. This is of crucial importance for processes such as cell spreading and migration ("outside-in" signaling). Conversely, integrin tail binding proteins can also induce conformational changes in the integrin ectodomain that alters the affinity for their ligands ("inside-out" signaling).

A number of recent studies have established the role of kindlins, together with the FERM domain-containing protein talin, as important regulators of integrin inside-out signaling. How kindlins and talin cooperate is still unknown. In addition, kindlin proteins have also been shown to play a major role in integrin "outside-in" signaling through binding and recruitment of actin regulatory proteins such as ILK and migfilin to focal adhesions/cell-matrix adhesion sites. Two human diseases have been associated with loss of kindlin function so far and are reflected in the corresponding kindlin knock-out mouse: Kindler syndrome (kindlin-1) and leukocyte adhesion deficiency type III (kindlin-3).

Kindlins also colocalize with their interaction partners ILK and migfilin at subcellular structures such as the nucleus and cell-cell contact sites. However, the function of focal adhesion proteins at these structures remains to be determined.

**Publication III: Integrin-linked kinase is an adaptor with essential functions during mouse development**

Integrin binding to extracellular matrix catalyses the assembly of multiprotein complexes, which transduce mechanical and chemical signals that regulate many aspects of cell physiology. Integrin-linked kinase (ILK) is a multifunctional protein that binds β-integrin cytoplasmic domains and regulates actin dynamics by recruiting actin binding regulatory proteins such as α- and β-parvin. ILK has also been shown to possess kinase activity and to regulate signaling downstream of integrins through phosphorylation of substrates such as Akt and Gsk-3β in mammalian cells; however, genetic studies in flies and worms failed to confirm the importance of ILK as a kinase.

Here we report the generation of ILK mutant mice harboring point mutations that have been shown to affect the kinase activity *in vitro*. These mice were generated in order to determine whether the catalytic activity of ILK might be specific for mammals or certain cell types. Mice carrying point mutations in the proposed autophosphorylation site (S343A/D) of the putative kinase domain and in the pleckstrin homology domain (R211A) are normal. In contrast, mice with point mutations in the conserved lysine residue of the potential ATP-binding site of the kinase domain die due to renal agenesis. The observed phenotype is not caused by altered kinase activity of the mutant ILK (K220A/M) but rather it affects ILK´s ability to act as a scaffold protein as this mutation specifically impairs binding of ILK to α-parvin. Similarly, renal effects also occur in α-parvin deficient mice.

Thus, we conclude that the kinase activity of ILK is dispensable for mammalian development and adult life; however, an interaction between ILK and α-parvin is crucial for kidney development.

**Publication IV: The ILK/PINCH/parvin complex: the kinase is dead, long live the pseudokinase!**

Genetic studies have firmly established a role for the IPP complex in adhesion strengthening and organization of the actin cytoskeleton downstream of integrins. This review focuses on the recent advances made towards the understanding of the function of the ILK/PINCH/parvin complex regarding the specialized roles this complex and its individual components have acquired during evolution.

It is now clear that the putative kinase activity of ILK is non-existent neither in invertebrates nor in vertebrates and thus not required for its function *in vivo*, and that the kinase homology domain is a critical mediator of several protein-protein interactions.

Recently, the analysis of the α-parvin-null mice has revealed a specific function of this protein in regulating cell contractility in a subset of cell types. Despite the ubiquitous expression pattern of α-parvin, these mice survive to E14.5, because of the ability of β-parvin to compensate during early development, and die as a result of severe cardiovascular defects. The absence of α-parvin causes impaired investment of vascular smooth muscle cells to developing vessel walls resulting in defective stabilization of the vasculature and subsequent dilation of vessels, formation of microaneurysms, and vessel rupture. At the molecular level, these defects are caused by increased RhoA activity that leads to elevated MLC phosphorylation and aberrant actomyosin contractility. Interestingly, α-parvin-null fibroblasts or endothelial cells do not display a hypercontractile phenotype, suggesting that the function of α-parvin as a negative regulator of RhoA is cell-type specific. A similar role for ILK in the regulation of Rho activity has been observed in Schwann cells of the nervous system, where ablation of ILK leads to upregulation of Rho/Rock signaling, resulting in the inability of the Schwann cells to extent cytoplasmic processes to envelope the nerves. In addition, point mutations in the potential ATP-binding site of ILK which selectively disrupts its interaction with α-parvin induce contractile cell behavior as well as enhanced random motility and loss of directional cell migration in collecting duct epithelial cells.

In conclusion these studies collectively identify the ILK/α-parvin complex as a negative regulator of cell contractility in certain cell types.

**Publication V: Integrin-mediated signals control microtubule dynamics required for plasma membrane targeting of caveolae**

Caveolae are cell surface organelles involved in signalling, endocytosis and cholesterol transport and have been shown to suppress tumor formation. Formation of caveolae requires the transport of caveolin-1 from the endoplasmatic reticulum where it is synthesized to the golgi apparatus where caveolae assembly is believed to start. Finally, caveolae are transported to the plasma membrane.

Interestingly, mice lacking either integrin β1 or ILK in the epidermis show reduced numbers of plasma membrane caveolae *in vivo*. The lack of plasma membrane caveolae is due to an impaired transport of caveolin-1 containing vesicles along microtubules caused by destabilzed microtubules at the cell cortex.

At the molecular level, the integrin/ILK complex recruits the F-actin binding protein IQGAP1 to nascent focal adhesions, which in turn recruits mDia leading to microtubule stabilization.

**Publication VI: Bacteria hijack integrin-linked kinase to stabilize focal adhesions and block cell detachment**

Cell-matrix attachment is crucial for normal development. Therefore bacterial pathogens often target cell adhesion molecules to attach to host cells, to invade them and to spread.

One of these pathogens is Shigella, a bacterium that infects the digestive tract and induces an intense inflammatory reaction. Using the type III secretion system Shigella injects virulence proteins into the host, which then manipulate host cell signalling cascades allowing them to colonize the gastric epithelium efficiently. In this study, it has been found that the effector protein OspE, which is secreted into the host cell, binds to ILK. This interaction causes stabilization of integrin-containing adhesion sites due to reduced adhesion turnover and suppression of detachment of infected cells from the basement membrane.

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# Curriculum Vitae

## Personal details


| | |
|---|---|
| Name: | Anika Lange |
| Address: | Steiermarkstrasse 9, 81241 Munich, Germany |
| Date and place of birth: | 11.08.1980 in Osterburg/Altmark, Germany |
| Nationality: | German |
| E-mail: | lange@biochem.mpg.de |


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## Education

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|---|---|
| 2005 – present | **Max Planck Institute of Biochemistry,** Martinsried, Germany; Department of Molecular Medicine<br>PhD thesis "Genetic analysis of the catalytic activity of integrin-linked kinase *in vivo*"<br>Supervisor: Prof. Dr. Reinhard Fässler |
| 2004 – 2005 | **Friedrich-Schiller University of Jena,** Germany<br>Department of Medical Biochemistry<br>Diploma thesis "Adjustable expression of oligomeric Ras-Raf binding domains to block Ras-mediated cellular signaling in a reversible manner"<br>Supervisor: Prof. Dr. Karlheinz Friedrich |
| 2002 – 2003 | **University of Lund, Sweden**<br>Graduate Studies in biology |
| 1999 – 2004 | **Friedrich-Schiller University of Jena,** Germany<br>Graduate studies in biology |
| 1992 – 1999 | **Markgraf-Albrecht-Gymnasium, Osterburg/Altmark,** Germany |
| 1987 – 1992 | **Primary School in Werben/Altmark,** Germany |

## Supplements

In the following, papers I to VI are reprinted.